Phytoliths as proxies for plant water availability. An experimental approach on selected C_4 species and its archaeological application in drylands

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Abstract

This work investigated the relationship between phytoliths from finger millet, pearl millet and sorghum and the water conditions under which these species grow. The archaeological debate on the use of millets remains open and finding a proxy to help recognise their growing conditions is crucial for the reconstruction of their dispersal. In this research phytolith concentration, ratio of sensitive to fixed morphotypes, phytolith assemblage composition, morphometry, and Si isotopic composition of sensitive morphotypes were analysed as potential indicators of plant water availability. The results showed changes in both the assemblage composition, the bulliform dimensions and isotopic content, depending on the species. Intra- and inter-specific differences were highlighted. In view of the results a predictive model based on morphotype concentration was elaborated and applied to four case-studies of the Indus Valley Civilization, proving that phytoliths can be efficient proxies to assess the water condition of plant growth in the archaeological record.

Keywords

Phytoliths; water availability; C_4 plants; millet archaeology; finger millet; pearl millet; sorghum; drylands; dryland agriculture; transpiration

Résumé

Ce travail porte sur la relation entre phytolithes du millet du doigt, du millet perlé et du sorgho et les conditions hydriques dans lesquelles ces espèces poussent. Le débat portant sur l'utilisation des millets en contexte archéologique reste ouvert. Aussi trouver un proxy pour identifier leurs conditions de croissance est crucial pour la reconstruction de leur dispersion. Dans cette recherche, la concentration de phytolithes, le rapport entre les morphotypes sensibles et fixes, la composition de l'assemblage de phytolithes, la morphométrie et la composition isotopique du Si des morphotypes sensibles ont été étudiés comme indicateurs potentiels de la disponibilité en eau des plantes. Les résultats ont montré des différences à la fois dans la composition de l'assemblage, les dimensions du *bulliforms* et le contenu isotopique, au niveau des espèces. Des différences intra et interspécifiques ont été mises en évidence. Ces résultats ont permis de développer un modèle prédictif basé sur la concentration des morphotypes, qui a été testé sur quatre cas d'études de la Civilisation de la Vallée de l'Indus. Les phytolithes peuvent ainsi être des indicateurs efficaces pour évaluer comment les conditions hydriques impactent la croissance des plantes dans les archives archéologiques.

Mots clés

Phytolithes; disponibilité de l'eau; C_4 plantes; archéologie des millets; millet du doigt; millet perlé; sorgho; zones arides; agriculture des zones arides; transpiration

Resumen

Este trabajo investigó la relación entre los fitolitos de mijo de dedo, mijo perla y sorgo, y las condiciones hídricas en las que crecen estas especies. El debate arqueológico sobre el uso de los mijos sigue abierto y encontrar indicadores que reflejen sus condiciones de crecimiento es crucial para reconstruir su cultivo. Como posibles indicadores de la disponibilidad de agua de las plantas se analizó: concentración de fitolitos, relación entre morfotipos sensibles y fijos, composición de conjuntos de fitolitos, morfometría y composición isotópica de los morfotipos sensibles. Los resultados mostraron cambios en composición dimensiones y contenido isotópico, dependiendo de la especie, así como grandes diferencias intraespecíficas. Se elaboró y aplicó un modelo predictivo a cuatro sitios de la civilización del valle del Indo, demostrando que los fitolitos son indicadores eficaces para evaluar la condición hídrica del cultivo de plantas en el registro arqueológico.

Palabras clave

Fitolitos; disponibilidad de agua; plantas C_4; arqueología de los mijos; mijo de dedo; mijo perla; sorgo; tierras áridas; agricultura de secano

Preface

The beginning of this PhD experience dates back to April 2018 when I applied to participate in Carla Lancelotti's ERC project RAINDROPS as a doctoral student in charge of the experimental work package. At that time, I had just finished my MSc thesis dedicated to the study of ancient *Vitis vinifera* seeds by both morphometric and aDNA analyses. The preparation of this master thesis reawakened in me a great interest in archaeobotany and prompted me to seek a doctorate that dealt with this subject. Thanks to the advice given by my supervisor at the time Dr Mauro Rottoli, I encountered Carla Lancelotti and her project. I began my PhD experience dedicated to the study of phytoliths, under the supervision of Dr Carla Lancelotti, Dr Vincent Vadez and Dr Marco Madella. The project developed into a co-tutoring programme between two universities Universitat Pomepu Fabra and Univeristé de Montpellier. At the Université de Montpellier (and at ICRISAT), with Vincent, I delved into crop physiology, studying plant growth under precise conditions of water availability. At Universitat Pompeu Fabra, under the supervision of Carla and Marco, I dedicated myself to the extraction of phytoliths from the plants grown during experimental cultivations, and to their study, with a focus on archaeology. The results achieved have broad application ranging from archaeology to paleoecology, from plant physiology to agronomy, and for that, I can say, I am satisfied with all the way so far.

As a result of the co-teaching programme, this thesis is developed following both Pompeu Fabra's and the Univerisitè de Montpellier's standards, both in terms of content and format, trying to make them coincide as much as possible. For this same reason, much of the content is proposed in three languages English, French and Spanish.

Francesca D’Agostini
Barcelona, October 2022

Résumé de la thèse en français

Contexte

Aujourd'hui, les zones sèches couvrent plus de 40% de la surface de la Terre et abritent environ 2,3 milliards de personnes (International Institute for Environment and Development - https://www.iied.org consulté le 23 avril 2022). Néanmoins, les zones sèches arides et hyperarides ont toujours été considérées comme des régions périphériques qui, aujourd'hui comme hier, ne permettent pas à l'homme de s'installer de manière prospère (Biagetti et al., 2022). Au contraire, les recherches anthropologiques et archéologiques ont montré la grande variabilité des systèmes socio-écologiques traditionnels dans les terres arides, hautement résilients et très riches en ressources adaptatives (Balbo et al., 2016 ; Critchley et al., 1994 ; D'Odorico et Bhattachan, 2012). La gestion de l'eau dans les zones arides est un sujet central. Dans les zones arides, diverses pratiques ont été adoptées pour collecter les précipitations et les eaux souterraines, canaliser les inondations et profiter des ressources hydriques disponibles (Prinz, 2002). La rareté des ressources hydriques a fait de l'eau une question clé dans l'étude des zones les plus arides du monde (Cooper et al., 2008 ; Osman et al., 2021 ; Salmon et al., 2015), s'avérant être un bien d'une importance capitale, notamment pour le développement des pratiques agricoles (Manning et Timpson, 2014 ; Marshall et Weissbrod, 2011). En effet, l'histoire a confirmé que ces zones ont été non seulement le berceau de la domestication de diverses cultures (Manning et al., 2011 ; Winchell et al., 2018) mais aussi de technologies spécifiques, aptes à développer des systèmes agricoles élaborés (Reynolds et al., 2007 ; Winslow et al., 2004). Mais l'étude du développement de ces pratiques est complexe car les *proxies* de la disponibilité en eau sont difficiles à trouver dans les archives archéologiques et archéobotaniques.

Les régions arides sont dominées par les biomes de savane et de prairie composés principalement d'espèces C_4 qui représentent environ 25% de la production primaire sur l'ensemble de la planète (Sage et Zhu, 2011). Quant aux millets, 95% de la production mondiale provient d'Asie et d'Afrique (Saleem et al., 2021). Plusieurs grandes cultures de millet comme le sorgho (*Sorghum bicolor* (L.) Moench) (Reddy, 2017a), dont l'appartenance à la catégorie des millets est débattue. Dans cette thèse sont désignées comme tels, le millet proso (*Panicum miliaceum* L.) (Gomashe, 2017), le millet à queue de renard (*Setaria italica* (L.) Beauv.). (Hariprasanna, 2017), et le millet perlé

(*Pennisetum glaucum* (L.) R.Br.) (Reddy, 2017b) ont été introduits dans toutes les régions les plus arides du monde. Les millets cultivés à plus petite échelle, mais toujours d'un intérêt central, incluent le millet à doigts (*Eleusine coracana* L. Gaertn.) et le millet de basse-cour indien (*Echinochloa frumentacea* Link) (Rudov et al., 2020). Avec des précipitations très minimes et peu d'intrants pendant la croissance, les champs de petits millets donnent généralement des rendements décents et fiables (Rudov et al., 2020). Le sorgho, le millet perlé et le millet à doigts, qui sont les céréales d'intérêt dans cette étude, ont été classés par la FAO parmi les 150 premières cultures produites dans le monde (Organisation des Nations unies pour l'alimentation et l'agriculture, 2018) et, dans les régions où les précipitations sont imprévisibles, ces trois cultures sont considérées comme les plus fiables (Weber et Kashyap, 2016).

Problématiques scientifiques et hypothèse de travail

L'objectif principal de cette recherche est de comprendre s'il est possible, et dans quelle mesure, de reconstruire la disponibilité de l'eau dans un contexte archéologique en étudiant des restes archéobotaniques spécifiques, à savoir les phytolithes. Les phytolithes ont été choisis après un examen minutieux des indicateurs directs et indirects de l'eau disponibles. Les indicateurs indirects comprennent toutes les structures artificielles (par exemple, les puits, les canaux, les réservoirs) qui peuvent être rencontrées dans les archives archéologiques et qui indiquent, par leur présence, une sorte de système de gestion de l'eau. Cependant, la découverte de ces structures artificielles présente certaines difficultés. En effet, bien souvent, les champs, les crêtes, les barrages ou les puits ne sont plus visibles dans les archives archéologiques. Deux explications sont possible: ces systèmes se sont détériorés au fil du temps ou des humains, ou n'ont pas été entretenus car étaient utilisés de façon éphémère ou bien encore n'étaient pas suffisamment efficaces, surtout à l'époque préhistorique (Jenkins et al., 2020). En outre, la simple découverte d'installations d'irrigation ou de stockage de l'eau n'est qu'un moyen indirect d'indiquer la disponibilité de l'eau et ne donne aucune indication sur la quantité d'eau utilisée. Par conséquent, l'essentiel de mes recherches a été orienté vers les vestiges archéobotaniques, qui peuvent fournir une évaluation directe de l'arrosage. La plupart des recherches archéobotaniques évaluant la disponibilité en eau des cultures se concentrent à ce jour sur les espèces C_3 car elles sont considérées comme les cultures fondatrices de l'agriculture moderne dans certaines des régions les plus étudiées du monde. Il faut préciser que l'étude de la disponibilité de l'eau à partir de *proxies* archéobotaniques est encore à ses débuts et que peu de zones ont été testées à ce jour, principalement autour du Croissant fertile et de l'Amérique centrale/nord. Pour C_3, l'étude de la composition isotopique des graines s'est avérée efficace pour indiquer la disponibilité en eau (Araus et al., 1997 ; Ferrio et al., 2005 ; Nitsch et al., 2015). La discrimination isotopique décrit l'assimilation préférentielle des isotopes les plus légers par rapport aux plus lourds lors du processus de photosynthèse, et reflète principalement la disponibilité en eau (Eggels, 2021). La gestion de l'eau peut, ainsi, être déduite en intégrant les données isotopiques aux indicateurs paléoclimatiques pour la période en question (Flohr et al., 2019). Si le signal isotopique des cultures indique une plus grande disponibilité en eau que celle attendue selon les *proxies* climatiques, il est probable que les cultures ont reçu de l'eau supplémentaire en plus des précipitations, comme par l'irrigation, l'arrosage artificiel ou par la culture sur des cônes de déjection (Ferrio et al., 2005). Cependant, les zones arides sont principalement cultivées avec des plantes C_4, dont le système photosynthétique est beaucoup plus complexe, tant sur le plan anatomique que physiologique, que celui des C_3. En effet, chez les plantes C_4, il existe une fuite de Carbone hors des cellules de la gaine des faisceaux (où la majorité du carbone est fixée) pendant la photosynthèse, ce qui est considéré comme influençant la discrimination du Carbone (ΔC_4) (Farquhar, 1983). Bien que des recherches plus récentes aient montré que le ΔC_4 répond à la sécheresse et a une relation directe avec l'efficience de l'eau (Buchmann et al., 1996 ; Cernusak et al., 2013), la nature exacte de cette relation doit encore être clarifiée. De plus, l'étude des C_4 caryopses provenant d'archives archéologiques est encore compliquée par les questions liées à leur morphologie. En effet, les caryopses de la plupart des petits millets (par exemple, le millet à doigts ou le millet perlé) sont de taille très réduite. Il faut préciser que les restes organiques tels que les graines sont conservés dans les archives archéologiques suite à la carbonisation ou à l'engorgement (Araus et al., 1997 ; Yang et al., 2011). Les phénomènes taphonomiques et de carbonisation sur les graines de petites dimensions compliquent considérablement leur découverte et leur reconnaissance dans le registre archéologique. Les phytolithes représentent donc un bon substitut aux graines puisque dans les zones arides, ce type de vestiges est souvent mal conservé (Fuller, 2002).

Les phytolithes, étant de nature inorganique, se conservent mieux que les graines et leur conservation est beaucoup moins affectée par les problèmes pré- et post-dépositionnels (Piperno, 2006). Cependant, la capacité des plantes C_4 à économiser l'eau en réduisant le taux de transpiration pourrait représenter un défi dans l'utilisation des phytolithes comme proxy de la disponibilité en eau. Cette recherche se concentre sur trois espèces en particulier : le millet à doigts (*Eleusine coracana* (L.) Gaertn.), le millet perlé (*Pennisetum glaucum* (L.) R.Br.) et le sorgho (*Sorghum bicolor* (L.) Moench). Selon l'Institut international de recherche sur les cultures des zones tropicales semi-arides (ICRISAT), ces trois espèces figurent parmi les millets les plus cultivés en Afrique de l'Est et du Sud et en Asie du Sud. Le millet à doigts, le millet perlé et le sorgho sont extrêmement résistants à la sécheresse, ils germent même en cas d'humidité résiduelle et constituent un aliment de base pour plus d'un demi-milliard de personnes dans le monde. Ces trois espèces ont été sélectionnées (a) en raison de leurs voies biochimiques C_4: NADP-ME pour le sorgho et NAD-ME pour le millet et le millet perlé. Leur diversité anatomique et biochimique permet d'évaluer pleinement les éventuelles différences entre les espèces C_4. (b) En termes de silicification, ils ont déjà été étudiés et classés comme accumulateurs de biosilice (Coskun et al., 2021 ; Kumar et al., 2017 ; Lux et al., 2002 ; Ma et Yamaji, 2015 ; Out et Madella, 2017 ; Sangster et Parry, 1976) (c) L'étude agronomique de ces trois espèces a été largement approfondie par des enquêtes sur leur taux de transpiration (par exemple, Vadez et al., 2011). En outre (d) les trois espèces ont été trouvées dans plusieurs assemblages archéobotaniques de sites arides d'intérêt à la fois en Asie et en Afrique (par exemple le sorgho à Al Khiday et Mezber par D'Andrea et al. (2015) ; et le millet à Harappa par Weber et Kashyap (2016)).

Dans cette recherche, des modèles de phytolithe basés sur l'abondance relative de morphotypes spécifiques ont été testés comme *proxies* de la disponibilité en eau, ainsi que la concentration de l'accumulation de biosilice dans les organes de transpiration, la taille des squelettes de silice, le contenu isotopique (δ^{30}Si, δ^{29}Si) et la dimension des morphotypes sensibles distincts. L'étude des phytolithes comme indicateurs de l'eau a déjà été explorée et ce n'est pas une question entièrement nouvelle (Bremond et al., 2005 ; Jenkins et al., 2016, 2020 ; Madella et al., 2009 ; Miller Rosen, 1994 ; Weisskopf et al., 2015). Madella et al. (2009), Weisskopf et al. (2015) et Jenkins et al. (2016 ; 2020), ont suggéré l'utilisation du rapport entre les morphotypes fixes (génétiques) et les morphotypes sensibles (contrôlés par l'environnement) pour détecter la disponibilité de l'eau directement à partir des assemblages de phytolithes. Ils ont émis l'hypothèse que dans des contextes d'abondance d'eau, les morphotypes sensibles sont produits plus abondamment que dans des conditions de stress hydrique et donc que le rapport entre les deux classes change lorsque la disponibilité en eau varie. Plus récemment, on a découvert que les signatures isotopiques stables du silicium et de l'oxygène dans les phytolithes pouvaient également servir d'indicateurs des conditions environnementales. Plus précisément, il a été démontré que le $\delta^{30}Si$ dans le phytolithe est un indicateur des conditions du sol, en particulier de l'altération et de la teneur en matière organique (Leng et al., 2009). Ces études sont basées sur l'hypothèse que dans les tissus ayant une activité photosynthétique, le silicium se polymérise principalement en raison de la sursaturation (Schaller et al., 2013). Ainsi, les phytolithes, qui sont l'effet phénotypique de la précipitation du silicium dans l'épiderme, sont considérés comme un proxy potentiel de la disponibilité en eau. Cependant, le rôle et la fonction du Si dans les plantes ne sont pas encore totalement compris, ce qui nuit à la compréhension des mécanismes qui conduisent à la formation des phytolithes. En effet, récemment, Kumar et ses collègues (2017) et Hodson (2019) ont montré que la classification des phytolithes est plus complexe qu'on ne le pensait et que les caractéristiques du morphotype ne dépendent pas seulement du mécanisme génétique/environnemental de dépôt mais que leur formation est également modifiée par le district cellulaire où la silice a commencé à précipiter. Par conséquent, l'étude des phytolithes de C_4 en relation avec la disponibilité en eau est encore un sujet de recherche ouvert. Une telle étude, en plus de répondre à une question archéologique, a également des implications importantes pour une exploration plus approfondie du rôle du dépôt de silice chez les espèces considérées comme de grands accumulateurs. L'approche expérimentale qui caractérise cette recherche a été construite sur la base de ces pilotes, répondant à la nécessité d'un travail expérimental supplémentaire pour réaliser toutes les capacités des études sur les phytolithes.

Objectifs

Cette recherche de thèse s'est développée dans le cadre du projet de subvention de démarrage ERC (ERC-StG nº 759800) RAINDROPS. Le projet combine la recherche sur la physiologie des plantes avec des applications archéobotaniques originales et l'enregistrement de TEK (de l'anglais, *traditional ecological knowledge*) sur les systèmes de culture dans les zones arides afin de fournir une méthodologie innovante et fiable pour comprendre les pratiques passées de gestion de l'eau dans les paysages arides.

Dans le cadre plus large du projet, le but de cette thèse est de produire des données détaillées et complètes sur la production de phytolithes en réponse à la sécheresse de trois espèces fondamentales de C_4 et de fournir des outils fiables pour enquêter sur les pratiques passées de culture de l'eau par comparaison avec des répliques modernes.

Les objectifs de l'étude sont (1) d'étudier les processus d'accumulation de biosilice dans le sorgho, le millet perlé et le finger millet, afin de comprendre si oui ou non les phytolithes produits par les cultures de C_4 peuvent être utilisés comme *proxies* pour la disponibilité en eau. (2) Étudier la variabilité intra et inter échantillon dans la production de phytolithes (3) Élaborer un modèle pour appliquer les résultats obtenus dans les cultures expérimentales aux assemblages de phytolithes archéologiques.

Pour atteindre les objectifs proposés, deux cultures expérimentales (en 2019 et 2020) des trois cultures étudiées ont été réalisées à l'ICRISAT (Inde). Les plantations ont été placées dans des conditions constamment contrôlées d'eau, de température, d'humidité, d'évapotranspiration et de composition du sol pendant la période de l'année dépourvue de précipitations (janvier-mai), dans des lysimètres protégés par des précipitations naturelles. Les lysimètres offrent des conditions similaires à celles des champs mais permettent un contrôle expérimental de la transpiration, de l'imposition du stress hydrique et des paramètres physiologiques. Les expériences ont été réalisées afin d'obtenir des données de base pour évaluer la production de phytolithes en relation avec l'arrosage et la variation génotypique. Afin d'assurer la signification statistique et le contrôle des mécanismes génétiques, les expériences ont été réalisées sur 10 cultivars pour chaque espèce, avec 5 répétitions dans 2 cultures répetées. Pour chaque culture, 5 cultivars ont été sélectionnés et 3 répétitions pour chaque traitement ont été analysées pour l'accumulation de biosilice dans les différents tissus. La production de phytolithes (concentration gramme/matière végétale sèche) ; l'assemblage des morphotypes (plus de 250 phytolithes ont été comptés pour chaque échantillon analysé) ; les rapports entre les formes contrôlées par l'environnement et les formes contrôlées génétiquement ; la taille du squelette de silice et les dimensions spécifiques (par exemple la longueur, la largeur, la longueur des pics) pour les formes *stomata*, *bulliforms* et *elongates* ont été collectées. En outre, le δ^{30}Si des feuilles et du phytolithe des paillettes a également été testé par des techniques de spectrométrie de masse par ablation au laser femtoseconde et source de plasma à couplage inductif.

Principaux résultats et discussion

Premièrement, les résultats expérimentaux amènent à la conclusion que, malgré la proximité taxonomique, les trois espèces possèdent des différences substantielles dans les mécanismes d'accumulation de Si. Ma et Yamaji (2006) ont suggéré que la quantité d'absorption de silice, attribuable à la génétique, dépend de deux facteurs principaux qui concourent tous deux à la charge du xylème: la présence/absence de canaux pour transporter le Si des cellules corticales au xylème (Lsi1-Lsi2-Lsi6 et orthologues), et la densité avec laquelle ils se produisent. La combinaison de ces deux facteurs détermine la classification en accumulateurs de silice actifs, passifs ou réjecteurs (non-accumulateurs). Bien que toutes les graminées soient considérées comme des accumulateurs élevés (Hodson et al., 2005), leur capacité à accumuler du Si par le biais de transporteurs spécifiques reste non vérifiée pour de nombreuses espèces. Chez *Sorghum bicolor*, certaines recherches ont déjà été menées et l'espèce a été testée pour la séquence de la protéine Lsi1 par Vatansever et ses collègues (2017) qui ont découvert deux gènes homologues codifiant pour les canaux du transporteur. Cependant, des mutations ont également été découvertes récemment par Markovich et al. (2019) qui ont prouvé la présence de variétés incapables d'absorber de grandes quantités de silice. Slp1, une protéine à la composition unique en acides aminés impliquée dans la précipitation de la silice dans les cellules de silice, a également été localisée dans le sorgho (Kumar et al., 2020, 2017a, 2017b). Dans cette étude, le sorgho a non seulement accumulé une grande quantité de Si par rapport aux deux autres espèces testées, mais le taux d'accumulation s'est avéré indépendant des facteurs environnementaux tels que la transpiration. Ainsi, ce résultat est en accord avec la présence d'un mécanisme dans le sorgho qui transporte l'acide silicique activement jusqu'aux feuilles, indépendamment de la transpiration (absorption de Si gouvernée par le transporteur) et a placé l'espèce parmi les accumulateurs élevés de silice. Pour le millet perlé et le millet à doigts, l'information semble plus complexe à interpréter. Bien que la quantité de Si accumulée situe également les deux espèces parmi les grands accumulateurs (Hodson et al., 2005), il est également vrai que la production relative (g de Si/g de matière sèche) par rapport au sorgho est plus faible. Les travaux de Ma et Yamaji (2006) indiquent que la densité des transporteurs facilitant l'absorption de Si est plus faible (bien qu'existante) dans le millet perlé et le millet à doigts. Ces millets dépendent largement du transport passif le long du flux de transpiration, bien que la dynamique du mécanisme reste à clarifier. Malheureusement, il n'est pas possible de l'étayer par des données sur la génétique de l'absorption de Si car aucune information sur les *loci* correspondants chez *Pennisetum glaucum* et *Eleusine coracana* n'est disponible à ce jour. L'accumulation de biosilice chez le millet à doigts et le millet perlé semble dépendre davantage de facteurs environnementaux (transpiration). Ces deux espèces sont alors plus appropriées que le sorgho pour étudier comment ces plantes répondent à la disponibilité de l'eau.

Les résultats des assemblages expérimentaux de phytolithes indiquent que de nombreuses valeurs de concentration de morphotypes pour les trois espèces sont prévisibles en fonction de l'approvisionnement en eau, ce qui démontre que les assemblages de phytolithes peuvent être utilisés comme *proxies* pour la disponibilité de l'eau. En outre, des analyses isotopiques préliminaires effectuées sur des phytolithes individuels provenant de feuilles de sorgho ont mis en évidence que les *bulliforms* et les *elongates* peuvent discriminer le traitement de l'eau sur la base de la composition δ^{30}Si et δ^{29}Si. La différence de discrimination de l'isotope plus léger ^{28}Si et des deux cunterparts plus lourds ^{30}Si et ^{29}Si, entre les deux traitements, est bornée par 0,5‰ et 1‰ pour les deux morphotypes et semble avoir une relation négative significative avec l'eau transpirée. Les *bulliforms* augmentent également leur robustesse (largeur + longueur + longueur du bec) avec l'augmentation des niveaux de transpiration, ce qui confirme qu'il s'agit d'un morphotype particulièrement efficace pour évaluer la disponibilité en eau, car cela se reflète dans la composition chimique des *bulliforms,* leur concentration dans l'assemblage et la taille moyenne des morphotypes. Jusqu'à présent, il a été démontré que seuls les *bulliforms* changent de taille en fonction de l'arrosage, par conséquent il semble approprié de supposer que, généralement, lorsque l'approvisionnement en silice change, la concentration des phytolithes déposés change mais pas leurs dimensions (Schulz-Kornas et al., 2017). À cet égard, il a été observé dans les échantillons analysés pour cette étude que certains morphotypes augmentent leur concentration dans des conditions WW (grande disponibilité de l'eau) tandis que d'autres dans des conditions WS (faible disponibilité de l'eau), et que cela dépend de l'espèce mais aussi du tissu où les morphotypes sont déposés. Cette particularité met en évidence que pour estimer la disponibilité de l'eau il faut observer l'assemblage complet des morphotypes et non un groupe réduit, pour que chaque concentration de morphotype, augmentant ou non suite à l'arrosage, soit prise en compte.

Tous les morphotypes ne se sont pas révélés aussi efficaces pour prédire l'arrosage. La régression par paliers appliquée pour sélectionner les morphotypes les plus significatifs pour l'identification de l'arrosage indique les formes *acute bulbosus*, *bulliformes*, *elongates clávate*, *dentate*, *entire* et *sinuate*, *crosses*, *polylobates*, *rondels* et *saddles* comme prédictives et exclut les autres morphotypes. Il faut souligner que les formes ayant le plus haut degré de signification sont les cellules courtes, qui sont normalement considérées comme des morphotypes fixes, c'est-à-dire des morphotypes pour lesquels la silicification est contrôlée par des facteurs génétiques et qui, dans des études précédentes, n'ont pas montré de changements de concentration par rapport à la disponibilité de l'eau (Ermish et Boomgarden, 2022 ; Jenkins et al., 2016, 2020 ; Madella et al., 2009). En considérant les résultats de la présente étude, il est possible de conclure que la concentration (par g de feuille sèche en millions) de certains morphotypes est influencée par la disponibilité en eau, mais que tous (selon l'espèce) n'augmentent pas leur concentration lorsque le taux de transpiration augmente. Ceci conduit à la conclusion que, puisque les mécanismes de dépôt des phytolithes sont complexes (et d'une certaine manière, ils échappent encore à notre compréhension), afin de produire un modèle prédictif basé sur la composition des assemblages de phytolithes provenant de nombreuses espèces différentes, il est nécessaire de prendre en compte tous les groupes de morphotypes, de tous les tissus, de sorte que les variations positives ou négatives de la disponibilité en eau dans chacun soient enregistrées. En conséquence, les catégories fixes et sensibles doivent être repensées, considérant que car si la concentration de certains morphotypes augmente avec l'arrosage, d'autres peuvent être stimulés par des conditions de stress hydrique.

Les espèces et les génotypes ont mieux prédit la quantité de silice accumulée que le traitement de l'eau seul. Les espèces et les tissus ont prédit la production de la plupart des morphotypes mieux que le traitement. Cependant, il faut mentionner que la plus grande variance dans l'accumulation de biosilice est expliquée par l'effet interactif des génotypes et de l'arrosage, ce qui suggère que les deux variables sont significatives. À cet égard, on peut supposer que la demande en biosilice dépend d'une manière ou d'une autre des conditions physiologiques liées à la disponibilité de l'eau, qui dépendent à leur tour de l'efficacité de l'espèce/du génotype à répondre à la disponibilité de l'eau. Par conséquent, on peut également supposer que certains morphotypes spécifiques, peut-être plus efficaces que d'autres dans l'exécution de fonctions structurelles ou biochimiques, pourraient augmenter en réponse à la sécheresse ou parce qu'ils sont stimulés par d'autres événements environnementaux (facteurs de stress abiotiques ou biotiques) ayant un fort impact sur la physiologie de la plante elle-même. Cette hypothèse est soutenue dans cette étude car les morphotypes dont la production augmente dans les réplications de sorgho WS (*acute bulbosus*, *crosses*, *elongates clavate* et *entire* et *stomata* dans les feuilles et *elongates sinuate* et *polylobates*) pourraient jouer un rôle structurel clé en réponse au stress hydrique. En effet, la silicification des *stomata* pourrait contribuer à réduire la transpiration en régulant la perte d'eau (Cooke et Leishman, 2016 ; Gao et al., 2020 ; Goto et al., 2003 ; Hosseini et al., 2017 ; Wang et al., 2021). Les *crosses* selon Kumar et al. (2017a) pourraient se déposer sur le côté des veines mineures et les durcir, et permettre au flux du xylème de continuer (Kaufman et al., 1985). Les *élongates*, disposées à la surface de l'épithélium, augmenteraient l'entrée de la lumière (Cooke et Leishman, 2016) et durciraient le tissu épidermique (particulièrement fragile dans les conditions WS) et empêcheraient simultanément à la fois l'attaque des pathogènes et le froissement supplémentaire du limbe (Meunier et al., 2017 ; Rodrigues et al., 2003 ; Yoshida et al., 1962). Les *trichomes*, dont il a été prouvé qu'ils répondent aux attaques de pathogènes (Daoud et al., 2018 ; Fauteux et al., 2005 ; Mateos-Naranjo et al., 2013 ; Oliva et al., 2021), ont également démontré qu'ils constituent une réponse constante à l'environnement xérique (Olsen et al., 2013), et qu'ils contribuent peut-être à maintenir un film d'humidité autour de la feuille ou de la panicule en développement.

Jusqu'à présent, l'effet taxonomique a été discuté sur l'accumulation de biosilice et sur la production de phytolithe, qui à leur tour répondent à des facteurs environnementaux tels que l'arrosage, augmentant peut-être la capacité à répondre aux stress biotiques et abiotiques de l'extérieur. En conséquence, la génétique et l'environnement ne semblent pas être deux facteurs complètement indépendants et non liés dans l'accumulation de Si. Katz (2019) a suggéré, dans une perspective d'écologie évolutive, que si la teneur en silice est persistante dans le règne végétal à partir des embryophytes (comme l'ont démontré Coskun et al., 2019), elle doit conférer certains avantages à la condition physique de la plante. Tous les résultats obtenus dans le cadre de ce travail semblent appuyer cette même déduction, ce qui implique dans une certaine mesure que la teneur en Si des plantes pourrait représenter un trait fonctionnel. Les traits fonctionnels ont été définis comme des caractéristiques morphologiques, biochimiques, physiologiques, structurelles, phénologiques ou comportementales qui sont exprimées dans les phénotypes des organismes individuels, pertinentes pour la réponse de ces organismes à l'environnement et/ou affectant les propriétés de l'écosystème (Díaz et al., 2013). Cette hypothèse est encouragée par différentes études indiquant que l'accumulation de biosilice pourrait être organisée en réponse à la demande interne de la plante (par exemple, Hosseini et al., 2017 ; Li et al., 2020 ; Oliveira et al., 2022) par la régulation de l'expression des gènes Lsi1-Lsi2-Lsi6 ou de leurs orthologues (par exemple, Mitani et al., 2011 ; Sun et al., 2017 ; Vatansever et al., 2017 ; Yamaji et al., 2008). S'il a également été établi que le dépôt de biosilice dans les plantes réagit aux changements environnementaux (par exemple, Daoud et al., 2018 ; Hartley et al., 2015 ; Mandlik et al., 2020 ; Schaller et al., 2013), dépend du milieu environnant (par exemple, la composition du sol) et affecte simultanément le cercle de Si sur l'écosystème (par exemple, Cornelis et Delvaux, 2016 ; de Tombeur et al., 2020 ; Grasic et al., 2020 ; Keller et al., 2021).

Au cours de ce projet de thèse, j'ai pu appliquer le modèle développé à partir des phytolithes modernes sur des échantillons provenant de 4 sites archéologiques dispersés entre le sud-est du Pakistan et le nord-ouest de l'Inde, tous appartenant à la civilisation de la vallée de l'Indus. Les couches comparées à l'ensemble des données modernes sont datées de la période Harappan mature (2500-1900 avant notre ère) et proviennent de quatre établissements archéologiques différents: Harappa, Kanmer, Shikarpur et Alamgirpur. L'utilisation d'échantillons de phytolithe de millet provenant de couches de la période Harappan mature semblait intéressante car, à ce jour, le débat sur la consommation et la diffusion de ces cultures dans la civilisation de l'Indus reste ouvert. Au cours de la période Mature Harappan, les petits millets représentent entre 3 et plus de 50% des céréales récupérées (Pokharia et al., 2014). Cependant, leur utilisation est difficile à justifier car le traitement de leur grain est très intensif en main d'œuvre (Arunachalam et al., 2005), outre le fait que leur productivité est plus faible par rapport à celle des céréales C_3 (comme l'orge et le blé) (Pearcy et Ehleringer, 1984). Nous supposons qu'une explication possible pourrait être directement liée à la disponibilité et à la gestion de l'eau, et pour cette raison, j'ai décidé d'appliquer le modèle développé pour fournir une interprétation possible du phénomène dans ces zones. En outre, une autre question importante qui a conduit à l'application du modèle est la rareté des preuves archéologiques de la disponibilité de l'eau dans la zone indiquée (Petrie et al., 2017).

Tout d'abord, le modèle a été appliqué en tant que comparaison avec les enregistrements archéologiques qui avaient déjà prouvé l'existence des espèces considérées au moyen également de *proxies* indépendants des phytolithes (par exemple, les caryopses). La présence d'espèces domestiques C_4 a été signalée dans chacun de ces établissements (Harappa: *Panicum* L. Sp. Pl. ; Kanmer: *Pennisetum galucum* et *Setaria* Sp. P. Beauv.; Shikarpur: *Brachiaria ramosa* L. Stapf., *Coix lacryma-jobi* L., *Digitaria* Sp. Haller, *Eleusine coracana*, *Setaria* Sp. P. Beauv. ; Alamgirpur: petits millets en général) (Bates 2019 ; Bates et al., 2021). Deuxièmement, la présence de C_3 blé et d'orge a également été attestée (Bates et Petrie, 2016). En raison des caractéristiques agronomiques de C_3, ces céréales, en plus d'avoir besoin d'une bonne quantité d'eau pour pousser (beaucoup plus par rapport à C_4), étaient très probablement cultivées pendant la période hivernale (les espèces nécessitant des températures basses) (Kalra et al., 2008) en dehors de la saison des pluies. Par conséquent, la présence de céréales C_3 indique que les colonies disposaient d'eau pour l'agriculture, qu'elle provienne de systèmes d'irrigation plus ou moins élaborés ou de l'agriculture fluviale.

Le modèle développé pour les phytolithes modernes a été appliqué aux archives archéologiques. Les résultats indiquent que la plupart des céréales ont poussé dans des conditions de pénurie d'eau. Tous les sites analysés ont fourni des résultats similaires, malgré le fait que les conditions hydriques et climatiques soient quelque peu différentes d'un site à l'autre. Harappa et Alamgirpur étaient situés près de cours d'eau, avec de vastes *doabs* (étendues de terres alluviales entre deux rivières éventuellement convergentes) qui ont pu éventuellement supporter des systèmes d'irrigation et être avantageux pour l'agriculture (Kenoyer, 1998). En revanche, Kanmer et Shikarpur étaient très probablement situés dans des zones plus sèches, sans accès à des plaines alluviales substantielles à exploiter à des fins agricoles, mais et/ou desservis par des *nullah* (cours d'eau) de petites dimensions (Lancelotti, 2010) ou, comme dans le cas de Kanmer, où des bassins d'eau artificiels (c'est-à-dire des réservoirs) ont été observés (Madella et Lancelotti 2022).

Sur la base de ces informations, j'ai proposé des hypothèses pour expliquer les résultats obtenus, qui semblent montrer que les espèces C_4 ont été cultivées en régime d'étiage dans toutes les localités. Tout d'abord, le résultat obtenu tend à exclure l'option de la polyculture (c'est-à-dire la culture de deux ou plusieurs cultures ensemble sur la même terre) dans le même espace agricole. En effet, sur la base du résultat obtenu par le modèle, il y a environ 60% de chances que les céréales C_4 soient cultivées avec moins de moins de 300 mm d'eau disponible. Cette quantité d'eau ne permettrait ni aux céréales C_3 ni aux légumineuses de se développer, et me pousse à plaider pour des options alternatives à la polyculture.

Dans les zones où l'eau est abondante (par exemple, Harappa et Alamgirpur), il pourrait y avoir un système de double culture (*rabi* ou hiver & *kharif* ou saison de la mousson) avec des céréales C_3 (par exemple, blé et orge ou riz) et des légumes cultivés sous un régime hydrique en hiver tandis que d'autres espèces plus résistantes à la sécheresse, comme C_4 employées pendant la période chaude et moins pluvieuse (Petrie et al., 2016). Ainsi, une première hypothèse conduit à penser que si pendant la saison froide, les céréales C_3 étaient cultivées à un rythme régulier d'approvisionnement en eau, pendant la saison la plus chaude, avant l'arrivée des pluies de mousson, les céréales C_4 (qui ont également une croissance très rapide) étaient plantées, ne gaspillant pas les terres disponibles. Ceci est soutenu par des preuves ethnographiques de la région, où les cultures de mil sont principalement des cultures d'été alimentées par la pluie (Fuller 2006 ; Giosan et al., 2012). Cette stratégie garantirait également la possibilité pour les populations semi-nomades de quitter le champ de mil une fois semé et de ne revenir qu'au moment de la récolte, consacrant ainsi la saison au pastoralisme (García-Granero et al., 2016). L'option de la double culture (*rabi* & *kharif*) est cependant largement débattue et plusieurs chercheurs affirment que les preuves archéologiques trouvées à ce jour ne peuvent assurer la certitude de l'existence d'un système de double culture (voir Petrie et al., 2017). Pour vérifier l'existence d'un véritable système de double culture, il pourrait être intéressant de développer un "modèle jumeau" basé sur la concentration des phytolithes C_3 et de l'appliquer aux assemblages archéologiques récupérés dans les zones. Si les espèces C_3 s'avéraient également être cultivées dans des conditions de pénurie d'eau, alors, peut-être, l'option pourrait être écartée. Bien que le résultat de l'application de ce modèle n'exclût pas l'éventualité d'un système de double culture, il est également vrai qu'il existe plus d'options pour justifier la présence d'espèces C_4 cultivées en situation de pénurie d'eau. Une autre stratégie possible, inclurait la culture de céréales C_4 aux côtés de C_3 tout au long de l'année, mais sur des terres où l'irrigation/inondation/humidité résiduelle des sols (en raison de l'éloignement des sources d'eau telles que les rivières) était complexe et donc presque absente. Cela justifierait pourquoi les échantillons archéologiques auraient poussé avec une certaine disponibilité de l'eau (peut-être due aux précipitations) mais pas en abondance.

Les résultats du modèle n'excluent pas non plus l'hypothèse selon laquelle la zone comprise entre le sud du Pakistan et le nord-ouest de l'Inde a connu une certaine forme d'assèchement et que les cultures se sont donc adaptées à des périodes sèches plus constantes dans le temps, comme le suggèrent Wright et al. (2008). Les auteurs ont suggéré qu'après un millénaire d'agriculture fluviale basée sur l'inondation des berges, vers 2800-2500 avant notre ère, les rivières n'ont pas réussi à fournir l'équilibre habituel en eau et ont forcé les agriculteurs à baser leur système agricole sur les précipitations. La même étude suggère qu'autour de 2000 avant notre ère, la réduction des précipitations pourrait conduire à un pic moyen minimum de 240 mm. Toutes ces informations correspondent aux résultats obtenus dans cette étude, qui a émis l'hypothèse de la présence possible de C_4 céréales cultivées dans des conditions de stress hydrique avec moins de 300 mm d'eau disponible. Que l'adoption des millets soit due à une question d'aridification progressive, qu'il y ait eu une culture opportuniste de C_4 pour exploiter des zones plus sèches ou que C_4 ait poussé à l'état sauvage dans des parcelles arides des sites, ce qui ressort de ces résultats est que très probablement C_4 a poussé dans des conditions de manque d'eau.

Conclusions

Sur le plan archéologique, de nombreuses recherches ont été consacrées à l'étude du développement à long terme des zones sèches, considérées comme le berceau du système social et agricole de l'Ancien Monde. La domestication de l'eau dans les zones arides a permis la consolidation et la diffusion de modes de vie agricoles qui ont favorisé ou permis une croissance démographique exponentielle (Manning et al., 2011 ; Manning et Timpson, 2014). Les régions arides sont dominées par des biomes de savane et de prairie composés principalement d'espèces C_4. L'étude des cultures de millet C_4, dont l'histoire de la dispersion en Afrique, en Asie et en Europe n'a pas encore été démêlée (Motuzaite-Matuzeviciute et al., 2016 ; Stevens et al., 2021 ; Winchell et al., 2018), a un rôle clé dans le débat archéologique sur la trajectoire d'amélioration de l'utilisation des terres. Les questions archéologiques qui sont à l'origine de l'étude des restes de millet, ne sont pas seulement liées à la dynamique de consommation des espèces impliquées, mais aussi au type de gestion de ces espèces nécessaire (Briggs et al., 2006 ; Fuller, 2011 ; Stephens et al., 2019). La gestion des cultures de C_4 est étroitement liée au concept de disponibilité de l'eau, car ces plantes sont réputées pour leur capacité à résister à des conditions extrêmes, notamment à des températures élevées et à la pénurie d'eau (Sage et Zhu, 2011 ; Yang et al., 2021). Pour cette même raison, les cultures C_4 sont considérées comme des "producteur" de *proxies* difficiles, jugés moins sensibles aux changements de précipitations ou de température (Ghannoum et al., 2002). La capacité des espèces C_4 à maintenir basse la tendance de la transpiration constitue un réel problème lorsque l'intérêt réside dans l'observation des effets de la fluctuation des variables climatiques sur le taux isotopique, les phytolithes ou le rendement de plantes.

Dans cette thèse, Il a été décidé d'approfondir l'étude des assemblages de morphotypes pour observer l'efficacité du phytolithe produit par le millet à doigts, le millet perlé et le sorgho comme indicateurs environnementaux. En étudiant l'accumulation de biosilice dans ces trois espèces C_4 différentes, soumises à différents régimes hydriques, il a été démontré que les phytolithes peuvent être de bons *proxies* pour la disponibilité en eau, ajoutant de nouvelles espèces à celles qui avaient déjà été testées dans le même but (Ermish et Boomgarden, 2022 ; Jenkins et al., 2016, 2020 ; Madella et al., 2009 ; Weisskopf et al., 2015). Grâce à la comparaison de l'ensemble de données avec des assemblages de phytolithes récupérés sur des sites archéologiques spécifiques, des hypothèses sur la gestion des espèces C_4 dans le passé ont été suggérées, mettant en évidence le potentiel des résultats obtenus appliqués aux contextes archéologiques.

Les résultats obtenus soutiennent l'hypothèse que le dépôt de phytolithe dépend, au moins dans une certaine mesure, des facteurs environnementaux. La disponibilité en eau influence le dépôt de phytolithes dans le millet à doigts, le millet perlé et le sorgho en affectant les concentrations et les compositions des assemblages de morphotypes. Ces différences, cependant, dépendent de l'espèce et des tissus de dépôt de phytolithes et sont plus évidentes dans le millet à doigts et le millet perlé que dans le sorgho. Plus en détail, il a été démontré que la disponibilité en eau joue un rôle fondamental dans la détermination de l'accumulation de biosilice dans le millet à doigts et le millet perlé, qui semblent être plus proches d'accumulateurs passifs. Au contraire, le sorgho est apparemment caractérisé par un mécanisme fort régi par les transporteurs (Lsi1-Lsi2-Lsi6), ce qui détermine également une grande variabilité entre les génotypes. Bien que l'assemblage hétéroclite de cultivars ait montré différentes tendances dans la production de phytolithes, l'ensemble des données permet de faire des prédictions sur la disponibilité de l'eau. En relation avec cette considération, il est évident que la génétique liée à l'absorption de Si devrait être mieux examinée à l'avenir, à la lumière des variations dans l'accumulation de biosilice entre les espèces, les variétés et les tissus de dépôt, découverts au cours de cette étude. L'élargissement de l'ensemble des variétés analysées est fondamental, surtout en archéologie, afin de se rapprocher le plus possible de l'assemblage retrouvé dans les sédiments anciens. Le concept de morphotypes sensibles et fixes devrait également être révisé. Les résultats présentés ici ont démontré que les *stomata* peuvent difficilement appartenir à la catégorie sensible alors que les *bulliforms* et les *acute bulbosus*, qui n'ont pas été pris en compte jusqu'à présent, le sont. Une information clé qui ressort de cette étude est qu'il est difficile de séparer les morphotypes dont le dépôt est contrôlé par la génétique de ceux dont le dépôt est déclenché par la disponibilité en eau, car les deux dépendent très probablement d'une régulation dépendant de l'environnement.

Tous les résultats présentés dans cette thèse semblent indiquer que la teneur en silice est un trait fonctionnel. Cette déduction entraîne l'idée que l'abondance de certains morphotypes, stratégiques dans ce type d'études, pourrait augmenter en réponse à la sécheresse, surtout chez des espèces comme le sorgho où l'absorption de Si est très probablement régulée. Cependant, les causes et les effets du dépôt de silice dans les plantes restent à élucider. Un dernier résultat crucial de cette étude est que la méthode appliquée pour la culture des plantes expérimentales s'est avérée fiable et capable de recréer des conditions de terrain tout en contrôlant les variables physiologiques comme la transpiration. Ceci est extrêmement important pour produire du matériel avec le plus grand contrôle sur les variables de croissance et son utilisation standard facilitera la création d'ensembles de données entièrement comparables dans les études futures.

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Resumen de la tesis en español

Contexto

En la actualidad, las tierras áridas cubren más del 40% de la superficie de la Tierra y en ellas viven aproximadamente 2.300 millones de personas (International Institute for Environment and Development - https://www.iied.org consultado el 23 de abril de 2022). Sin embargo, las tierras áridas e hiperáridas siempre se han considerado regiones periféricas que suponen un reto, tanto ahora como en el pasado, para el establecimiento de asentamientos prósperos (Biagetti et al., 2022). Por el contrario, las investigaciones antropológicas y arqueológicas han demostrado una gran variabilidad de sistemas socioecológicos tradicionales que se encuentran en las tierras áridas, altamente resilientes y muy ricos en recursos (Balbo et al., 2016; Critchley et al., 1994; D'Odorico y Bhattachan, 2012). Un tema de interés central es la gestión del agua en las zonas áridas. En estas regiones se han adoptado diversas prácticas para recoger el agua proveniente de precipitaciones y acceder a aguas subterráneas, canalizar las inundaciones y aprovechar los pocos recursos hídricos disponibles (Prinz, 2002). La escasez de recursos hídricos ha hecho que el agua sea un tema clave en el estudio de las zonas más áridas del mundo (Cooper et al., 2008; Osman et al., 2021; Salmon et al., 2015), demostrando ser un bien de importancia central, especialmente para el desarrollo de prácticas agrícolas (Manning y Timpson, 2014; Marshall y Weissbrod, 2011). De hecho, la historia ha confirmado que estas zonas no solo han sido la cuna de la domesticación de diversos cultivos (Manning et al., 2011; Winchell et al., 2018), sino incluso de tecnologías específicas aptas para el desarrollo de sistemas agrícolas elaborados (Reynolds et al., 2007; Winslow et al., 2004). Sin embargo, el estudio del desarrollo de estas prácticas se complica por el hecho de que es difícil identificar *proxy* de disponibilidad de agua en el registro arqueológico y arqueobotánico.

Las regiones áridas están dominadas por biomas de sabana y pastizales compuestos principalmente por especies de C_4 que representan aproximadamente el 25% de la producción primaria de todo el planeta (Sage y Zhu, 2011). En cuanto a los mijos, el 95% de la producción mundial procede de Asia y África (Saleem et al., 2021). Varios de los principales cultivos de mijo, como el sorgo (*Sorghum bicolor* (L.) Moench) (Reddy, 2017a), cuya pertenencia a la categoría de mijo es objeto de debate, pero para mayor claridad en esta tesis se denominará como tal, el mijo proso (*Panicum miliaceum* L.)

(Gomashe, 2017), el mijo cola de zorra (*Setaria italica* (L.) Beauv.) (Hariprasanna, 2017), y el mijo perla (*Pennisetum glaucum* (L.) R.Br.) (Reddy, 2017b) se han introducido en todas las zonas más áridas del mundo. Entre los mijos cultivados a menor escala, pero que siguen siendo de gran interés, se encuentran el mijo de dedo (*Eleusine coracana* L. Gaertn.) y el mijo de corral indio (*Echinochloa frumentacea* Link) (Rudov et al., 2020). Con una pluviometría mínima y poco consumo de agua durante el cultivo, los campos de pequeños mijos suelen dar un rendimiento decente (Rudov et al., 2020). El sorgo, el mijo perla y el mijo de dedo, que son los cereales de interés en este estudio, han sido clasificados por la FAO entre los 150 principales cultivos producidos a nivel mundial (Organización de las Naciones Unidas para la Alimentación y la Agricultura, 2018) y, en las regiones donde las precipitaciones son imprevisibles, estos tres cultivos se consideran los más fiables (Weber y Kashyap, 2016).

Cuestiones científicas e hipótesis de trabajo

El objetivo principal de esta investigación es comprender si es posible, y en qué medida, reconstruir la disponibilidad de agua en un contexto arqueológico mediante la investigación de restos arqueobotánicos específicos, concretamente los fitolitos. Los fitolitos fueron elegidos tras una cuidadosa revisión de los posibles indicadores de agua directos e indirectos disponibles. Los indicadores indirectos incluyen todas aquellas estructuras artificiales (por ejemplo, pozos, canales, depósitos) que pueden encontrarse en el registro arqueológico y que indican, por su presencia, algún tipo de sistema de gestión del agua. Sin embargo, el estudio de estas estructuras artificiales presenta ciertas dificultades. En efecto, los campos, los camellones, las presas o los pozos la mayoría de las veces ya no son visibles en el registro arqueológico, en parte por su deterioro debido al tiempo o destruidos por los humanos, en parte porque habrían sido efímeros e ineficaces, especialmente en la prehistoria (Jenkins et al., 2020). Además, el mero hallazgo de instalaciones de riego o de almacenamiento de agua solo informan sobre gestión hídrica y no dan ninguna indicación de la cantidad de agua en uso. En consecuencia, el enfoque principal de mi investigación se ha dirigido a los restos arqueobotánicos, que pueden proporcionar una evaluación directa del riego. La mayor parte de las investigaciones arqueobotánicas hasta la fecha, que evalúan la disponibilidad de agua en los cultivos, se centran en las especies de C_3 porque se consideran los cultivos fundacionales de la agricultura moderna en algunas de las zonas más estudiadas del mundo. Hay que decir, en este sentido, que el estudio de la disponibilidad de agua a partir de *proxies* arqueobotánicos está aún en sus inicios y son pocas las zonas que se han investigado, principalmente en torno al Creciente Fértil y América Central/Norte. Para las gramíneas C_3, el estudio de la composición isotópica de las semillas ha demostrado ser efectivo para indicar la disponibilidad de agua (Araus et al., 1997; Ferrio et al., 2005; Nitsch et al., 2015). La discriminación isotópica describe la asimilación preferente de los isótopos más ligeros sobre los más pesados durante el proceso de fotosíntesis, y refleja principalmente la disponibilidad de agua (Eggels, 2021). Por lo tanto, la gestión del agua puede inferirse integrando los datos isotópicos con los indicadores paleoclimáticos para el período de tiempo en cuestión (Flohr et al., 2019). Si la señal isotópica de los cultivos indica una mayor disponibilidad de agua que la esperada según los *proxies* climáticos, es probable que los cultivos recibieran agua de otras fuentes además de las precipitaciones, como por ejemplo a través de la irrigación, el riego artificial o mediante el cultivo en abanicos aluviales (Ferrio et al., 2005). Sin embargo, las zonas áridas se cultivan principalmente con plantas C_4, cuyo sistema fotosintético es mucho más complejo que el C_3, tanto anatómica como fisiológicamente. De hecho, en las plantas C_4 hay una fuga de Carbono fuera de las células de la vaina del haz (donde se fija la mayor parte del Carbono) durante la fotosíntesis, lo que se considera que influye en la discriminación del mismo (ΔC_4) (Farquhar, 1983). Aunque investigaciones más recientes han demostrado que la ΔC_4 responde a la sequía y tiene una relación directa con la eficiencia hídrica (Buchmann et al., 1996; Cernusak et al., 2013), la naturaleza exacta de esta relación aún debe ser aclarada. Además, el estudio de las cariópsides de C_4 procedentes de registros arqueológicos se complica aún más por cuestiones relacionadas con su morfología. Las cariópsides de la mayoría de los mijos pequeños (por ejemplo, el mijo de dedo o el mijo perla) son de tamaño muy reducido. Además, hay que precisar que los restos orgánicos como las semillas se conservan en el registro arqueológico como resultado de la carbonización o del anegamiento (Araus et al., 1997; Yang et al., 2011). Los fenómenos tafonómicos y de carbonización de las semillas de pequeñas dimensiones complican considerablemente su preservación y reconocimiento en el registro arqueológico. Por ello, los fitolitos representan un buen sustituto de las semillas, ya que en las zonas áridas este tipo de restos suele estar mal conservado (Fuller, 2002).

Los fitolitos, al ser de naturaleza inorgánica, se conservan mejor que las semillas y su conservación se ve mucho menos afectada por los problemas previos y posteriores a su depósito (Piperno, 2006). Sin embargo, la capacidad de las plantas de C_4 para ahorrar agua mediante la reducción de la tasa de transpiración podría representar un reto en el uso de los fitolitos como indicador de la disponibilidad de agua. Esta investigación se centra en tres especies en particular: el mijo de dedo (*Eleusine coracana* (L.) Gaertn.), el mijo perla (*Pennisetum glaucum* (L.) R.Br.) y el sorgo (*Sorghum bicolor* (L.) Moench). Según el International Crop Research Institute for the Semi-Arid Tropics (ICRISAT), las tres especies se encuentran entre los mijos más cultivados en el este y sur de África, y en el sur de Asia. El mijo de dedo, el mijo perla y el sorgo son extremadamente resistentes a la sequía, germinan incluso con humedad residual y son un alimento básico para más de 500 millones de personas en el mundo. Estas tres especies han sido seleccionadas (a) por sus vías bioquímicas de C_4: NADP-ME para el sorgo y NAD-ME para el mijo de dedo y el mijo perla. Su diversidad anatómica y bioquímica permite la evaluación completa de las eventuales diferencias entre las especies de C_4. (b) En términos de silicificación, ya han sido estudiadas y clasificadas como acumuladoras de biosílice (Coskun et al., 2021; Kumar et al., 2017; Lux et al., 2002; Ma y Yamaji, 2015; Out y Madella, 2017; Sangster y Parry, 1976). (c) El estudio agronómico de estas tres especies ha sido ampliamente profundizado por investigaciones sobre su tasa de transpiración (por ejemplo, Vadez et al., 2011). Además, (d) las tres especies se han encontrado en varios conjuntos arqueobotánicos de sitios áridos de interés tanto en Asia como en África (por ejemplo, el sorgo en Al Khiday y Mezber por D'Andrea et al. (2015) y el mijo de dedo en Harappa por Weber y Kashyap (2016)).

En esta investigación se han probado modelos de fitolitos basados en la abundancia relativa de morfotipos específicos como *proxies* de la disponibilidad de agua junto con la concentración de acumulación de biosílice en los órganos de transpiración, el tamaño de los esqueletos de sílice, el contenido isotópico (δ^{30}Si, δ^{29}Si) y la dimensión de algunos morfotipos sensibles. El estudio de los fitolitos como indicadores de agua ya ha sido explorado y no es un tema totalmente nuevo (Bremond et al., 2005; Jenkins et al., 2016, 2020; Madella et al., 2009; Miller Rosen, 1994; Weisskopf et al., 2015). Madella et al. (2009), Weisskopf et al. (2015) y Jenkins et al. (2016; 2020) sugirieron el uso de la proporción de morfotipos fijos (genéticos) frente a los sensibles (ambientales) para detectar la disponibilidad de agua directamente a partir de los conjuntos de fitolitos. Ellos plantearon la hipótesis de que, en contextos de abundancia de agua, los morfotipos sensibles se producen en mayor cantidad que en condiciones de estrés hídrico y, por lo tanto, que la proporción entre las dos clases cambia al variar la disponibilidad de agua.

Más recientemente, se ha descubierto que las señales isotópicas estables de silicio y oxígeno en los fitolitos también tienen potencial como indicadores de las condiciones ambientales. En concreto, se ha demostrado que el $\delta^{30}Si$ en los fitolitos es un indicador de las condiciones del suelo, especialmente de la meteorización y del contenido de materia orgánica (Leng et al., 2009). Estos estudios se basan en la suposición de que, en los tejidos con actividad fotosintética, el silicio se polimeriza principalmente como resultado de la sobresaturación (Schaller et al., 2013). Así, los fitolitos, que son el efecto fenotípico de la precipitación de silicio en la epidermis, se consideran un *proxy* potencial de la disponibilidad de agua. Sin embargo, el papel y la función del Si en las plantas aún no se comprenden del todo y esto dificulta la comprensión de los mecanismos que impulsan la formación de fitolitos. De hecho, recientemente Kumar et al. (2017) y Hodson (2019) han demostrado que la clasificación de los fitolitos es más compleja de lo que se pensaba y que las características de cada morfotipo no dependen solo del mecanismo genético/ambiental de deposición, sino que su formación también se ve alterada por el espacio celular en el que comenzó a precipitar la sílice. Por lo tanto, el estudio de los fitolitos de C_4 en relación con la disponibilidad de agua sigue siendo un tema de investigación abierto. Dicho estudio, además de responder a una cuestión arqueológica, tiene también importantes implicaciones para profundizar en el papel de la deposición de sílice en especies consideradas como altas acumuladoras. El enfoque experimental que caracteriza esta investigación se ha construido a partir de los estudios anteriores, abordando la necesidad de realizar más trabajos experimentales para aprovechar todas las capacidades de los estudios de fitolitos.

Objetivos

Esta investigación de tesis se desarrolló en el marco del proyecto ERC Starting Grant RAINDROPS (ERC-StG nº 759800). El proyecto combina la investigación sobre la fisiología de las plantas con aplicaciones arqueobotánicas y el registro de TEK (del inglés, *traditional ecological knowledge*) sobre los sistemas de cultivo en las zonas áridas para ofrecer una metodología innovadora y fiable para comprender las prácticas de gestión del agua en el pasado en las regiones áridas.

Dentro del ámbito más amplio del proyecto, el objetivo de esta tesis es producir datos detallados y exhaustivos sobre la producción de fitolitos en respuesta a la sequía de tres especies de C_4 y proporcionar herramientas fiables para investigar las prácticas de cultivo de agua del pasado por comparación con réplicas modernas.

Los objetivos de esta investigación son (1) estudiar los procesos de acumulación de biosílice en el sorgo, el mijo perla y el mijo de dedo, con el fin de comprender si los fitolitos producidos por los cultivos de C_4 pueden ser utilizados como *proxies* de la disponibilidad de agua. (2) Investigar la variabilidad intra- e inter- muestra en la producción de fitolitos. (3) Elaborar un modelo para aplicar los resultados obtenidos en los cultivos experimentales a los conjuntos de fitolitos arqueológicos.

Para alcanzar los objetivos propuestos se han llevado a cabo dos cultivos experimentales (en 2019 y 2020) en ICRISAT (India) de las tres especies seleccionadas. Las plantaciones se han establecido bajo condiciones constantemente controladas de agua, temperatura, humedad, evapotranspiración y composición del suelo durante el periodo del año desprovisto de lluvias (enero-mayo), en lisímetros protegidos de precipitaciones. Los lisímetros proporcionan condiciones similares a las de los campos, pero permiten el control experimental de la transpiración, la imposición de estrés hídrico y los parámetros fisiológicos. Se han realizado experimentos para obtener datos de referencia que permitan evaluar la producción de fitolitos en relación tanto con el riego como con la variación genotípica. Para garantizar la significación estadística y el control de los mecanismos genéticos, se han realizado experimentos con 10 variedades locales de cada especie, con 5 repeticiones en 2 cultivos replicados. Para cada cultivo, se han seleccionado 5 variedades locales y se han analizado 3 repeticiones de cada tratamiento para la acumulación de biosílice en diferentes tejidos. Se ha recogido la producción de fitolitos (concentración gramo/material vegetal seco); el conjunto de morfotipos (se han contado más de 250 fitolitos por cada muestra analizada); las proporciones entre las formas controladas por el medio ambiente y las genéticas; el tamaño de esqueletos de sílice y las dimensiones específicas (por ejemplo, longitud, anchura, longitud de los picos) para las formas de *stomata*, *bulliforms* y *elongates*. Además, también se ha analizado el δ^{30}Si de los fitolitos de las hojas y de las cascarillas mediante técnicas de espectrometría de masas de plasma acoplado inductivamente por ablación láser de *femtosecond*.

Principales resultados y discusión

En primer lugar, los resultados experimentales permiten concluir que, a pesar de la proximidad taxonómica, las tres especies poseen diferencias sustanciales en los mecanismos de acumulación de Si. Ma y Yamaji (2006) sugirieron que la cantidad de absorción de sílice, atribuible a la genética, depende de dos factores principales que concurren a la carga del xilema: la presencia/ausencia de canales para transportar Si desde las células corticales al xilema (Lsi1-Lsi2-Lsi6 y ortólogos), y la densidad con la que se producen. La combinación de estos dos factores determina la clasificación en acumuladores de sílice activos, pasivos o rechazantes (no acumuladores). Aunque todas las gramíneas se consideran altas acumuladoras (Hodson et al., 2005), su capacidad de acumular Si a través de transportadores específicos sigue sin verificarse para muchas especies. En *Sorghum bicolor* ya se realizaron algunas investigaciones y la especie ha sido analizada para la secuencia de la proteína Lsi1 por Vatansever et al. (2017), quienes descubrieron dos genes homólogos que codifican los canales del transportador. Sin embargo, también se han descubierto recientemente mutaciones por parte de Markovich et al. (2019) que demostraron la presencia de variedades incapaces de absorber altas cantidades de sílice. Slp1, una proteína de composición de aminoácidos única que participa en la precipitación de sílice en las células silicificantes, también se ha localizado en el sorgo (Kumar et al., 2020, 2017a, 2017b). En este estudio, el sorgo no solo acumuló una gran cantidad de Si en comparación con las otras dos especies analizadas, sino que la tasa de acumulación resultó ser independiente de factores ambientales como la transpiración. Por lo tanto, este resultado está en línea con la presencia de un mecanismo en el sorgo que transporta el ácido silícico activamente hasta las hojas, independientemente de la transpiración (absorción de Si gobernada por el transportador), lo que hace de esta especie una gran acumuladora de sílice. En el caso del mijo perla y el mijo de dedo, la información parece más compleja de interpretar. Aunque la cantidad de Si acumulado también sitúa a las dos especies en el grupo de grandes acumuladores (Hodson et al., 2005), también es cierto que la producción relativa (g de Si/g de materia seca) en comparación con el sorgo es menor. Basándose en lo propuesto por Ma y Yamaji (2006), podría especularse que la densidad de los transportadores que facilitan la captación de Si es menor (aunque existente) en el mijo perla y el mijo de dedo, y que estos mijos dependen en gran medida del transporte pasivo a lo largo de la corriente de transpiración, aunque la dinámica del mecanismo está por aclarar. Desgraciadamente, no se puede corroborar con datos genéticos sobre la captación de Si porque hasta ahora no se dispone de información sobre los *loci* correspondientes en *Pennisetum glaucum* y *Eleusine coracana*. Sin embargo, la acumulación de biosílice en el mijo de dedo y el mijo perla parece depender más de los factores ambientales (transpiración), por lo que se puede proponer que sean más adecuados que el sorgo para investigar su respuesta a la disponibilidad de agua.

Los resultados de los conjuntos de fitolitos experimentales indican que muchos de los valores de concentración de morfotipos para las tres especies pueden predecirse mediante el tratamiento del agua, lo que demuestra que los conjuntos de fitolitos pueden utilizarse como indicadores de la disponibilidad de agua. Además, los análisis isotópicos preliminares en fitolitos individuales de hojas de sorgo destacaron que los *bulliforms* y los elongados pueden discriminar el tratamiento del agua sobre la base de la composición de δ^{30}Si y δ^{29}Si. La diferencia en la discriminación del isótopo más ligero ^{28}Si y de las dos partes más pesadas ^{30}Si y ^{29}Si, entre los dos tratamientos, está limitada por el 0,5‰ y el 1‰ para ambos morfotipos, y parece tener una relación negativa significativa con el agua transpirada. *Los bulliforms* también aumentan su tamaño (anchura + longitud + longitud del pico) con el aumento de los niveles de transpiración, confirmando ser un morfotipo particularmente eficiente para evaluar la disponibilidad de agua, ya que esto se refleja en la composición química de los *bulliforms*, la concentración en el conjunto y el tamaño medio del morfotipo. Hasta ahora, con esto estudio, se ha demostrado que solo los *bulliforms* cambian de tamaño en función del riego. En consecuencia, parece adecuado asumir que, en general, al cambiar el suministro de sílice, cambia la concentración de fitolitos depositados, pero no sus dimensiones (Schulz-Kornas et al., 2017). En este sentido, se ha observado en las muestras analizadas para este estudio que algunos morfotipos aumentan su concentración en condiciones WW (alta disponibilidad de agua) mientras que otros en condiciones WS (baja disponibilidad de agua), y que esto depende de la especie, pero también del tejido donde se forman los morfotipos. Esta peculiaridad pone de manifiesto que para estimar la disponibilidad de agua debe observarse el conjunto completo de morfotipos y no un grupo reducido, para que cada concentración de morfotipos, aumente o no como consecuencia del riego, sea tenida en cuenta.

No todos los morfotipos resultaron ser igualmente eficaces para predecir el riego. La regresión por pasos aplicada para seleccionar los morfotipos más predictivos en la identificación del tratamiento del agua indican como predictivos los *acute bulbosus*, los *bulliformes*, los *elongates clávate*, *dentate*, *entire* y *sinuate*, los *crosses*, los *polylobates*, los *rondels* y *saddles*, y excluyen los morfotipos restantes. Cabe destacar que las formas con mayor grado de significación son las células cortas, que normalmente son consideradas como morfotipos fijos, es decir, morfotipos para los que la silicificación está controlada por factores genéticos y que en estudios anteriores no mostraron cambios de concentración respecto a la disponibilidad de agua (Ermish y Boomgarden, 2022; Jenkins et al., 2016, 2020; Madella et al., 2009). Teniendo en cuenta los resultados del presente estudio, es posible concluir que la concentración (por g de hoja seca en millones) de algunos morfotipos está influenciada por la disponibilidad de agua, pero que no todos ellos (dependiendo de la especie) aumentan su concentración cuando aumenta la tasa de transpiración. Esto lleva a la conclusión de que, dado que los mecanismos de deposición de fitolitos son complejos (y en cierto modo todavía se escapan a nuestra comprensión), para elaborar un modelo predictivo basado en la composición de conjuntos de fitolitos procedentes de muchas especies diferentes, es necesario tener en cuenta todo el grupo de morfotipos, de todos los tejidos, de modo que se registren variaciones positivas o negativas a la disponibilidad de agua en cada uno de ellos. Como consecuencia, deben replantearse las categorías fijas y sensibles, considerando que mientras la concentración de algunos morfotipos aumenta con el riego, otros pueden ser estimulados por las condiciones de estrés hídrico.

Las especies y los genotipos predicen la cantidad de sílice acumulada mejor que el tratamiento de agua solo. Sin embargo, cabe mencionar que la mayor varianza en la acumulación de biosílice se explica por el efecto interactivo de los genotipos y el riego, lo que indica/muestra que ambas variables son significativas. En este sentido, se puede especular que la demanda de biosílice depende de alguna manera de las condiciones fisiológicas relacionadas con la disponibilidad de agua, que a su vez dependen de la eficiencia de la especie/genotipo para responder a la disponibilidad de agua. En consecuencia, también se puede hipotetizar que algún morfotipo específico, posiblemente más eficiente que otros en la realización de funciones estructurales o bioquímicas, podría aumentar en respuesta a la sequía o porque son estimulados por otros eventos ambientales (estresores abióticos o bióticos) con un fuerte impacto en la fisiología de la propia planta. La hipótesis se apoya en este estudio, ya que los morfotipos cuya producción aumenta en las réplicas de sorgo WS (*acute bulbosus*, *crosses*, *elongates clavate* y *entire* y *stomata* en las hojas y *elonagates sinuate* y *polylobates* en la inflorescencia) podrían desempeñar un papel estructural clave en respuesta al estrés hídrico. De hecho, la silicificación de los *stomata* podría ayudar a reducir la transpiración regulando la pérdida de agua (Cooke y Leishman, 2016; Gao et al., 2020; Goto et al., 2003; Hosseini et al., 2017; Wang et al., 2021). Las *crosses*, según Kumar et al. (2017a), podrían depositarse al lado de las venas menores y endurecerlas, y permitir que el flujo de la xilema continúe (Kaufman et al., 1985). Los *elongates*, dispuestos en la superficie epitelial, aumentarían la incepción de la luz (Cooke y Leishman, 2016) y endurecerían el tejido epidérmico (particularmente frágil en condiciones de WS) y simultáneamente prevendrían tanto el ataque de patógenos como un mayor arrugamiento de la hoja (Meunier et al., 2017; Rodrigues et al., 2003; Yoshida et al., 1962). Los *trichomes*, que han demostrado responder al ataque de patógenos (Daoud et al., 2018; Fauteux et al., 2005; Mateos-Naranjo et al., 2013; Oliva et al., 2021), también han demostrado ser una respuesta consistente al ambiente xérico (Olsen et al., 2013), y posiblemente ayudan a mantener una película de humedad alrededor de la hoja o panícula en desarrollo.

Hasta ahora se ha discutido cómo el efecto taxonómico tiene un fuerte impacto en la acumulación de biosílice y en la producción de fitolitos, que a su vez responden a factores ambientales como el riego, aumentando posiblemente la capacidad de respuesta a estreses tanto bióticos como abióticos del exterior. En consecuencia, la genética y el medio ambiente parecen no ser dos factores completamente ajenos e independientes en la acumulación de Si. Katz (2019) sugirió, desde la ecología evolutiva, que si el contenido de sílice es persistente en el reino vegetal desde las embriofitas en adelante (como demostraron Coskun et al., 2019), tiene que conferir algunas ventajas adaptativas. Los resultados obtenidos en este trabajo parecen apoyar esta misma deducción, implicando en cierta medida que el contenido de Si en las plantas podría representar un rasgo funcional. Los rasgos funcionales se definen como características morfológicas, bioquímicas, fisiológicas, estructurales, fenológicas o de comportamiento que se expresan en fenotipos de organismos individuales, relevantes para la respuesta de dichos organismos al medio ambiente y/o que afectan a las propiedades del ecosistema (Díaz et al., 2013). La hipótesis es alentada por diferentes estudios que indican que la acumulación de biosílice podría estar dispuesta en respuesta a la demanda interna de la planta (por ejemplo, Hosseini et al., 2017; Li et al., 2020; Oliveira et al., 2022) mediante la regulación de la expresión de los genes Lsi1-Lsi2-Lsi6 u ortólogos (por ejemplo, Mitani et al., 2011; Sun et al., 2017; Vatansever et al., 2017; Yamaji et al., 2008). Aunque también se ha establecido que la deposición de biosílice en las plantas reacciona a los cambios ambientales (por ejemplo, Daoud et al., 2018; Hartley et al., 2015; Mandlik et al., 2020; Schaller et al., 2013), depende del entorno (por ejemplo, la composición del suelo) y afecta simultáneamente al círculo de Si en un ecosistema (por ejemplo, Cornelis y Delvaux, 2016; de Tombeur et al., 2020; Grasic et al., 2020; Keller et al., 2021).

Durante este proyecto, he podido aplicar el modelo desarrollado a partir de fitolitos modernos en muestras procedentes de 4 yacimientos arqueológicos repartidos entre el sureste de Pakistán y el noroeste de la India, todos ellos pertenecientes a la Civilización del Valle del Indo. Los estratos comparados con el conjunto de datos modernos están datados en el periodo Harappa Maduro (2500-1900 a.C.) y proceden de cuatro asentamientos arqueológicos diferentes: Harappa, Kanmer, Shikarpur y Alamgirpur. La utilización de muestras de fitolitos de mijo procedentes de estratos del periodo Harappa Maduro es interesante porque, hasta la fecha, existe un debate abierto sobre el consumo y la difusión de estos cultivos en toda la civilización del Indo. Durante el periodo Harappa Maduro, los mijos pequeños representaban entre el 3 y más del 50% de los cereales recuperados (Pokharia et al., 2014). Sin embargo, su uso es difícil de justificar, ya que el procesamiento de su grano requiere mucha mano de obra (Arunachalam et al., 2005), aparte de que su productividad es menor en comparación con la de los cereales C_3 (como la cebada y el trigo) (Pearcy y Ehleringer, 1984). Suponemos que una posible explicación podría estar directamente relacionada con la disponibilidad y gestión del agua, y por ello decidí aplicar el modelo desarrollado para dar una posible interpretación al fenómeno en estas zonas. Adicionalmente, otra cuestión importante que llevó a la aplicación del modelo es la escasa evidencia arqueológica de disponibilidad de agua en la zona indicada (Petrie et al., 2017).

En primer lugar, el modelo se aplicó como comparación con los registros arqueológicos que ya habían demostrado la existencia de las especies consideradas por medio también de *proxies* independientes de los fitolitos (por ejemplo, cariopsis). En cada uno de los asentamientos seleccionados se ha señalado la presencia de especies de C_4 domesticadas (Harappa: *Panicum* L. sp. Pl.; Kanmer: *Pennisetum galucum* y *Setaria* sp. P. Beauv.; Shikarpur: *Brachiaria ramosa* L. Stapf., *Coix lacryma-jobi* L., *Digitaria* sp. Haller, *Eleusine coracana*, *Setaria* sp. P. Beauv.; Alamgirpur: pequeños mijos en general) (Bates 2019; Bates et al., 2021). En segundo lugar, también se atestiguó la presencia de C_3 de trigo y cebada (Bates y Petrie, 2016). Debido a las características agronómicas de los cultivos C_3, estos cereales, además de necesitar una buena cantidad de agua para crecer (mucho más en comparación con el C_4), se cultivaban muy probablemente durante el periodo invernal (especies que necesitan temperaturas bajas) (Kalra et al., 2008) fuera de la estación de lluviosa. Por lo tanto, la presencia de cereales C_3 indica que los asentamientos disponían de agua para la agricultura, independientemente de que ésta procediera de sistemas de riego más o menos elaborados o de la agricultura fluvial.

Una vez aplicado el modelo desarrollado sobre fitolitos modernos al registro arqueológico, los resultados indican que la mayoría de los cereales crecieron en condiciones de escasez de agua. Todos los yacimientos analizados proporcionaron resultados similares, a pesar de que las condiciones hídricas y climáticas son algo diferentes entre ellos. Harappa y Alamgirpur estaban situados cerca de cursos de agua, con extensos *doabs* (extensiones de tierras aluviales entre dos ríos posiblemente convergentes) que probablemente podrían soportar sistemas de riego ventajosos para la agricultura (Kenoyer, 1998). Por el contrario, Kanmer y Shikarpur se encontraban posiblemente en zonas más secas, sin acceso a importantes llanuras aluviales para explotarlas con fines agrícolas, pero/o servidas por *nullah* (cursos de agua) de pequeñas dimensiones (Lancelotti, 2010) o, como en el caso de Kanmer, donde se han observado tanques artificiales (Madella y Lancelotti 2022).

El resultado obtenido tras aplicar el modelo predictivo permite excluir la posibilidad de los policultivos (es decir, el cultivo de dos o más cultivos juntos en la misma tierra) en el mismo espacio agrícola. De hecho, según el resultado obtenido del modelo, hay aproximadamente un 60% de posibilidades de que los cereales C_4 se cultiven con menos de 300 mm de agua disponible. Esta cantidad de agua no permitiría el crecimiento ni de los cereales C_3 ni de las leguminosas, y me hace abogar por opciones alternativas al policultivo.

En zonas con amplia disponibilidad de agua (por ejemplo, Harappa y Alamgirpur) podría haber un sistema de doble cultivo (*rabi* o invierno y *kharif* o monzón) con cereales C_3 (por ejemplo, trigo y cebada o arroz) y legumbres cultivadas en régimen de agua en invierno mientras que otras especies más resistentes a la sequía, como las C_4, podría haber sido empleadas durante el periodo cálido y menos lluvioso (Petrie et al., 2016). Por lo tanto, es plausible sugerir que durante la estación fría se cultivaban cereales C_3 en régimen de agua constante, en la estación más calurosa, antes de la llegada de las lluvias monzónicas, se plantaban cereales C_4 (que además son de muy rápido crecimiento), no desperdiciando la tierra disponible. Esto se ve respaldado por los datos etnográficos de la zona, donde los cultivos de mijo son en su mayoría cultivos de verano de secano (Fuller 2006; Giosan et al., 2012). Esta estrategia también garantizaría la posibilidad de que las poblaciones seminómadas pudieran abandonar el campo de mijo una vez sembrado y regresar solo en el momento de la cosecha, dedicando la temporada al pastoreo (García-Granero et al., 2016). La opción del doble cultivo (*rabi* y *kharif*) es, sin embargo, ampliamente debatida y varios investigadores sostienen que las evidencias arqueológicas encontradas hasta la fecha no pueden asegurar que existiera un sistema de doble cultivo (véase Petrie et al., 2017). Para verificar la existencia de un verdadero sistema de doble cultivo podría ser interesante desarrollar un "modelo gemelo" basado en la concentración de los fitolitos de C_3 y aplicarlo a los conjuntos arqueológicos recuperados en las zonas. Si se descubriera que las especies de C_3 también crecen en condiciones de escasez de agua, entonces, tal vez, se podría descartar la opción. Aunque el resultado de la aplicación de este modelo no excluye la posibilidad de un sistema de doble cultivo, también es cierto que hay más opciones para justificar la presencia de especies C_4 cultivadas en condiciones de escasez de agua. Otra estrategia posible, incluiría el cultivo de cereales C_4 junto a C_3 durante todo el año, pero en tierras donde el riego/inundación/humedad residual de los suelos (por la distancia a fuentes de agua como los ríos) era difícil y por tanto casi inexistente. Esto justificaría que las plantas identificadas en las muestras arqueológicas hubieran crecido con cierta disponibilidad de agua (quizás por las lluvias) pero no abundante.

Los resultados del modelo tampoco descartan la hipótesis de que la zona comprendida entre el sur de Pakistán y el noroeste de la India haya experimentado algún tipo de desecación y que, por lo tanto, los cultivos se hayan adaptado a períodos secos más constantes en el tiempo, como sugieren Wright et al. (2008). Los autores propusieron que, tras un milenio de agricultura fluvial basada en las inundaciones de las riberas, en torno al 2800-2500 a.C., los ríos dejaron de aportar la habitual cantidad de agua y obligaron a los agricultores a basar su sistema agrícola en las precipitaciones. El mismo estudio sugiere que hacia el año 2000 a.C. la reducción de las precipitaciones podría llevar a un pico medio mínimo de 240 mm. Toda esta información coincide con los resultados obtenidos en este estudio, que plantea la posible presencia de cereales C_4 cultivados en condiciones de estrés hídrico con menos de 300 mm de agua disponible. Tanto si la adopción del mijo se debió a una cuestión de aridificación progresiva, como si hubo un cultivo oportunista de C_4 para aprovechar las zonas más secas o que plantas C_4 crecieron de forma natural en las parcelas áridas de los sitios, lo que se desprende de estos resultados es que muy probablemente las plantas C_4 de los contextos arqueológicos analizados crecieron en condiciones de escasez de agua.

Conclusiones

Las regiones áridas han sido centrales en la investigación arqueológica entre otras razones porque se consideran la cuna del sistema social y agrícola del Viejo Mundo. El control/La gestión del agua en las zonas áridas permitió la consolidación y difusión de estilos de vida agrícolas que iniciarion un crecimiento demográfico exponencial (Manning et al., 2011; Manning y Timpson, 2014). Las regiones áridas están dominadas por biomas de sabana y pastizales compuestos principalmente por especies de C_4. El estudio de los cultivos de mijo C_4, cuya historia de dispersión en África, Asia y Europa aún no ha sido desentrañada (Motuzaite-Matuzeviciute et al., 2016; Stevens et al., 2021; Winchell et al., 2018), tiene un papel clave en el debate arqueológico sobre los cambios de uso de la tierra en dichas regiones. Las cuestiones arqueológicas que están detrás del estudio de los restos de mijo no sólo están relacionadas con la dinámica de consumo de las especies implicadas, sino también con el tipo de gestión de dichas especies que se requiere (Briggs et al., 2006; Fuller, 2011; Stephens et al., 2019). La gestión de los cultivos de C_4 está estrechamente relacionada con la disponibilidad de agua, ya que estas plantas son notorias por su capacidad de soportar condiciones extremas, incluyendo altas temperaturas y escasez de agua (Sage y Zhu, 2011; Yang et al., 2021). Por esta misma razón, los cultivos de C_4 se consideran "productores" de *proxies* de agua difíciles, ya que se cree que son menos sensibles a los cambios en las precipitaciones o en la temperatura (Ghannoum et al., 2002). La capacidad de las especies C_4 de mantener baja la tendencia de transpiración constituye un verdadero problema cuando el interés se centra en observar qué efectos tuvo la fluctuación de las variables climáticas sobre la tasa isotópica, los fitolitos o el rendimiento de las plantas.

Esta tesis se centró en el estudio de los conjuntos de morfotipos producidos por el mijo de dedo, el mijo perla y el sorgo para observar la eficacia de los fitolitos como indicadores ambientales. Mediante el estudio de la acumulación de biosílice en estas tres especies diferentes de C_4, sometidas a distintos regímenes hídricos, se ha demostrado que los fitolitos pueden ser buenos indicadores de la disponibilidad de agua, añadiendo nuevas especies a las que ya se habían probado con el mismo fin (Ermish y Boomgarden, 2022; Jenkins et al., 2016, 2020; Madella et al., 2009; Weisskopf et al., 2015). A través de la comparación del conjunto de datos con conjuntos de fitolitos recuperados en sitios arqueológicos específicos, se han sugerido hipótesis sobre la gestión de las especies de C_4 en el pasado, lo que pone de manifiesto el potencial de los resultados obtenidos aplicados a contextos arqueológicos.

Los resultados obtenidos indican que la deposición de fitolitos depende, al menos en cierta medida, de factores ambientales. La disponibilidad de agua influye en la deposición de fitolitos en el mijo de dedo, el mijo perla y el sorgo al afectar a las concentraciones y composiciones de los conjuntos de morfotipos. Estas diferencias, sin embargo, dependen de la especie y de los tejidos donde se forman los fitolitos, y son más evidentes en el mijo de dedo y el mijo perla que en el sorgo. La disponibilidad de agua juega un papel fundamental en la determinación de la acumulación de biosílice en el mijo de dedo y el mijo perla, que los resultados demonstrar ser más afines a los acumuladores pasivos. Por el contrario, el sorgo parece caracterizarse por un mecanismo gobernado por la genética (los transportadores Lsi1-Lsi2-Lsi6) que también determinan una gran variabilidad entre genotipos. Aunque el variado conjunto de genotipos autóctonos mostró diferentes tendencias en la producción de fitolitos, el conjunto de datos completo permite hacer predicciones sobre la disponibilidad de agua. En relación con esta consideración, es evidente que la genética relacionada con la absorción de Si debería ser mejor examinada en el futuro, a la luz de las variaciones en la acumulación de biosílice entre especies, variedades locales y tejidos de deposición, descubiertas durante este estudio. También debería revisarse el concepto de morfotipos sensibles y fijos. Los resultados presentados aquí demostraron que los *stomata* difícilmente pueden pertenecer a la categoría sensible, mientras que los *bulliforms* y los *trichomes*, que no se han tenido en cuenta hasta ahora, sí lo son. Una información clave que se desprende de este estudio es que es difícil separar los morfotipos cuya deposición está controlada por la genética de aquellos cuya deposición está provocada por la disponibilidad de agua, ya que ambos dependen muy posiblemente de una regulación dependiente del medio ambiente.

Todos los resultados presentados en esta tesis parecen indicar que el contenido de sílice es un rasgo funcional. Esta deducción conlleva la idea de que la abundancia de ciertos morfotipos, estratégicos en este tipo de estudios, podría aumentar en respuesta a la sequía, especialmente en especies como el sorgo donde la absorción de Si está muy probablemente regulada genéticamente. El método aplicado para el cultivo de las plantas experimentales ha demostrado ser fiable y capaz de recrear condiciones similares a las del campo, teniendo bajo control las variables fisiológicas como la transpiración. Esto es extremadamente importante para producir material con mayor control sobre las variables de crecimiento y su uso estandarizado facilitaría la creación de conjuntos de datos totalmente comparables en futuros estudios.

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List of abbreviations

2D: two-dimensional
3D: three-dimensional
AI: aridity index
AIC: akaike information criterion
Ala: alanine
AlaAT: alanine transaminase
ANOVA: analysis of variance
Asp: aspartate
AspAT: aspartate transaminase
ATP: adenosine triphosphate molecules
BCE: before common era
BP: before present
CAPEPC: carbonic anhydrase phosphoenolpyruvate carboxylase
CCA: canonical correspondence analysis
CK: carboxykinase
ERC: European research council
ET0: Evapotranspiration
FAO: food and agriculture organisation
FM: finger millet
FM1: IE2511 genotype
FM10: IE2822 genotype
FM2: IE3476 genotype
FM3: IE5335 genotype
FM4: IE6717 genotype
FM5: IE7509 genotype
FM6: IE4450 genotype
FM7: IE4456 genotype
FM8: IE4458 genotype
FM9: IE3193 genotype
fsLA-MC-ICP-MS: femtosecond laser ablation multi-collector inductively coupled plasma-mass spectrometry
GFZ: GeoForschungsZentrum
GLM: generalised linear models

List of abbreviations

HI: harvest index
HSD: honest significant difference
ICPN: international code for phytoliths nomenclature
ICPT: international committee for phytolith taxonomy
ICRISAT: International Crops Research Institute for the Semi-Arid Tropics
IPS: inner pericranial surface
IRMS: gas isotope ratio mass spectrometer
Lsi1: low silicon channel-type 1
Lsi2: low silicon channel-type 2
Lsi6: low silicon channel-type 6
Mal: malate
MC-ICP-MS: multi-collector inductively coupled plasma-mass spectrometry
ME: malic enzyme
NAD-MDH: nicotinamide adenine dinucleotide-dependent malate dehydrogenase
NAD-ME: nicotinamide adenine dinucleotide-dependent malic enzyme
NAD: nicotinamide adenine dinucleotide
NADP-MDH: nicotinamide adenine dinucleotide phosphate - dependent malate dehydrogenase
NADP-ME: nicotinamide adenine dinucleotide phosphate -dependent malic enzyme
NADP: nicotinamide adenine dinucleotide phosphate
OAA: oxaloacetate
PEP-CK: phosphoenolpyruvate carboxykinase
PEP: phosphoenolpyruvate
PEPC: phosphoenolpyruvate carboxylase
PM: pearl millet
PM1:IP13327 genotype
PM10: IP18022 genotype
PM2: IP9859 genotype
PM3: IP9888 genotype
PM4: IP13329 genotype
PM5: IP2367 genotype
PM6: IP18119 genotype
PM7: IP18019 genotype
PM8: IP18020 genotype
PM9: IP18021 genotype

PMMA: poly-methyl methacrylate

PPDK: pyruvate dehydrogenase kinase

PPNA: pre-pottery neolithic A

PVC: polivinilcloruro

Pyr: pyruvic acid

RDA: redundancy analysis

RH: relative humidity

Rubisco: ribulose-1,5-bisphosphate carboxylase/oxygenase

S: sorghum

S1: IS23074 genotype

S10: IS35217 genotype

S2: IS23075 genotype

S3: IS3450 genotype

S4: IS11060 genotype

S5: IS11061 genotype

S6: IS38025 genotype

S7: IS17415 genotype

S8: IS35215 genotype

S9: IS35216 genotype

SD: standard deviation

TE: transpiration efficiency

TEK: traditional ecological knowledge

TWT: total water transpired

TWU: total water used

UNEP: united nations environment program

UV: ultraviolet

VPD: vapour pressure deficit

WP: work package

WS: water-stress

WW well-watered

Appendices

Appendices available at Zenodo.org https://doi.org/10.5281/zenodo.7220967

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Chapter 7

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Chapter 1 - General introduction

Today, drylands cover over 40% of the Earth's surface and are home to approximately 2.3 billion people (International Institute for Environment and Development - https://www.iied.org accessed on 23 April 2022). Nevertheless, arid and hyper-arid drylands have always been considered peripheral regions challenging now, as in the past, for prosperous human settlement (Biagetti et al., 2022). On the contrary, anthropological and archaeological researches have shown the high variability of traditional socio-ecological systems in arid lands, highly resilient and very rich in adaptive resources (Balbo et al., 2016; Critchley et al., 1994; D'Odorico and Bhattachan, 2012). A topic of central interest concerns dryland water management. In arid areas, various practices have been adopted to collect rainfall and groundwater, to channel floodings, and profit from the few hydric resources available (Prinz, 2002). The scarcity of water resources has made water a key issue in the study of the most arid areas of the world (Cooper et al., 2008; Osman et al., 2021; Salmon et al., 2015), proving to be a commodity of central importance, especially for the development of agricultural practices (Manning and Timpson, 2014; Marshall and Weissbrod, 2011). Indeed, history has confirmed that these areas not only have been the cradle of domestication of various crops (Manning et al., 2011; Winchell et al., 2018) but even of specific technologies, suitables for the development of elaborate agricultural systems (Reynolds et al., 2007; Winslow et al., 2004). However, the study of the development of these practices is complicated by the fact that trustful proxies for water availability are hard to find in the archaeological and archaeobotanical record.

The main aim of this research is to understand whether it is possible, and to what extent, to reconstruct water availability in an archaeological context by investigating specific archaeobotanical remains, namely phytoliths. Phytoliths were chosen after a careful review of the possible direct and indirect water indicators available. Indirect proxies include all those artificial structures (e.g., wells, canals, tanks) that can be encountered in the archaeological record and that indicate, by their presence, some sort of water management system. However, the discovery of these artificial structures presents certain difficulties. Indeed, fields, ridges, dams or wells most of the time are no longer visible in the archaeological record, partly because of their deterioration due to time, partly because they would have been ephemeral and inefficient, especially in prehistoric times (Jenkins et al., 2020). Furthermore, the mere finding of irrigation or water storage facilities are only indirect ways of indicating water availability and give no indication of the amount of water in use. As a consequence, the main focus of my research has been directed on archaeobotanical remains, which may provide a direct assessment of watering. Most of the archaeobotanical research assessing crop water availability to date focuses on C_3 species because they are considered the foundational crops of modern agriculture in some of the most studied areas of the world. It must be said, in this regard, that the study of water availability from archaeobotanical proxies is still in its infancy and few areas have been tested to date, primarily around the Fertile Crescent and Central/North America. For C_3, the study of the isotopic composition of seeds has proven efficient in indicating water availability (Araus et al., 1997; Ferrio et al., 2005; Nitsch et al., 2015). Isotopical discrimination describes the preferential assimilation of the lighter isotopes over the heavier during the process of photosynthesis, and primarily reflects water availability (Eggels, 2021). Water management can be, thus, inferred by integrating isotopic data with palaeoclimate indicators for the time period in question (Flohr et al., 2019). If the crop isotopic signal indicates greater water availability than expected according to the climate proxies, it is likely that the crops received extra water in addition to rainfall, such as through irrigation, artificial watering or by cultivation on alluvial fans (Ferrio et al., 2005). However, arid areas are mainly cultivated with C_4 species, whose photosynthetic system is much more complex, both anatomically and physiologically than the C_3 Indeed, in C_4 species there is a leakage of carbon out of the bundle-sheath cells (where most of the carbon is fixed) during photosynthesis, which is considered to influence carbon discrimination (ΔC_4) (Farquhar, 1983). While more recent research has shown that ΔC_4 responds to drought and has a direct relationship with water efficiency (Buchmann et al., 1996; Cernusak et al., 2013), the exact nature of this relationship still needs to be clarified. In addition, the study of C_4 seeds coming from archaeological records is further complicated by issues related to their morphology. Indeed, seeds of most of the small millets (e.g., finger millet or pearl millet) are very reduced in size. It must be specified that organic remains such as seeds are preserved in the archaeological record as a result of charring or waterlogging (Araus et al., 1997; Yang et al., 2011). Taphonomic and charring phenomena on seeds of small dimensions considerably complicate their discovery and recognition in the archaeological register. Therefore, phytoliths represent a good substitute to seeds since in dryland areas, this type of remains are often poorly preserved (Fuller, 2002).

Phytoliths, being inorganic in nature, preserve better than seeds and their conservation is much less affected by pre- and post-depositional issues (Piperno, 2006). However, the ability of C_4 species to save water by reducing transpiration rate might represent a challenge in the use of phytoliths as a proxy for water availability. This research focuses on three species in particular: finger millet (*Eleusine coracana* (L.) Gaertn.), pearl millet (*Pennisetum glaucum* (L.) R.Br.) and sorghum (*Sorghum bicolor* (L.) Moench). According to the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT), the three species are among the widest cultivated millets across Eastern and South Africa and South Asia. Finger millet, pearl millet and sorghum are extremely drought-resistant, germinating even on residual moisture and they are a staple food for over half a billion people in the world. These three species have been selected (a) because of their biochemical C_4 pathways: NADP-ME for sorghum and NAD-ME for finger millet and pearl millet. Their anatomical and biochemical diversity allows the full evaluation of eventual differences among C_4 species. (b) In terms of silicification they have already been studied and classified as biosilica accumulators (Coskun et al., 2021; Kumar et al., 2017; Lux et al., 2002; Ma and Yamaji, 2015; Out and Madella, 2017; Sangster and Parry, 1976) (c) The agronomic study of these three species has been extensively deepened by investigations on their transpiration rate (e.g., Vadez et al., 2011). Furthermore (d) the three species have been found in several archaeobotanical assemblages of arid sites of interest both in Asia and Africa (e.g., sorghum at Al Khiday and Mezber by D'Andrea et al. (2015); and finger millet at Harappa by Weber and Kashyap (2016)).

In this research phytolith models based on the relative abundance of specific morphotypes have been tested as proxies for water availability together with the concentration of biosilica accumulation in the transpiration organs, the size of the silica skeletons, the isotopic content (δ^{30}Si, δ^{29}Si) and the dimension of distinct sensitive morphotypes. The study of phytolith as water indicators has been already explored and it is not an entirely new issue (Bremond et al., 2005; Jenkins et al., 2016, 2020; Madella et al., 2009; Miller Rosen, 1994; Weisskopf et al., 2015). Madella et al. (2009), Weisskopf et al. (2015) and Jenkins et al. (2016; 2020), suggested the use of the ratio of fixed (genetic) *versus* sensitive (environmentally) - controlled morphotypes to detect water availability directly from phytolith assemblages. They hypothesised that in contexts of water abundance, sensitive morphotypes are produced more abundantly than in water stress conditions and that the ratio between the two classes changes at varying water availability. More recently both stable silicon and oxygen isotopic signatures in phytoliths have been found to have potential as proxies for environmental conditions too. Specifically, δ^{30}Si in phytolith was shown to be a proxy for soil conditions, particularly for weathering and organic matter content (Leng et al., 2009). The studies are based on the assumption that in tissues with photosynthetic activity, silicon mostly polymerises as a result of supersaturation (Schaller et al., 2013). Thus, phytoliths, which are the phenotypic effect of silicon precipitation in the epidermis of tissues, are considered a potential proxy for water availability. However, the role and function of Si in plants is not yet fully understood and this impairs the comprehension of the mechanisms that drive phytolith formation. Indeed, recently Kumar and colleagues (2017) and Hodson (2019) have shown that the classification of phytoliths is more complex than previously thought and morphotype characteristics do not depend only on the genetic/environmental mechanism of deposition, but their formation is also altered by the cell district where silica started to precipitate. Therefore, the study of C_4 phytoliths in relation to water availability is still an open topic for research. Such a study, apart from answering an archaeological question, has also important implications for a more in-depth exploration of the role of silica deposition in species considered high accumulators. The experimental approach that characterises this research has been built on these pilots, addressing the need for further experimental work to realise the full capabilities of phytolith studies.

1.1 Thesis objectives

This thesis research developed into the framework of the ERC starting grant project (ERC-StG nº 759800) RAINDROPS. The project combines research on plant physiology with original archaeobotanical applications and the recording of TEK on cultivation systems in drylands (figure 1.1) to deliver an innovative and reliable methodology to understand past water management practices in arid landscapes.

Within the wider scope of the project, the aim of this thesis is to produce detailed and comprehensive data on phytolith production in response to drought of three fundamental C_4 species and to provide reliable tools to investigate past water cultivation practices by comparison with modern replicas.

The objectives of the study are(1) to study biosilica accumulation processes in sorghum, pearl millet and finger millet, in order to understand whether phytoliths produced by C_4 crops can be used as proxies for plant water availability; (2) to investigate intra and inter specific variability in phytolith production, and with regards to water availability during crop development (3); to elaborate a model that can be applied for the interpretation of archaeological phytolith assemblages in respect to ancient water management. Therefore, the objective of this thesis was to be able to answer the following questions:

1) *Is the developed methodology suitable to answer the three initial aims?*
2) *Do finger millet, pearl millet and sorghum accumulate biosilica through the same pathway?*
3) *Are the phytolith assemblages of the three studied species of C_4 comparable?*
4) *Do water availability influence phytolith deposition?*
5) *Is the ratio of sensitive to fixed morphotypes an efficient indicator of water availability in the C_4 species under study?*
6) *Do genetic or water treatment have the most significant effect on plant silicon accumulation?*
7) *What are the implications of this work in archaeology?*
8) *Can this research contribute to a better understanding of agricultural water management in the past?*

To reach the proposed objectives two experimental cultivations (in 2019 and 2020) of the three crops under study have been executed in ICRISAT (India). The plantings have been set under constantly controlled conditions of water, temperature, humidity, evapotranspiration and soil composition during the period of the year devoid of rainfall (January-May), in lysimeters, protected by natural precipitation. Lysimeters provide similar conditions to fields but allow experimental control over transpiration, water stress imposition and physiological parameters. Experiments have been performed to obtain baseline data to assess phytolith production in connection both to watering and to genotypic variation. To ensure statistical significance, and the control of the genetic mechanisms, experiments have been run on 10 landraces for each species, with 5 repetitions in 2 replicated cultivations. For each cultivation, 5 landraces have been selected and 3 repetition for each treatment have been analysed for different tissue biosilica accumulation. Phytolith production (concentration gram/dry plant material); morphotypes assemblage (over 250 phytoliths have been counted for each sample analysed); ratios between environmentally and genetically controlled forms; silica skeleton size and specific dimensions (e.g., length, width, peaks length) for stomata, bulliforms and elongates shapes have been collected to be related with water availability. In addition, $\delta^{30}Si$ from leaf and chaff phytolith has also been tested by a femtosecond laser ablation inductively coupled plasma source mass spectrometry techniques.

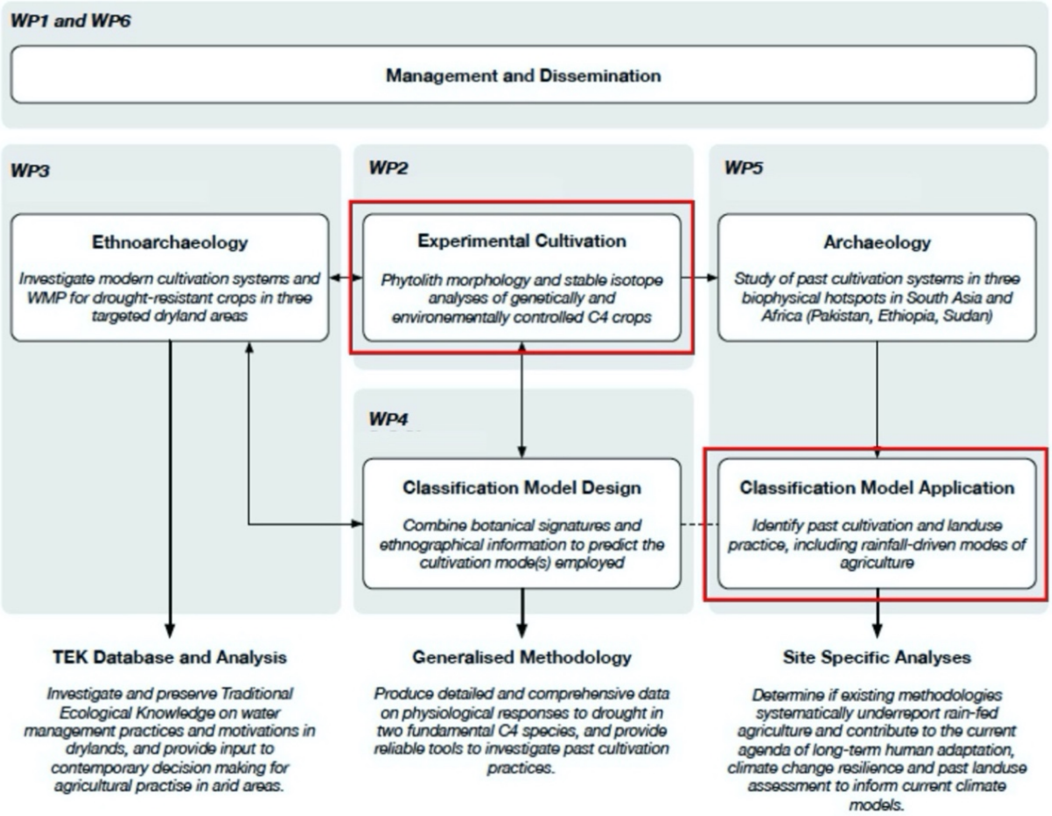

Figure 1.1 - RAINDROPS work packages (WP1-6) schematically represented. The order of the work packages is not casual but reflects the timeline scheduled. This research addresses methodological questions contributing to the experimental work package (WP2) and the analysis of the archaeobotanical samples (WP 5).

1.2 Thesis outline

The thesis is composed of 11 chapters. After this brief introduction (chapter 1), the following three chapters are a review of the available literature divided into subject areas. The problem of water management in drylands (chapter 2), the characteristics of C_4 species (chapter 3), and an in-depth description of the study of phytoliths (chapter 4) are treated with special attention in describing the state of the art in research. In the second chapter it is argued why it is essential to deal with the reconstruction of water management in drylands and what are the related issues. Chapter 3 is dedicated to the three species of interest and why they are historically considered by archaeologists difficult proxies producers. Phytoliths deposition, classification and compositional issues are treated in chapter 4. Next, chapter 5 is devoted to the description of the experimental methodology, arguing why it overcomes the above-mentioned problems. The body of the thesis is divided into 4 main chapters (chapter 6-7-8-9). Chapter 6-7-8 are represented by an article already published or still in the process of. The first article (chapter 6) published in April 2022 is dedicated to the study of biosilica accumulation in the three species under study. The second article (chapter 7), accepted in October 2022, reports the application of the model built on leaf phytoliths to the reconstruction of water availability in different archaeological sites of the Indus Valley Civilization. The last article (chapter 8) is devoted to the construction of a model to predict water availability by using a mix of C_4 tissue phytolith assemblages. Chapter 9 consists of results and discussion regarding investigations made on phytolith morphometry and δ^{30}Si and δ^{29}Si in sensitive morphotypes. Chapter 10 presents a general discussion, where a comprehensive reasoning is provided to answer the scientific questions that led to the development of this research. Chapter 11 is the concluding chapter where the main conclusions drawn from the research are discussed.

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Chapter 2 - Water managements in drylands

2.1 The narrative about water domestication in drylands

Under the assumption that "'water has reflected the image of society" (Clark, 1944) marking a key turning point in the cultural trajectory of each region of the world, archaeologists have long appreciated the importance of the identification of past water management systems thanks to which societies developed. Indeed, most of the ancient civilizations were dependent upon sophisticated techniques of water management for agricultural intensification (Boomgarden et al., 2019), technological development (Mithen, 2010) and settlement patterns (Schmitz, 1986). Hence, research on water techniques for agriculture provides important pieces of information on the evolution of land-use (Koohafkan, 2012), on the environmental limits and opportunities (Marshall and Weissbrod, 2011), and on the choices and decisions taken in the past, related to sociological and cultural attributes (Beckers et al., 2013).

Today drylands cover over 40% of the Earth's surface (https://www.iied.org accessed on 23 April 2022). Archaeologically, vast research has been devoted to the study of the drylands long-term development, considered the cradle of the Old World social and agricultural system. Drylands are defined by the United Nations Environment Program (UNEP) according to an aridity index (AI), which expresses the ratio between average annual precipitation and potential evapotranspiration (figure 2.1). According to this ratio, drylands are areas with an AI < 0.65 and are subdivided into four major categories: hyper-arid (AI < 0.05), arid (0.05–0.20), semi-arid (0.20–0.50), and dry sub-humid (0.50–0.65). Due to the water scarcity, the study of water domestication and the consequent agricultural rise in dry areas need to be addressed carefully.

The domestication of water in dry areas enabled the consolidation and spread of farming lifestyles that ignited an exponential population growth (Manning et al., 2011; Manning and Timpson, 2014). The Later Neolithic period (8300–6500 BP) has been identified as a key period when significant investment in water management occurred, laying the foundation of the first urban communities of the Early Bronze Age (Mithen, 2010). Even the absence of water management systems may have contributed to changes in the sociological and cultural choices, aside from lading to specific land use adaptation: for example, around 8500 years ago, many of the Pre-Pottery Neolithic A

(PPNA) villages and towns become abandoned. The settlement pattern shifted to a series of much smaller dispersed settlements, probably reflecting the rise of nomadic pastoralism, possibly caused also by the absence of water administration and control (Mithen, 2003). The same has been hypothesised by Giosan et al. (2012) who suggested a gradual decrease in flood intensity and the consequent decreasing water management as a possible cause of the collapse of the Bronze Age Harappan (between North India and Pakistan).

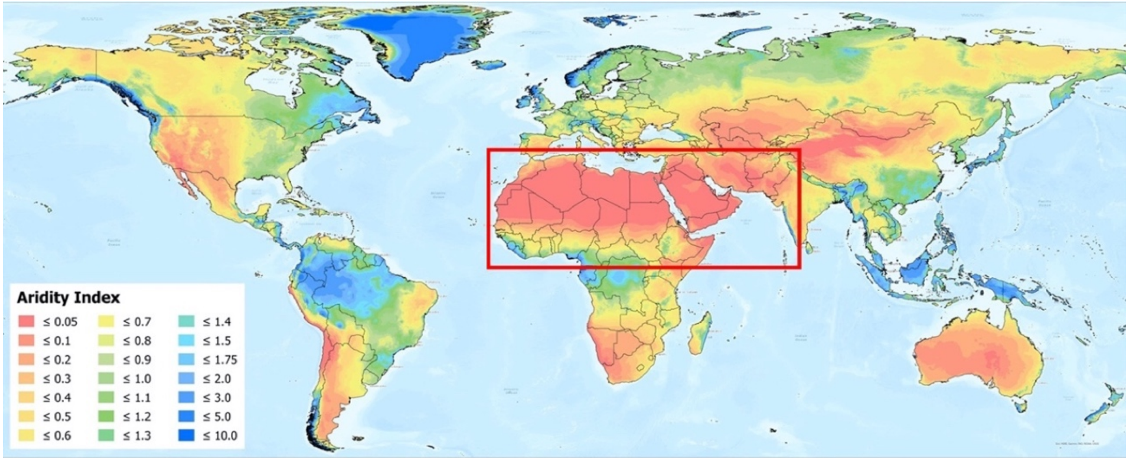

Figure 2.1 - Global Aridity Index based upon the FAO-56 Penman Monteith equation, referenced to the evapotranspiration (ET0) (Zomer et al., 2022). The drylands of interest for this study are marked by the red rectangle.

Focusing the attention on water management for agricultural purposes, is it possible to gather information from present communities to obtain a picture of the possible strategies put in place to handle the little water available. Indeed, a wide range of traditional water management techniques can be found in areas between 100 and 1500 mm annual precipitation and with population densities varying from 10- 500 persons/km² and (Prinz, 2002). According to Beckers and colleagues (2013) the principal strategies put in place can be divided into four categories:

1) Water wells (artificial holes that reach the groundwater table) were probably the first structures that allowed the settlement of drylands beyond locations close to natural perennial surface water. The tunnels usually channelled the groundwater to a reservoir, frequently connected by a covered canal (Hein, 2020). The strategy, in comparison with the alternatives, reduces evaporation losses and the risk of water contamination (Beckers et al., 2013). The origin of the technique, called traditionally "*quanat*", is dated to c. 3000 BP and has been first identified in Persia but probably rapidly spread to the whole North Africa and Spain (Prinz, 2002). Many *quanats* were constructed in India too, where they are still in use in Kerala ("*surangam*") and Madhya Pradesh ("*bhandara system*") (Agarwa and Narain, 1997).

2) *Micro and macro catchment runoff harvesting*. In many dryland areas, the "tank" system is traditionally the backbone of agricultural production (Prinz, 2002). In India, about 6 million hectares are irrigated from various forms of water harvesting. A traditional example is the "*ahars*" in the Bihar region (Uttar Pradesh, India), where unlike tanks, *ahars* beds are not dug out (Agarwa and Narain, 1997; Sengupta, 1993). In the Middle East archaeological evidence of water harvesting structures, like tanks or terraces to store the water in ephemeral canals, appear in Jordan, Israel, Palestine, Syria, Iraq, the Negev and in the Arabian Peninsula (mainly the Yemen) (Bruins et al., 1986). The oldest is believed to have been constructed c. 9000 BP (Prinz, 2002) but only during the Bronze Age (5500 BP) the archaeological evidence became more substantial (Mithen, 2010). In Wadi Faynan (Jordan) an extensive series of walls to collect run-off water for crop irrigation has been discovered. Probably the system has survived for centuries till sustaining the Roman and Byzantine settlement (Barker et al., 2007; Bennett and Barker, 2011). In North Africa micro and macro catchments are still used extensively and they have been reported with their traditional name in many regions of Sub- Saharan Africa (M. Abdeldayem et al., 2020), like the "*caag*" and the "*gawan*" systems in Somalia; various types of "*hafirs*" in Sudan (UNEP 1983) and the "*zay*" system in West Africa (Critchley and Gowing, 2012). Agricultural terraces also represent a system for water harvesting. Terraces are particularly widespread in the Mediterranean region (Beckers et al., 2013). All these strategies necessitate the construction of elaborate structures and an intensive maintenance labour (Koohafkan, 2012).

3) *Floodwater harvesting* is a technique that collects from during flood events. The management requires the construction of elaborate hydraulic structures, such as large dams or dikes together with distribution facilities (Beckers et al., 2013). In Balochistan (Pakistan), the technique has a long tradition and it is called the "*khuskaba*" system or "*sailaba*" system (Prinz, 2002).

Although recognising the different water management strategies is of fundamental importance for archaeology (e.g., for comparison, for the assessment of potential uses and for knowing what to look for/expect), it must also be mentioned that the categorisation of these techniques brings with it certain problems:

-Even if complex water management systems (as wells, tanks or terraces) allowed desert landscapes to be colonised (Braemer et al., 2010, 2009) and should, as a matter of fact, leave traces recognizable by archeologists, they were most of the time ephemeral (Jenkins et al., 2016, 2020) and when traces of them survived, they are generally not datable (Mithen, 2010).

-Water harvesting strategies have been considered to reflect the historical evolution and the specific hydrological conditions to which they had been adapted (Beckers et al., 2013; Hein, 2020; Mithen and Black, 2011). However, water harvesting has not only been for agriculture, especially in drylands where water management systems concerned reservoir management rather than irrigation (Mithen 2010). Moreover, not all societies which have developed sophisticated techniques of water management evolve into states through history, therefore the development of such techniques is not always a mirror of the society's progress. Thus, studies of water domestication practices can give indications on possible short- and long-term technological advances, which might have effects on the environment and on people (Beckers et al., 2013), but they need to be addressed with additional proxies.

-If social and agricultural progress is considered possible only with the above listed water harvesting strategies, settlements of all the areas without permanent rivers, where people had to rely only on rainfall, fog, dew, or subsurface flow of water are indirectly excluded by the narrative on the evolution of drylands occupation. Drylands are considered unsuitable places for mainstream practices of cultivation (Biagetti et al., 2022), characterised by low annual rainfall (<450 mm) labelled as non-productive, under the assumption that these levels of rainfall do not allow for non-irrigated agriculture (Rockström and Falkenmark, 2015). However, it is becoming clear that the evaporative demand/rate during the rainy season needs to be observed to assess the possibility of cultivation or not more than rainfall levels.

2.2 The RAINDROPS project - contributing in the changing of the paradigm

This thesis research developed into the framework of the ERC starting grant project (ERC-StG nº 759800) named RAINDROPS. The project combines ground-breaking research on plant physiology with original archaeobotanical applications and records of TEK on cultivation systems in drylands to deliver an innovative and reliable methodology to understand past water management practices in arid landscapes (figure 2.2). By doing so, RAINDROPS has the objective to: 1) identify past agricultural adaptation to drylands; 2) understand what mechanisms people put in place to create resilient socio-ecological systems in drylands; and 3) provide a more robust framework for designing approaches to resilience adaptation to future climate changes in vulnerable regions, using as tool past societies adaptive responses to drylands.

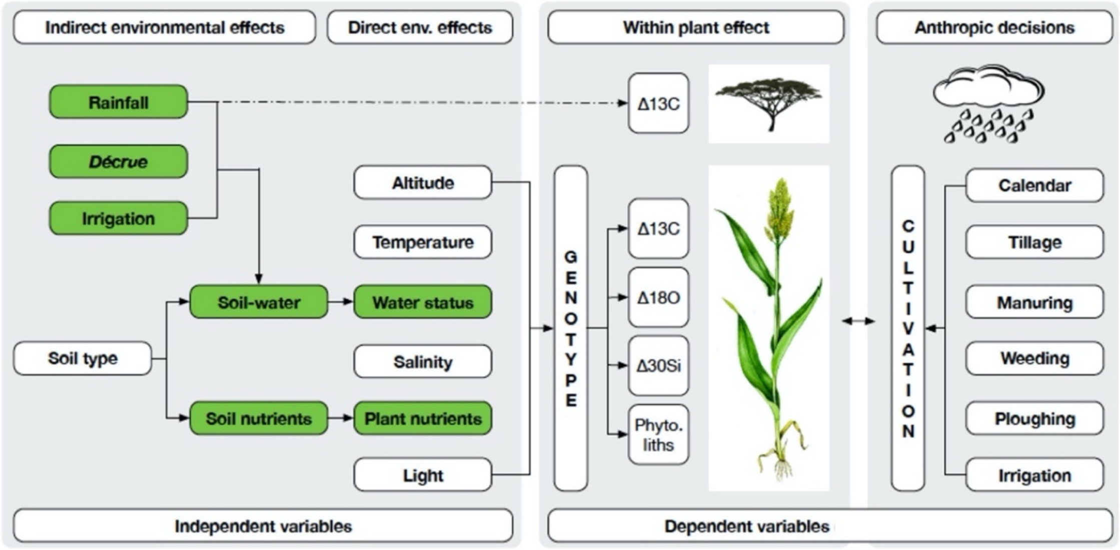

Figure 2.2 - Schematic representation (courtesy of RAINDROPS project) of the model used by RAINDROPS to interpret archaeobotanical samples. On the left-hand side, are listed the environmental factors affecting the phytolith content and isotopic signature in plants. In green are the variables RAINDROPS analyses. On the right-hand side, the experimental and ethnographic data (dependent variables) collected, that constitute the basis for archaeobotanical samples interpretation.

Several issues have prompted the development of such an interdisciplinary project. Most of them are connected to the type of information available on the adaptation of present and past societies to dry environments, which is sometimes partial and biased by a selective perspective. Indeed drylands, especially the more arid, are generally characterised by a poor narrative built on the (wrong) idea that it is impossible to create complex socio-ecological systems sustained by scarcity or absence of rainfall. The description of drylands is often dominated by the *struggle* for human populations to produce food given the general *harshness* of living conditions (Berge et al., 1995; Biagetti et al., 2022). As a consequence, arid and hyper-arid lands have often been considered peripheral regions unsuitable now, as in the past, for stable human settlement. On the contrary, the fluctuation of environmental parameters and the consequent high variability in food production (Koohafkan, 2012; Osman et al., 2021), have made traditional dryland socio-ecological systems highly resilient and very rich in adaptive resources (Balbo et al., 2016; Critchley and Gowing, 2012; D'Odorico and Bhattachan, 2012). As a result of the changing paradigm set in place in recent years, pastoral societies are no longer seen as fragile and irrational socio-economic systems (Rockström et al., 2010). Rather, they represent successful examples of adaptation, delineated by multiple strategies of land use, flexibility, and opportunistic resource management (Reynolds et al., 2007; Winslow et al., 2004). RAINDROPS, adding information on the narrative of the evolution of the human adaptation to dry environment, exploits the concept of "Usable pasts" as an approach to explore how the past can be relevant for the present. Bassey Andah, one of the first Africanist archaeologists who used the term, defined usable past as "*a past that does not merely instil pride but also helps Africans build socio-political units equipped to fight 'cultural poverty' and negotiate justice at both national and international levels* [...]" (Logan et al., 2019).

RAINDROPS developed within another topic, which has always characterised the debate on the development of land use and past societies in drylands: whether or not the rainfed cultivation practice could have existed or not. Either irrigation, permanent water sources or some form of water harvesting were deemed necessary for cultivation in drylands. Today several examples testify otherwise by showing the existence of successful rainfed strategy even in drylands (Cooper et al., 2008; Osman et al., 2021; Salmon et al., 2015). RAINDROPS entered the debate by devoting itself to the ethnographic/archaeological study of three sites specifically. Each site is located in one of the three ecological areas that broadly define the concept of drylands (Zomer et al., 2008): semi-arid (Harappa-India), dry sub-humid (Mezber-Ethiopia) and arid (Al Khiday-Sudan), covering the most of geo-climatic variability of drylands. Water management as irrigation, at these three sites, has been already hypothesised on the basis of geography, economy and degree of social organisation, but no test for rainfed or alternative water strategies have ever been provided. For example, both *décrue* and irrigation have been suggested at Harappa yet, but no visible irrigation structures have been observed archaeologically and recent studies tend to exclude its practice (Giosan et al. 2012). Ethnographic investigations were carried out by RAINDROPS members in the sites of interest apart from targeted archaeological excavations in the areas. Structured and unstructured interviews have been conducted with a statistically significant number of farmers to specifically assess water management practices, as well as to collect traditional ecological knowledge (TEK) on cultivation systems. These interviews targeted quantifiable data on cultivation methods (e.g., tillaging, weeding, manuring, ploughing methods) and information on decision-making related to where and when cultivation is carried out and how these processes are affected by climate variability. RAINDROPS used the following definition to describes the three different options to successfully cultivate in drylands: (a) irrigated cultivation is when water is provided to crops at regular intervals by human intervention, with the aim to supply enough moisture when rainfall is not adequate; (b) *décrue* cultivation occurs when water is provided by natural inundation, typically from major river systems; (c) rainfed cultivation develops when water is provided by rainfall alone, typically when plantation occurs far from rivers and any permanent water sources (Lancelotti et al., 2019).

The ethnographic research conducted so far has produced results which highlighted the existence of an extended rainfed and *décrue* management of the cultivation that go beyond the concept of irrigation (Biagetti et al., 2022; Lancelotti and Biagetti 2021; Lancelotti et al., 2019, Ruiz-Giralt et al., 2022). Not all results are available yet (those concerning Asia/India are still work in progress). The information recorded by the members of the project so far are here briefly summarised. In Sudan, in inland plains from 15 to 50 km west of the Nile River, rainfed pearl millet is the only cereal cultivated. Along the Nile Valley, sorghum is farmed on the floodplain following a *décrue* regimen, which exploits the water from seasonal flooding along the riverbanks. *Décrue* sorghum fields are plowed with mechanical tractors before the flooding, while no preparation is needed for millet fields in the upland areas. Once the flood retreats or right after the rain, seeds are set in small shallow holes dug manually using a simple hoe. In the case of rainfed millet fields, about 1 m between plants is required for each plant to absorb enough nutrients and humidity from the soil. The timing of rainfall strongly affects farmers' calendar, especially in the case of rainfed pearl millet. Sowing takes place normally in summer between July and September, and harvesting occurs in late November through late December. The amount and timing of rainfall determine whether rainfed millet is planted or not. Approximately 100 mm of annual rainfall is enough for farmers to have a successful growing season. Rainfed farming is the predominant watering system in Tigray, with only a single farmer (amongst those interviewed during the ethnographic fieldwork) irrigating both finger millet and sorghum cultivation. Finger millet and sorghum are usually planted in late April to May after the first rains of the short rainy season. According to the interviewees, harvesting occurs between September and October, but it can be prolonged as late as the end of December to early January, especially for slow-growing varieties of sorghum. However, other crops, especially fruits, legumes, and other vegetables, as well as wheat in northeast Tigray, follow different cycles, which allows farmers to keep producing for most of the year.

In Sindh, about 40% of the farmers interviewed rely exclusively on rainfall for watering their fields also because water can be expensive, and irrigation can be done at most once every 15 days. Eight farmers cultivate pearl millet in rainfed fields (75%). The growing cycle of sorghum lasts about three months, whereas millet grows faster, needing about two months. Mechanical tractors are used to prepare the fields before the rain, and once the fields are wet sorghum and pearl millet are sown.

Having brought to light both the existence of alternatives to the water harvesting techniques and the extended presence of crops growing with limited hydric resources, RAINDROPS suggests re-evaluating the archaeological records, trying to assess the presence of rainfed water management systems also in past agricultural societies. Research on past rainfed cultivations in drylands is still very limited. In Africa during the Early and Middle Holocene (10000–5000 BCE), patchy, savannah-like vegetation probably supported small communities of hunter-gatherers that exploited a range of wild millet grasses, including attempts at opportunistic cultivation without domestication (Mercuri et al., 2018). The shift to the present-day conditions occurred probably by the end of the Middle Holocene, accompanied by relevant socio-economic transformations (Gasse, 2000; Kuper and Kröpelin, 2006). After 3500 BCE, when the climate conditions became drier, some of the nomadic pastoral groups seem to have adopted cultivation in selected Saharan locations (Cremaschi and Zerboni, 2009). The role that rainfed agriculture may have played in such a critical transition has not been explored yet, and very few data are available on the earliest agricultural experiments in the Sahara

(Lancelotti et al., 2019). In more recent times, millet varieties were grown in the Ahaggar Mountains (Algeria), where rainfall varies between 0 and 100 mm per year (Duveyrier, 1864). The Tuareg peoples used a specific word (“*tawgest*”) to designate non-irrigated plots of the cultivated areas (Nicolaisen and Nicolaisen, 1997). The engagement of nomadic Tuareg groups with rainfed cultivation is further confirmed by Nicolaisen and Nicolaisen (1997), who observed rainfed wheat and millet fields in the Tassili n’Ajjer (Algeria) in the early 1950s. This last report is particularly surprising, given the very low (and mostly uneven) rainfall in this area, which ranges between 0 and 40 mm per year (Lancelotti et al., 2019). Cultivation has also been recorded in the Tadrart Acacus massif (Libya) and around the city of Ghat (Libya) (di Lernia et al., 2012), where annual rainfall is less than 20 mm. In the Tibesti Mountains of south-east Libya, close to the Guezendi area, rainfed cereals were observed in areas with less than 50 mm per year of precipitation (Desio, 1942). In the Asian region between modern Pakistan, North-West India and South Afghanistan (where Indus Valley Civilization developed - c. 2600-1300 BCE), the information available seems to point towards a different condition of agricultural development and water management than in Africa. The difference lies in the fact that a significant number of Indus sites are located on or near the banks of perennial and semi-perennial rivers, while in Africa this condition is rare. However, nothing rules out the possibility that the discrepancies may be caused by the fact that, to date, there is little (if any) information on the pre-urban period, and most of the areas studied relate to an already structured and complex period of urbanisation. Once sedentary and densely populated villages emerged the issues related to water resources and their management became fundamental to understanding the economy and social organisation of the Indus Valley Civilization. Miller provides a detailed discussion of the possible models of water management, mentioning possible strategies of water management, from rainfed agriculture to well or lift irrigation, according to an increasing level of organisational complexity and labour intensity (Miller 2006). Most probably, during the Urban period, floodplains constituted some of the most favourable areas for Indus Civilization agriculture. Nonetheless, the data highlight a mixed settlement pattern, with many sites located along river channels, while others at some distance from the modern relict watercourses (Conesa et al., 2014) that can be connected more to a type of rainfed agriculture. Farther away from the floodplains, where water availability was less predictable and mostly associated with seasonal rains, some structures for water retention and storage were probably built (Madella and Lancellotti 2022), but no information excludes the existence of a complete rainfed system. The case in Dholavira, in Kachcch (Gujarat) is exemplary. It stands in an area that today receives less than 200 mm of rainfall per year. Around the sites, a series of small dams along the stream have been encountered, but an impressive system of water reservoirs has been documented as well, into which water was diverted and probably stored for many months after the rains (Madella and Lancellotti 2022), emphasising how a certain water management might have always existed.

2.3 Conclusions

The examples above mentioned about the development of water management in the African regions as well as in the Indus Civilization territory show that the cultivation of rainfed crops most possibly complemented pastoral and foraged resources and specific water reservoir systems in the present as in the past. Therefore, the reconstruction of the water management becomes more complex because observing only indirect proxies does not allow a clear and complete reading on land use. While in the recent past we can rely on written sources and direct testimonials, the main obstacle to recognize crop management and water availability in archaeological contexts is methodological. The identification of ancient fields, for example, is extremely difficult, even using techniques such as geoarchaeology or molecular footprints (Motuzaite-Matuzeviciute et al., 2016). Fields and different types of water management are usually inferred from the presence of related technology, such as ploughs, terracing or wells and tanks. Only recently environmental alterations pushed by human pressure caused by the adoption and spread of agricultural system have been identified in paleoenvironment and archaeological records for example in South American landscapes (Lombardo et al., 2020) in the Sahara region (Mercuri et al., 2018) in Israel (Shahack-Gross et al., 2014) in South East Asia (Madella et al., 2016), but the real challenge is to identify proxies capable of quantifying the extent of these phenomena. Indeed, the aim of this thesis work is to investigate a direct archaeobotanical proxy, namely phytoliths, in order to understand whether hypotheses regarding land use, even in arid areas of the world mainly cultivated with C_4, can be formulated through the use of botanical remains.

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Chapter 3 - The C_4 crops

3.1 The agricultural scenario in drylands

Arid regions are dominated by savannah and grassland biomes composed mainly by C_4 species which account for approximately 25% of the primary production on the entire planet (Sage and Zhu, 2011). As for millets, 95% of the world production comes from Asia and Africa (Saleem et al., 2021). Several major millet crops like sorghum (*Sorghum bicolor* (L.) Moench) (Reddy, 2017a), whose belonging to the millet category is debated but for clarity in this thesis will be referred to as such, proso millet (*Panicum miliaceum* L.) (Gomashe, 2017), foxtail millet (*Setaria italica* (L.) Beauv.) (Hariprasanna, 2017), and pearl millet (*Pennisetum glaucum* (L.) R.Br.) (Reddy, 2017b) have been introduced in all the most arid areas of the world. Millets cultivated on smaller scales, but still of central interest, include finger millet (*Eleusine coracana* L. Gaertn.) and indian barnyard millet (*Echinochloa frumentacea* Link) (Rudov et al., 2020). With very minimal rainfall and little input while growing, fields of small millets generally consent to decent and dependable yields (Rudov et al., 2020). Sorghum, pearl millet and finger millet, which are the cereals of interest in this study, have been ranked by the FAO among the 150 top crops produced globally (Food and Agriculture Organisation of the United Nations, 2018) and, in regions where rainfall is unpredictable, these three crops are considered the most reliable (Weber and Kashyap, 2016).

3.1.1 The use of millets today

Today millets are cultivated as source for food, for their grain rich in fibres and micro and macro nutrients, as well as source for firewood, fodder, construction material, medicine, and in recent times for desert reclamation programs and afforestation (Rudov et al., 2020). Indeed, millets have an additional potential as staple crops to be exploited in this present period of climate change, due to their comparatively lower carbon footprints (Muthamilarasan and Prasad, 2015). The distribution of the many races of sorghum suggests an extended adaptation of local forms (such as for example durras in India or guinea races in West African savannas). The enormous existing variety of sorghum races and the great specificity with which these varieties are used, prove how the species is not totally separated by the traditional indigenous use (Ragupathy et al., 2016). For example, people still like to chew the culm of sorghum like a sugarcane or to make beer with it because grains are rich in tannin (Harris and Hillman, 2014). The cultivation of small millets, such as finger millet, is mainly driven by cultural practices (Rudov et al., 2020). Ragupathy and colleagues (2016) reported that in India local farmers emphasise the nutritional importance of many landraces of small millets which should help to recover from stomach disorders and are claimed to control diabetes (Dharmaraj and Malleshi, 2011). Furthermore, Rengalakshmi (2005) reported that small millets are seen as sacred crops associated with social prestige or bride price. The vast use of millets today, mainly connected to climatic necessity and traditional use, hint at a long history of management and use.

3.2 A long history of management

The three crops considered in this study (finger millet, pearl millet and sorghum) are native to Africa, but they probably followed different trajectories of domestication (figure 3.1). Sorghum seems to have been domesticated relatively late (c. 50 BCE) even if it was probably cultivated in a wild form a few millennia earlier (Ball et al., 2016). The population of east Sahel most likely domesticated sorghum as a fodder grass after becoming sedentary (Winchell et al., 2018). The earliest report of sorghum in the Indian peninsula is from Kunal, assumed as early as the Late Harappan period (2000-1700 BCE) (Fuller and Boivin, 2009). Meanwhile, on the eastern side of the African continent finger millet was cultivated by Aksumite populations in the Ethiopian highlands by the beginning of the second millennium BP (D’Andrea, 2008). Based on the distribution of the wild progenitor and of landrace diversity, it has been suggested that finger millet was probably domesticated somewhere in the highlands stretching from Ethiopia through Uganda and western Kenya into northern Tanzania (Mueller et al., 2021). Finger millet arrival in India inland is disputed but the most accepted hypothesis places its introduction in the Urban period (Fuller, 2003). Pearl millet is the oldest domesticated crop of Africa (c. 3000 BCE) (Manning et al., 2011). Analysis coming from phylogeographical studies are consistent with a single domesticated centre either in western Sahel or in the region included between eastern Mali and western Niger (Dussert et al., 2015). Arguably wild pearl millet attracted the attention of the local non sedentary pastoralists for the prolific production of small grains (Mercuri et al., 2018), and the clump-forming features of wild pearl millet, which lend it to easy management (Fuller, et al., 2016). Cultivation of pearl millet millets probably preceded sedentarism in central and west Sahara. Archaeobotanical evidence from sub-Saharan West Africa suggests that pearl millet was the predominant, or even the only, cultivated cereal across the region including Mauretania, Mali, Ghana, Burkina Faso, and Cameroon (D'Andrea and Casey, 2002; Klee et al., 2004; Neumann, 2005). The southward dispersal of pearl millet may be interpreted as a part of the retreat to green Shara when climate dried and pastoral populations declined during the Middle Holocene (Manning and Timpson, 2014). In addition, pearl millet must have spread eastwards across the Sahelian zone and reached India within less than 1000 years of domestication (Manning et al., 2011). Domesticated pearl millet has been recovered south of the Indus Valley in the Saurashatra peninsula (Gujarat) by at least 1700 BCE and probably arrived even earlier around 1900 BCE (Madella and Fuller, 2006; Manning et al., 2011). The discovery of early small-grain varieties, which suggests the derivation from the Sahelian forms, denotes a rapid dissemination of the cultivar eastward, while the evolution of big-grain pearl millet probably happened independently in India (Winchell et al., 2018). Fuller (2011) argued that all the South Indian patterns of early domestication of local millets are reminiscent of Africa, presenting the hypothesis that not only the grains, but also management and practices knowledge could have moved towards East.

The arrival of these three species to the Indian peninsula is largely debated (figure 3.1). The current hypothesis places the arrival in the Harappan urban period (2600–2000 BCE), brought by Harappan "seafarers" (Fuller et al., 2011). Alternatively Fuller and colleagues (2011) suggested that this movement of African crops took place at the end of the Harappan era, perhaps as the urban Harappan civilisation was undergoing its transformative de-urbanisation process (Madella and Fuller, 2006). Probably the African cereal package was adopted without the divestment of the native Indian millets, as such they can be seen as an addition to the existing summer monsoon agricultural package (Fuller and Madella, 2001). Only sorghum has been probably added to the winter crop package (Fuller and Boivin, 2009). It has been hypothesised that in the Indian peninsula and all the surrounding regions, African millets prevails over the original species, preferred for the higher productivity under intensive cultivation and for the taste (Weber and Fuller, 2006). Archaeobotanical remains recovered demonstrated substantial changes in the agricultural package between the early and the mature Harappan period when the African package was adopted (Fuller et al., 2011; Fuller and Madella, 2001), and it has been pointed that native Indian small millets (e.g., foxtail millet) were maintained for over centuries only for traditional reasons.

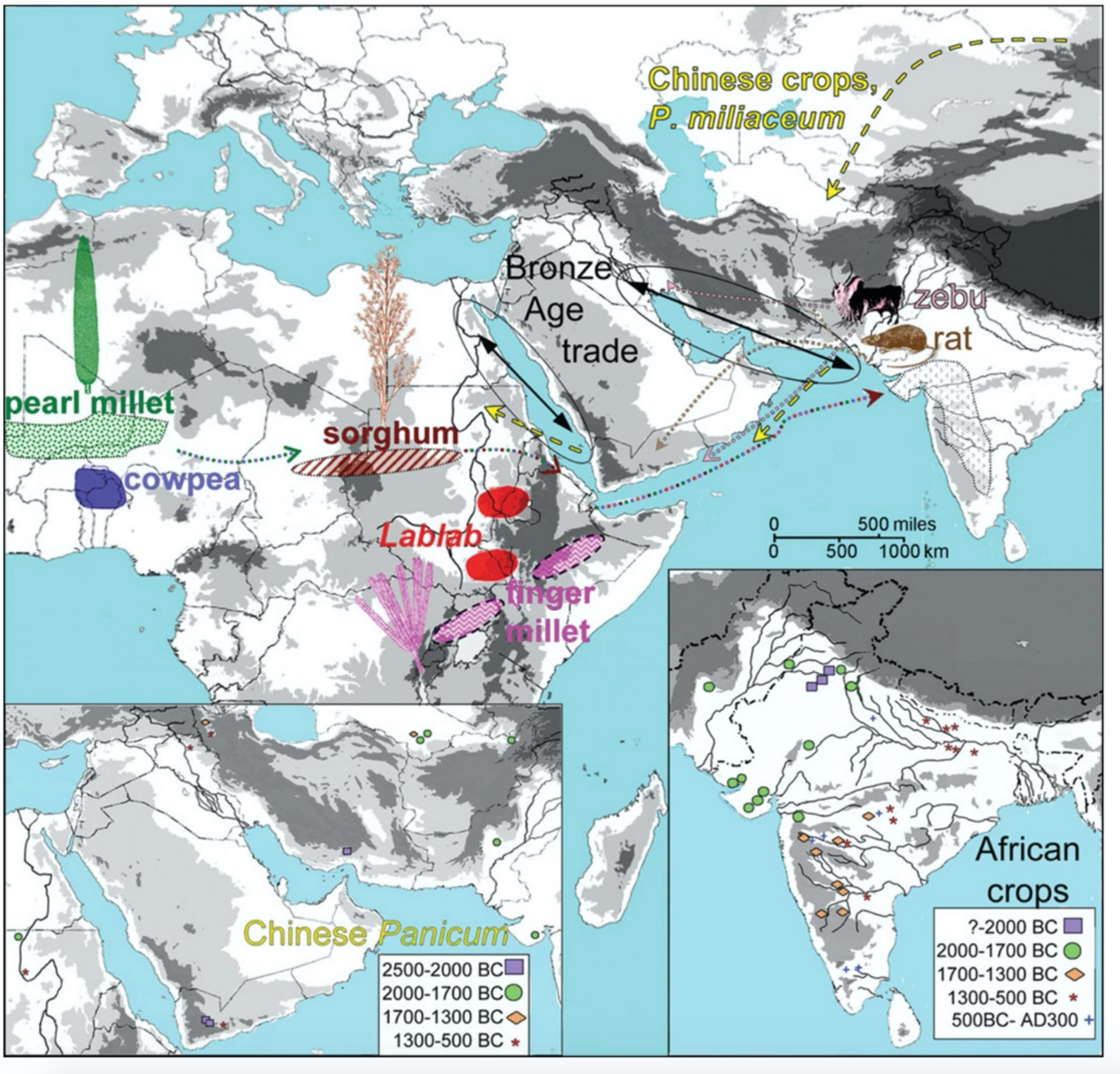

Figure 3.1 - Schematic map of centres of domestication and dispersion pathways of millets by Fuller et al. 2010.

The domestication process of these three species, the history of their use and the way they historically influenced the land use are difficult to identify. Today, the concept of domestication, agriculture and farming is a matter of debate (Zeder 2006). The difficulties stem from the fact that, although morphological changes occurred in a range of domesticated African plant taxa, it has been suggested that a number of African savanna plants were cultivated or intensively managed in ways that did not lead to morphological domestication (Neumann, 2005). The mechanisms proposed for late morphological changes include the possibility of continued crossing between cultivated and wild populations that made species morphologically domesticated only when they were removed geographically from its wild African range (Fuller, 2003; Haaland, 2007). In conclusion, most probably, millets have been used and managed even before the domestication gave rise to landscape modification or morphological changes, meaning that finger millet, pearl millet and sorghum have been probably exploited conspicuously in drylands since the beginning of their occupation. This statement is sustained for example by Marshal and Weissbord (2011) who argued that at the site of Um Direiwa (Sudan), evidence of sorghum usage was dating to the seventh millennium BP when there is no macrobotanical evidence or indication of landscape modification that supports claims for cultivation of African sorghum (domestication is dated c.50 BCE).

3.3 C_4 pathways - the challenge for archaeologists

Despite the widespread interest in the domestication and dispersal of millets, less is known about their management. Archaeobotanical research so far focuses on many C_3 species for several reasons. In the first place C_3 species are considered the foundational crops of the old-world agriculture, and the discovery of their macro and micro remains in the archaeological record has been ample. Secondly C_3 carbon cycle is less complex than that of C_4 species (Farquhar, 1983), making some of the C_3 proxies (e.g., isotopes) less problematic to interpret than than that of C_4. C_4 species have higher water-use efficiency than C_3 plants because C_4 photosynthesis can operate at low intracellular concentrations of CO_2, hence, at lower stomatal conductance (Furbank, 2017). Nitrogen and carbon-use efficiency in C_4 species is also improved because Rubisco is used more effectively, due to the suppression of photorespiration (Mu and Chen, 2021). Thus, C_4 crops are less affected by water constraints, nitrogen deficiency and high light intensity (Saleem et al., 2021). All these features make more complicated to interpret some C_4 derived proxies as environmental indicators in climate reconstruction or human managing of the land.

3.3.1 C_4 photosynthesis

C_4 species are distinguished for their physiological ability to withstand high temperatures and scarce and erratic rainfall patterns thanks to their specific photosynthetic pathway (Slack and Hatch, 1967). In contrast to C_3 photosynthesis, which evolved under high atmospheric CO_2 levels and mesic conditions, C_4 photosynthesis developed when CO_2 concentration were low and in arid environments (Rudov et al., 2020; Sage, 2004). The climatic changes occurring during the Oligocene, marked by dropping CO_2 levels and increasing seasonality, favoured the convergent evolution and diversification of various C_4 lineages united by a specific carbon circle (Christin et al., 2008; Osborne and Beerling, 2006; Sage, 2001). C_4 photosynthesis differentiate from C_3 for the increase in the assimilation rate and the reduction on photorespiration, which are made possible by concentrating CO_2 at the site of the Rubisco in the bundle sheath cells (Pearcy and Ehleringer, 1984). Indeed, C_4 species are characterised by a structural mechanism that distributes the Calvin circle between two compartments, the so called Kranz anatomy: the mesophyll cells for the CO_2 fixation by the PEPC (Phosphoenolpyruvate carboxylase enzyme) and the bundle sheath cells for its decarboxylation around Rubisco (Ribulose-1,5-bisphosphate carboxylase/oxygenase). This special leaf anatomy allows C_4 species to compensate the reduced stomatal opening that cut down CO_2 concentration, but simultaneously to prevent water loss by transpiration (Bräutigam et al., 2014). The C_4 engine, by concentrating CO_2 in the bundle sheath cells, allows Rubisco to operate close to its saturation point, suppressing photorespiration by >80% at the cost of two adenosine triphosphate (ATP) molecules (Sage and Zhu, 2011). Therefore, the entire process has a higher energetic cost in comparison to C_3 (Pearcy and Ehleringer, 1984). The maintenance of the energetic cost (2 ATPs) depends upon the temperature and light intensity to stimulate an intense photosynthetic production (Christin et al., 2011; Kanai and Edwards, 1999). Concurrently, because of this specific mechanism, C_4 species can have elevated photosynthetic capacities at warmer temperatures, compared with ecologically similar C_3 crops, and accomplish instantaneous water use efficiency (water used/ biomass produced) (Ghannoum et al., 2010; Kocacinar et al., 2008; Long, 1999).

3.3.1.1 Not all C_4 are the same

The biochemical subtypes of C_4

The trait which distinguishes the C_4 has convergently evolved at least 64 times and it is preserved in all the C_4 (Sage and Zhu, 2011). It encodes for the phosphoenolpyruvate carboxylase (PEPC) which serves to incorporate bicarbonate into phosphoenolpyruvate (PEP) in the bundle sheath, yielding the four-carbon molecule of oxaloacetate (OAA) (Bräutigam et al., 2014) which gave the name to C_4. However, different evolutionary lineages have developed distinct means and anatomical features to decarboxylate the organic carbon to release the CO_2 at the site of Rubisco (Pyankov et al., 2010) (figure 3.2). In the NADP-ME subtype a nicotinamide adenine dinucleotide phosphate (NADP)-

dependent malic enzyme (ME) decarboxylate malate to pyruvate in chloroplasts (Rao and Dixon, 2016). The layer of cells between the bundle sheath cells and the vascular bundle is absent, and suberin is deposited in the bundle sheath cell wall. Bundle sheath chloroplasts with reduced grana are arranged centrifugally in monocotyledons and centripetally in dicotyledons (Lundgren et al., 2014). NAD-ME subtypes (nicotinamide adenine dinucleotide-dependent malic enzyme) decarboxylate malate to pyruvate in mitochondria (Rao and Dixon, 2016). Comparatively, the vasculature of the NAD-ME subtype is usually surrounded by a double sheath, consisting of the outer bundle sheath and the inner non-photosynthetic mestome sheath (Lundgren et al., 2014). Suberin ubiquitously deposits in the mestome sheath rather than in bundle sheath cells, and bundle sheath chloroplasts with developed grana are arranged centripetally (Fouracre et al., 2014; Lundgren et al., 2014; Mertz and Brutnell, 2014). PEP-CK subtypes decarboxylate OAA to PEP in the cytosol thanks to phosphoenolpyruvate carboxykinase (PEP-CK) (Rao and Dixon, 2016). PEP-CK subtypes differ from NAD-ME for chloroplast position which is centrifugal or scattered and for the suberization of the sheath lamellae (Sage et al., 1999). Regarding the species of interest in this research, sorghum is a NADP-ME species whereas finger and pearl millets are NAD-ME subtypes (Cousins et al., 2008).

Therefore, NAD-ME and NADP-ME species differ in their typical Kranz anatomy, in the metabolite flow through mesophyll and bundle sheath cells, and in the plastid transporters (Dengler et al., 1994; Dengler and Nelson, 1999; Rao and Dixon, 2016). As a consequence, the biochemical differences between the two pathways involve both the water use adaptation, photosynthetic ability and leaf anatomy (Sage et al., 1999), which led to different environmental adaptations (Ghannoum et al., 2002). Indeed, with decreasing annual rainfall average (900–50 mm), abundance of NAD–ME grass species increases while that of NADP–ME grasses decreases (Ellis et al., 1980; Schulze et al., 1996; Taub, 2000). For the development of this research considering finger millet, pearl millet and sorghum was interesting because, not only the three species occupy different ecological niches that differ in moisture levels, but also because this fact suggests that the carbon fixation pathway somehow involves the internal water cycle and the capacities these species have to respond to drought. In order to test intra- and inter-subtype variability and to observe whether or not biochemical subtypes have an effect on the “production” of environmental proxies related to water use, such as phytoliths, it was decided to evaluate two subtypes NADP-ME and NAD-ME, and at least for one (NAD-ME), two different species belonging to the same category (finger millet and pearl millet).

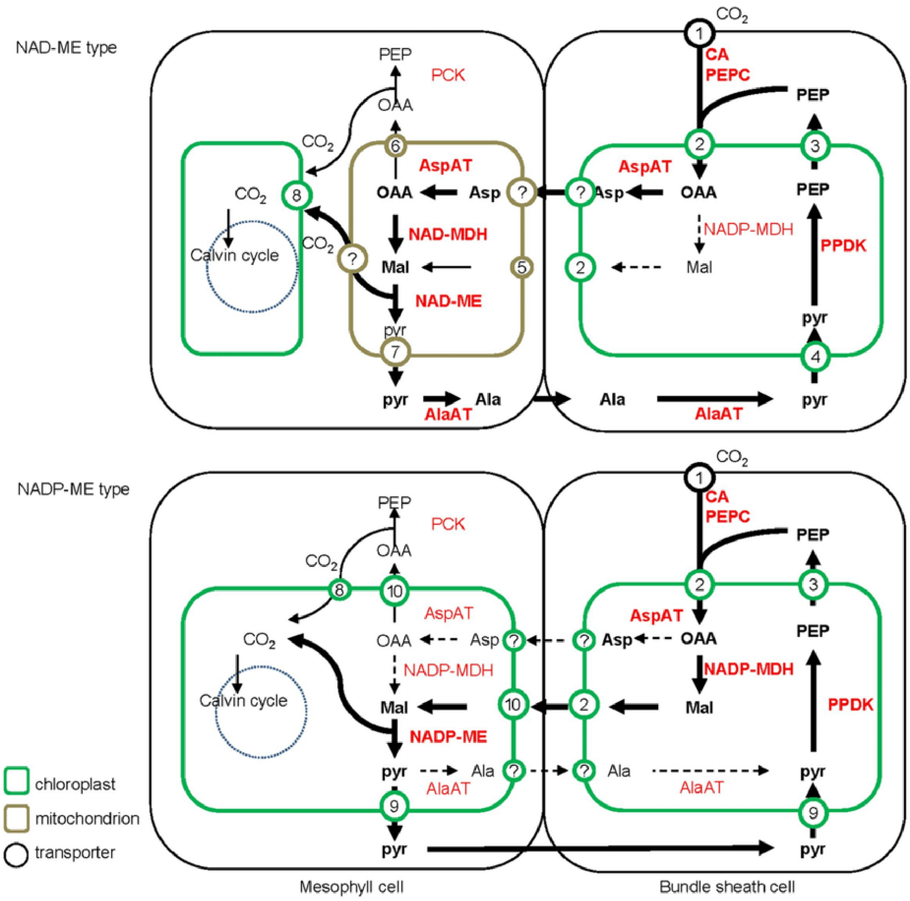

Figure 3.2 - Leaf structure and C4 metabolic pathways of NADP-Me and NAD-ME subtypes (Rao and Dixon, 2016).

However, two main problems exist and are related with the classification of C_4 into biochemicals subtypes. In the first instance, some cases occur of biochemical activities that do not match the anatomical patterns. For example, numerous species of the genus *Eragrostis*, *Enneapogon*, and *Triraphis* are anatomically PCK but biochemically NAD-ME (Kellogg, 1998; Soreng and Davis, 1998). Two single celled C_4 species have been also identified, *Bienertia cycloptera* (Bunge ex Boiss) and *Borszczowia aralocaspica* (Bunge) (Voznesenskaya et al., 2002). Secondly, mixed subtypes exist, having high activity of both NADP-ME and NAD-ME, for example *Neostapfia colusana* (Burtt Davy) (Keeley, 1998). This underlines the fact that biochemical research related to the photosynthetic process of C_4 species is a field that is still evolving and researching.

The taxonomic classification of C_4

The C_4 photosynthesis is restricted to just the angiosperms. It occurs in 19 families of flowering plants, 16 of which are dicots (among which *Asteraceae, Brassicaceae, Euphorbiaceae*) and 3 monocotyledons (*Poaceae, Cyperaceae and Hydrocharitaceae*) (figure 3.3). Most of C_4 species are found in the grass family (approximately 4600 species) (Ellis et al., 1980; Sage and Sultmanis, 2016; Soreng and Davis, 1998), which are estimated to have 11 independent lineages (while only 4 for *Cyperaceae*) (Sage 2004). For groups lacking a biochemical description, delineation of the subtype is still possible by considering that many taxa are classified into tribes exhibiting only one mode of decarboxylation (Sage and McKown, 2006). For example, species in one of the main tribes of the Chloridoideae, as finger millet, are most likely NAD-ME or PCK.

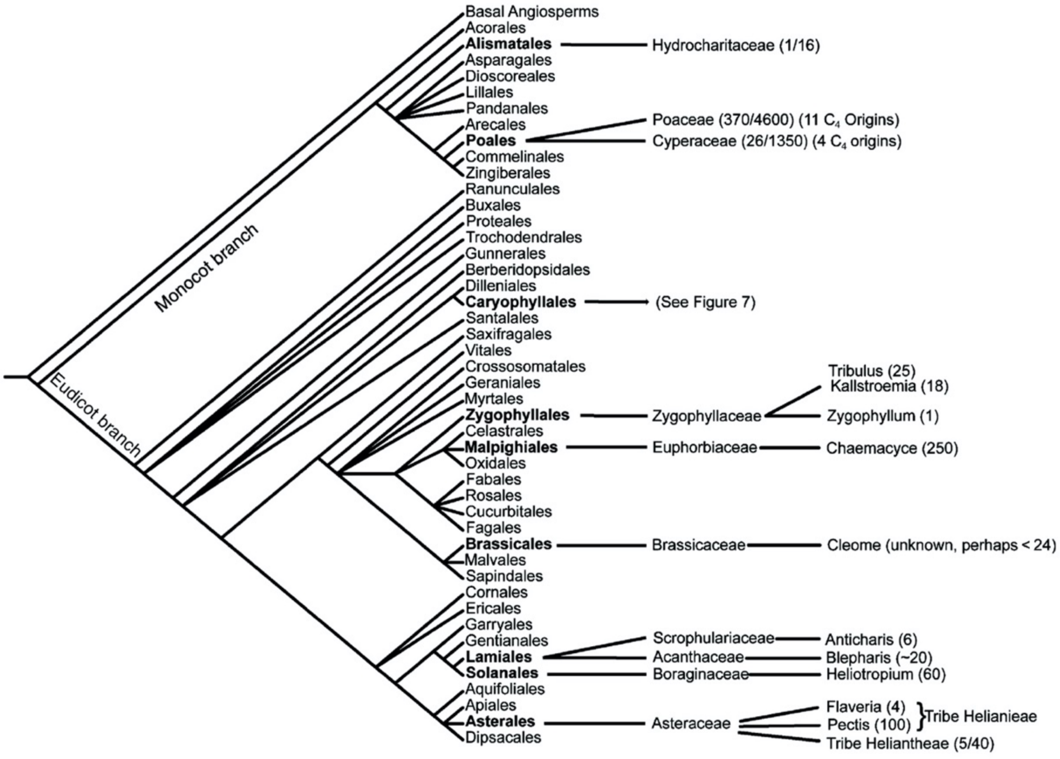

Figure 3.3 - Distribution of C_4 in taxonomic order for angiosperms (Sage 2004).

It has been shown that different taxonomic groups of C_4 species follow different distributions relating their photosynthetic capacity to climatic variables (Pyankov et al., 2010). The percentage of C_4 *Poaceae* (species of *Poaceae* in relation to the total number of species encountered) increases with the growing mean annual daily temperature, indeed they are the most abundant species in tropical and subtropical Asia and Africa (Cabido et al., 2007; Rudov et al., 2020; Schulze et al., 1996; Wooller et al., 2007).

Drought stress in C_4 *Poaceae* led to a significant reduction in plant height, plant biomass, plant weight and grain number (Debieu et al., 2018). Short life cycle, reduced plant heights, small leaf areas, thickened cell walls, and dense root systems are various traits that help millets in general in resisting stress (Saleem et al., 2021). These traits are highly advantageous as they increase the water use and nitrogen efficiency (Bandyopadhyay et al., 2017).

In the following paragraphs are illustrated the three C_4 species of interest in this study: finger millet (orden: *Poales*, family: *Poaceae*, subfamily: *Chloridoideae*), pearl millet (orden: *Poales*, family: *Poaceae*, subfamily: *Panicoideae*) and sorghum (orden: *Poales*, family: *Poaceae*, subfamily: *Panicoideae*). The descriptions of the characteristics of the three species have been provided by the ICRISAT genebank descriptive panels www.genebank.icrisat.org (accessed on 25 September 2022).

Eleusine coracana L. Gaertn.

Finger millet (*Eleusine coracana* (L.) Gaertn.) is originally native to the Ethiopian highlands and thus, it is highly adaptable to higher elevations. Finger millet in the Himalaya can grow up to 2300 m altitude. It is one of the most important small millet in the tropics (12% of global millet area) and is cultivated in more than 25 countries in Africa (eastern and southern) and Asia (from Near East to Far East), predominantly as a staple food grain. The major producers are Uganda, India, Nepal, and China. Finger millet has high yield potential (>10 t/ha under optimum irrigated conditions) and grain stores very well. Blast, caused by *Pyricularia grisea* (T.T Hbert) M.Monod. is the major production constraint. On the basis of inflorescence morphology (figure 3.4), the species *Eleusine coracana* is classified into two subspecies *(africana and coracana)*. *Eleusine coracana* is an annual crop. The height of a mature plant ranges from 30-150 cm in the cool, high-altitude regions of Africa and Asia. The seeds, which may be white, light brown, or dark brown, are consumed in a variety of forms including as unleavened bread made from milled flour. Finger millet is particularly well adapted to various high temperature and moisture ranges. It is mostly grown on reddish brown lateritic soils having good drainage and adequate water holding capacity. Its ability to bear waterlogging is limited, so good drainage of the soils and moderate water-holding capacity are optimal. Finger millet can tolerate moderately acidic soils (pH 5), but also moderately alkaline soils (pH 8.2).

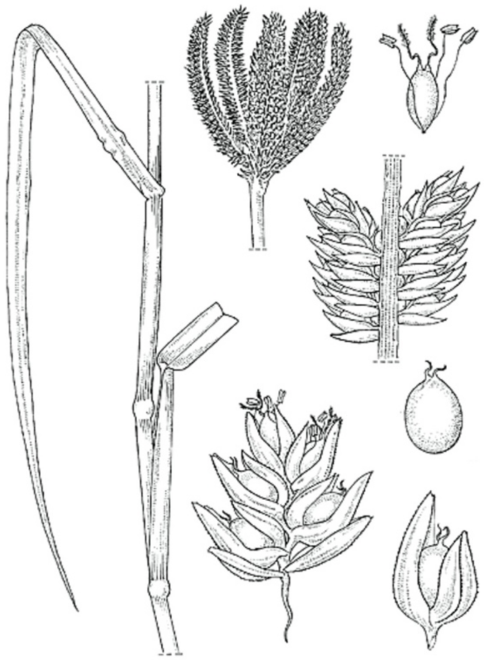

Figure 3.4 - *Eleusine coracana* botanical illustration form Pl@nt Use (www.plantnet.org accessed on 25 September 2022).

Pennisetum glaucum (L.) R.Br.

Pearl millet (Pennisetum glaucum (L.) R. Br.) is one of the main cereal crops grown in a wide range of latitudes (35ºS to 35ºN of equator). Normally pearl millet is grown in areas with rainfall of 125-900 mm. Pearl millet is a robust, quick growing, summer cereal with large stems, leaves, and panicles. The height of the plant ranges from 0.5-4 m. Plants are tall and vigorous, with exceptional grain and fodder yielding potential. Pearl millet plants usually have variable number of tillers, variable panicle length, seed size, seed colour, depending on the cultivars and but also environments. Pearl millet has ovoid grains of 3-4 mm length (figure 3.5). Seeds can be nearly white, pale yellow, brown, grey, slate blue or purple. The crop is cultivated for both forage and grain. It is an annual crop. It grows on a wide range of soil types but is best suited to light sandy soils. It is often grown in warm regions overlapping sorghum cultivation (above 400 mm isohyet). The crop is less tolerant to waterlogging and flooding than sorghum. It is often grown on poor soils, but the crop responds well to good management, such as irrigation and fertilisation. Pearl millet has a higher level of heat tolerance. It performs well in soils with high salinity or low pH.

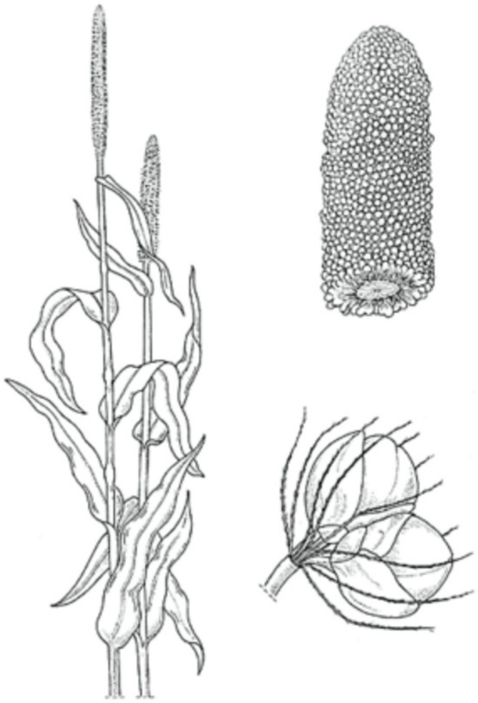

Figure 3.5 - *Pennisetum glaucum* botanical illustration form Pl@nt Use (www.plantnet.org accessed on 25 September 2022).

Sorghum bicolor (L.) Moench

Sorghum (*Sorghum bicolor* (L.) Moench) is the world's fifth most important cereal crop and grown throughout the arid and semi-arid tropics. Sorghum is now widely found in the drier areas of Africa, Asia, the Americas and Australia. Sorghum is adapted to a wide range of environmental conditions but is particularly adapted to drought. It has a number of morphological and physiological characteristics that contribute to its adaptation to dry conditions, including an extensive root system, waxy bloom on the leaves that reduces water loss, and the ability to stop growth in periods of drought and resume it again when conditions become favourable. It is also tolerant to waterlogging and can be grown in high rainfall areas. It is, however, primarily a crop of hot, semi-arid tropical environments with 400-600 mm rainfall that are too dry for maize. It is also widely grown in temperate regions and at altitudes of up to 2300 m in the tropics. Sorghum is a vigorous grass that varies between 0.5-5 m in height. It produces one or many tillers, which emerge initially from the base and later from stem nodes. The root system consists of fibrous adventitious roots that emerge from the lowest nodes of the stem. Roots are normally concentrated in the top 0.9 m of soil but may extend to twice that depth and can extend to 1.5 m in lateral spread. The stem is solid, usually erect and its centre can be dry or juicy, insipid or sweet to taste. Leaves vary in number from 7-24, depending on the cultivar. The leaf sheath often has a waxy bloom. Leaves are from 30-135 cm long and 1.5-13 cm wide, with flat or wavy margins. The flower is a panicle, usually erect, but sometimes recurved to form a gooseneck (figure 3.6). The panicle has a central rachis, with short or long primary, secondary and sometimes tertiary branches, which bear groups of spikelets. The length and closeness of the panicle branches determine panicle shape, which varies from densely packed conical or oval to spreading and lax. Grain is usually partially covered by glumes. The seed is rounded and bluntly pointed, from 4-8 mm in diameter and varying in size, shape and colour with cultivar. It is an annual crop. Sorghum can be successfully grown on a wide range of soil types. It is well suited to heavy vertisols found commonly in the tropics, where its tolerance to waterlogging is often required, but is equally suited to light sandy soils. It tolerates a range of soil pH from 5.0-8.5 and is more tolerant to salinity than maize.

Figure 3.6 - *Sorghum bicolor* botanical illustration form Pl@nt Use (www.plantnet.org accessed on 25 September 2022).

3.3.2 C_4 crops as proxies “producer”

As explained above C_4 species are different from C_3 biochemically and anatomically (Slack and Hatch, 1967) and, as a consequence, in their $\delta^{13}C$ values too (Buchmann et al., 1996; Farquhar et al., 1989; Hubick et al., 1990). These differences may be exploited as proxies in the geological/archaeological record to determine the presence of C_4 species and/or to make hypotheses about the paleoclimate or the paleodiet (Cerling, 1999; Fiorentino et al., 2015). However, C_4 species are thought to be less sensitive to changes in precipitation or in temperature and their capacity to keep low the transpiration trend constitute a real problem when the interest stands in observing which effects had the fluctuation of climatic variables on the C_4 species products as for example isotopical rate, phytoliths or yield.

3.3.2.1 Issues related to stable isotope measurements

The magnitude of carbon discrimination in plants is largely dependent on the photosynthetic pathway. However, carbon discrimination can be also affected by environmental factors, most notably water availability, as it has been confirmed by studies on modern plants such as wheat and barley (Araus et al., 1997; Ferrio et al., 2007, 2005; Wallace et al., 2013). In C_3 species it has been proved that carbon stable isotope composition of plant tissues, which describes the preferential assimilation of the lighter carbon isotope ^{12}C over the heavier ^{13}C during the process of photosynthesis, is determined by CO_2 assimilation rate and stomatal conductance, and it primarily reflects water availability (Eggels, 2021). Water management can be inferred by integrating the isotope data with palaeoclimate indicators for the time period in question (Flohr et al., 2019). If the crop isotope signal suggests that water availability was greater than expected according to the climate proxies, it is likely that the crops received water in a form other than rainfall, such as through irrigation, artificial watering or by cultivation on alluvial fans (Ferrio et al., 2005). C_4 species have Kranz anatomy, thus CO_2 has to go through a different path of fixation than C_2. CO_2 apart diffusing through stomata, it has to convert into HCO_3^-, and then to be fixed by phosphoenolpyruvate (PEP) carboxylase into oxaloacetate (Slack and Hatch, 1967). Various transformations occur, different for the C_4 subtypes, but the net result in all cases is that CO_2 is leaking in the bundle sheath cells and before being refixed by Rubisco (Farquhar, 1983). This C_4 pathway of CO_2 fixation has several consequences, including different $\delta^{13}C$ values, which is about –12‰

for C_4 species while $\delta^{13}C$ averages about –26‰ to –27‰ for C_3 plants (Cerling, 1999). The unequivocal stable isotope evidence can be used to identify C_4 species or C_4 ecosystems in the geological record (Araus et al., 1999, 1997; Caracuta et al., 2012; Ferrio et al., 2007, 2005; Fiorentino et al., 2008; Masi et al., 2013; Riehl, 2008; Riehl et al., 2008) but it becomes an issue of central interest when environmental fluctuations must be distinguished on the basis of isotopic discrimination of only C_4 species. PEP carboxylase (2.2‰), CO_2 diffusion through the stomata (4.4‰) and Rubisco (29‰) discrimination have been evaluated for C_4. However, it is known that some CO_2 and HCO_3^- is likely to leak out of bundle sheath into the mesophyll cells where they mix with other CO_2 diffused through the stomata (Farquhar, 1983). The quantification of this phenomenon as well as its associated fractionation remains unknown to date. The leakage (Φ) defined as the rate of CO_2 which leaks out of the bundle sheath divided by the rate of phosphoenolpyruvate carboxylation, depends on (1) the fraction of CO_2 previously fixed by PEPC, that (2) leaks back to the mesophyll cells and on (3) the Rubisco carboxylation rates (Ghannoum et al., 2002). The amount of CO_2 leaking establishes also the differences in carbon discrimination between the different subtypes of C_4 (Cousins et al., 2008). The conductance to leakage is substantially reduced in bundle sheath cell walls which contain a suberized lamella, which are found in NADP-ME and PCK subtypes of grasses but not in NAD-ME (Pearcy and Ehleringer, 1984). Furthermore, it appears that NADP-ME subtypes require a lower CO_2 pressure in the bundle sheath to saturate carboxylation and inhibit oxygenation of Rubisco (Farquhar, 1983). It is likely that the leakiness, is lowest in NADP-ME type grasses, and greatest for NAD-ME grasses and NAD-ME and PCK dicots, with PCK-type grasses and NADP-ME dicots intermediate (Ghannoum et al., 2002). Shulze and colleagues (1996) tried to quantify the differences in $\delta^{13}C$ among the subtypes of C_4 and arrived at the conclusion that NADP-ME-type exhibit the highest $\delta^{13}C$ values (-11.7 ‰) and it occurs mainly in regions with high rainfall. NAD-ME-type C_4 species have significantly lower $\delta^{13}C$ values (-13.4‰) and dominate in the most arid part of the precipitation regime. PCK-type C_4 species play an intermediate role (-12.5‰) and reach a maximum abundance in areas of intermediate precipitation. In conclusion, the major problem related to carbon discrimination in C_4 is the leakiness (Φ) which has no fixed value but it governs by great extent the slope of $\Delta^{13}C$. While it has been argued that there is no theoretical basis for considerable impact of water availability on the $\Delta^{13}C$ values of C_4 species (Farquhar et al., 1989) because of the uncertain value of Φ, such an effect has been observed in several different C_4 taxa (Ghannoum et al., 2002; Henderson et al., 1998; Reid et al., 2018). Several scientists are putting efforts in trying to model the $\Delta^{13}C$ for different C_4, including RAINDROPS members which are evaluating $\Delta^{13}C$ in seeds of sorghum and pearl millet coming from the experimental and ethnographic fields. The difficulty in modelling the change of $\Delta^{13}C$ in C_4 species is also aggravated by the fact $\delta^{13}C$ values have been found to vary within plant parts (Lightfoot et al., 2016). Studies suggest that maize grains have $\delta^{13}C$ values that are c. 1.5‰ higher than those of leaves (Gleixner et al., 1993), whereas the roots of C_4 species tend to have similar or slightly lower $\delta^{13}C$ values than leaves (Schweizer et al., 1999; Spain and Le Feuvre, 1997). Alkanes and lipids have been shown to have $\delta^{13}C$ values that are 8–10‰ lower than those of bulk leaf matter (Collister, 1994) and plant cellulose $\delta^{13}C$ values tend to be higher than those of lignin (Benner et al., 1987). Grains are suggested to have $\delta^{13}C$ values on average 0.8‰ higher than those of leaves (Lightfoot et al., 2016).

Oxygen isotope composition ($\delta^{18}O$) can be used to determine the origin of the water source in cultivated crops (Williams et al., 2005) and it reflects the transpiration history of the plant by detecting the stomatal behaviour (Barbour, 2007). Therefore, assuming little variation in the isotope composition of the source water during plant growth (e.g., irrigation together with rainfall), $\delta^{18}O$ may be used as an indicator of crop water status in a way similar as $\delta^{13}C$ (Cabrera-Bosquet et al., 2009b, 2009a). As stated above, in C_4 species the effect of the water regime on photosynthesis is minimal and also the evaluation of $\delta^{18}O$ has some complications. Helliker and Ehleringer (2002) hypothesise that the parallel veinal structure of C_4 grass leaves creates a gradient of water which increases $\delta^{18}O$ along the leaf length and decreased between the veins. Researchers supposed that the C_4 leaf structure is critical in determining water dependency of $\delta^{18}O$ because the effect of the gradient discrimination along the leaves blade seems to override the impact of stomatal closure (Webb and Longstaffe, 2003). Indeed, leaf $\delta^{18}O$ of C_4 grasses was found to have no consistent response to drought (Ghannoum et al., 2002). In addition, the average leaf $\delta^{18}O$ was found significantly more enriched in NADP–ME than NAD–ME grasses, as a consequence of the shorter interveinal distance of the former subtypes (Dengler et al., 1994). Even though the theoretical framework indicates no effect on oxygen discrimination due to the watering, many efforts have been made to understand and model the fractionation of oxygen in C_4 species, to better understand whether or not it can be used as marker of stomatal functioning. With time leaf dry matter samples have been abandoned for the purpose and few pilot studies have preferred to investigate $\delta^{18}O$ in phytoliths. Silica in the non- transpiring tissues (roots, rhizomes, and stem) of grasses is formed in equilibrium with the water, which, in this case, is unfractionated from soil water (Shahack-Gross et al., 1996; Webb and Longstaffe, 2002). Indeed leaf silica has been discovered to be consistently enriched in ^{18}O in comparison to stem phytoliths due to relative humidity which in turns depends on transpiration (Webb and Longstaffe, 2003). Unfortunately, what remains difficult to untangle for archaeologists is to separate stem phytoliths from leaf phytoliths when analysing soil assemblage composed by a mixture of species and tissue morphotypes.

3.3.2.2 Issues related to C_4 seeds production

A fundamental difference between C_3 and C_4 species is the quantum yield produced for CO_2 uptake (Ehleringer and Björkman, 1977). Under optimal conditions it is expected that C_4 species should have a lower quantum yield than C_3 plants because of the additional energy expense of the C_4 cycle, which means less and smaller grains in comparison to C_3 (Pearcy and Ehleringer, 1984). When temperatures exceed 35°C and the atmospheric CO_2 concentrations decrease, C_4 species exhibit an advantage over C_3 plants (Cerling, 1999). This, as also explained above, means that there are particularly arid and hot areas that specialise in C_4 production, while others, more temperate, are C_3 grains predominate. When archaeological assemblages composed by both C_3 and C_4 seeds are analysed, it must be considered that the production of C_4 grains cannot be bought with that of the C_3, especially if they are part of the assembly of the same context and period. Additionally, it is also true that yield did not differ significantly among C_4 under a well irrigated scenario, but some C_4 have an advantage over others depending on the stress conditions. Under drought conditions sorghum and pearl millet yield is considered comparable but their genetic richness (high number of specific varieties) has been shown to exhibit highly significant agro-morphologic variation in seeds which came across varied agro-climate conditions (Wondimu et al., 2020). This indicates that seed production is governed by both a broad spectrum of environmental variables and by genetics. Moreover, there has been little systematic characterization of millet traits and not all the varieties have been indexed yet to be recognized.

The fact that C_4 species produced a high variable (for size and morphology) range of seeds could also compromise the use of the C_4 grains as proxy for water availability. Indeed, it was initially suggested that grain size could be used to assess past crop water levels of irrigation. For example, Helbaeck (1960) used the size of charred flax seeds to determine how much water crops received during growth. This method can be problematic especially when observing charred C_4 seeds coming from an archaeological context. The size and the yield productivity of C_4 species has not interfered with different water levels yet and bigger and more vigorous seedlings have not directly been related to the ability of seeds to germinate under stress in C_4 (Qi and Redmann, 1993). However, because taphonomic processes, such as charring, can distort the size and shape of grains (Märkle and Rösch, 2008), seed remains may not be the most direct way to establish how much water the plant received during growth. Indeed, most of the archaeological seeds are found in a charred (carbonised) state. Although carbonization prevents microbial degradation, this process might involve some shifts in shape and composition (isotopes) (Fiorentino et al., 2015). Some taxa have narrow ranges of conditions that will result in a good carbonization while others can be preserved under a wide range of arrangements (Märkle and Rösch, 2008). Until these biases are completely understood, taxa that are poorly preserved by carbonization (e.g., many C_4 millets) may be misinterpreted as unimportant to ancient agricultural systems and diets (Wright et al., 2008) simply because they are not retrieved frequently. C_4 charring conditions are more restrictive when compared with other cereals (Yang et al., 2011). Finger millet is only well preserved when carbonised at temperatures lower than 220°C, which may contribute to its scarcity in the archaeological record (Mueller et al., 2021). In addition, millet's small size could make flotation and recognition very complex, especially when charred (Madella et al., 2016). Although carbonization experiments have been carried over on wide ranges of C_4 cereals at different temperatures (Braadbaart, 2007; Braadbaart et al., 2005; Ferrio et al., 2004; Märkle and Rösch, 2008; Mueller et al., 2021), a lot of work remains to be done to model the effect of fire on several C_4 species. The impact of carbonization on millets depends mostly on temperature, heat exposure time and on the morphological characteristics of grains, which directly reflect changes in the components during carbonization (Mueller, 2017). Indeed, the decrease of $\delta^{13}C$ observed in common millet is probably caused by changes in starch composition, that tend to have $^{13}C/^{12}C$ ratios similar to that of the carbon initially fixed in photosynthesis, whereas cellulose and hemicellulose are heavier (Farquhar et al., 1989).

The process of domestication is another issue related to the recognition of C_4 seeds in an archaeological context. Not all the wild ancestors of C_4 have been satisfactorily identified and this prevents the complete comprehension of their dispersal and domestication pathway (Zohary and Hopf, 2000). Compared to the wild form, domesticated C_4 millets exhibit reduced branching and tillering, loss of the shattering ability, size increase of the seeds and spike (Poncet et al., 2000). Pearl millet is highly outcrossing, hybrids between wild and domesticated forms can be viable and fertile (Brunken et al., 1977) and they can produce seeds of uncertain shape and size. Finger millet has significantly more genetic variation, suggesting that the size of the founding population of domesticated *Eleusine* was small compared to that of its wild progenitor (Gimode et al., 2016). Because crops are constantly evolving, domesticated plants cannot be maintained in a consistent form without an equally consistent management regime and indicization. Several studies have indicated that there is more diversity between landraces grown by a single community than there is between landraces grown in different regions (Gimode et al., 2016) and archaeologists comparing for shape and size modern grains with ancient ones must keep a close eye on the type of modern grains they use for identification. Ultimately, the problem of seed size and shape during domestication is an issue but does not remain limited to C_4 alone being common to all plant species.

3.4 Conclusion

By learning which plants were utilised and the context of both use and production, researchers can evaluate the economic decisions that were made by past humans (Ermish and Boomgarden, 2022). Acknowledging the plasticity of C_4 species anatomy and biochemistry, it is crucial to understand which type of phenomena can be observed through them (Ferrio et al., 2020) and so analyse the environmental context in which individual decisions were taken. Indeed, C_4 species are the main crops involved in the agricultural system in arid areas (Harris and Hillman, 2014; Kirleis et al., 2022; Mercuri et al., 2018; Rockström et al., 2010) and the study of their remains turns out to be crucial when trying to develop a model on the evolution of the past arid land-use. C_4 species are well adapted to extreme climates, high temperatures, and water scarcity (Sage and Zhu, 2011; Yang et al., 2021). However, for this same reason, they are thought to be difficult proxies' producers, less sensitive to changes due to precipitation or temperature (Ghannoum et al., 2002). The study of phytoliths, described in the next chapter, is an excellent alternative to the evaluation of the isotopic signatures of seeds and leaf tissues, and they have been proposed as possible indicator of watering even in for C_4 species.

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Chapter 4 - Phytoliths

4.1 Phytolith formation: a trade-off-mechanism between taxonomy and environmental parameters

Phytoliths are solid deposits of amorphous biosilica ($SiO_2 \cdot nH_2O$) produced by living plants in and among cells (Pearsall, 2016). Silicon accumulation in plant tissues could vary from 0.1% up to 10% of the dry weight (Cornelis and Delvaux, 2016). These differences are connected to taxonomic as well as environmental parameters that when undergo changes they suppose a physiological adjustment connected to biosilica deposition. In this paragraph the silica accumulation process within plant tissues will be described to clarify how phytolith formation can be considered a trade-off mechanism where both genetics and water availability could play a fundamental role.

Soluble Si uptake, in the form of monosilicic acid $Si(OH)_4$, depends to a great extent on water availability in the soil (Sahebi et al., 2014). The entry of the orthosilicic acid takes place by root cortical cells following water, via both an apoplastic (space outside the plasma membrane) and a symplastic (inner side of plasma membrane) route (Guerriero et al., 2016). Thus, Si is distributed along the plant organs either by diffusion (passive) with the transpiration stream acting as the main motive force (Nawaz et al., 2019) or in an energy dependent (active) manner (Ma et al., 2001). A channel-type Si transporter gene Lsi1 translocates Si across the plasma membrane from apoplast to cells (Deshmukh and Bélanger, 2016) and Si is transported to proximal side apoplastic connections by the efflux transporter Lsi2 (Ma et al., 2006). The xylem loading of silica is mediated by another transporter gene Lsi6 found in the xylem parenchyma cells of the leaf sheath and blades (Gaur et al., 2020). These transporter channels were first identified in rice (*Oryza sativa* L.) but the same translocation mechanism has been detected in the same three (orthologues) transporter genes in maize (*Zea Mays* L.) (Mitani et al., 2009), and in vegetables such as pumpkin (*Cucurbita pepo* L. Dumort.) (Mitani et al., 2011) and cucumber (*Cucumis sativus* L.) (Sun et al., 2017). Ma and Yamaji (2006) suggested that the amount of silica uptake attributable to genetics depends on two principal factors which both contribute to the xylem loading: the presence/absence of channels to transport Si from cortical cells to the xylem (Lsi1-Lsi2-Lsi6 and orthologs), and the density with which they occur (Ma and Yamaji, 2006). The combination of these two factors determines the classification into active, passive or rejective (non-accumulators) silica accumulators. According to Tubaña and Heckman (2015), Si content in the shoot of high-accumulators ranges from 1.0% to 10% of the dry weight. The species recognised as high accumulators are primarily monocotyledons such as bamboo (Bambuseae), barley (*Hordeum vulgare* L.), rice (*Oryza sativa* L.), sorghum (*Sorghum bicolor* L.Moench), sugarcane (*Saccharum officinarum* L.), and wheat (*Triticum aestivum* L.). The intermediate-accumulation category are mostly dryland Gramineae with shoot Si content that ranges between 0.5% and 1.5% of the dry weight. The low-accumulation group are dicots which accumulate in the shoots <0.2% of the dry weight (Tubaña and Heckman, 2015). Once in the plant shoots, after passing through xylem loading determined by the distribution of carrier channels, monosilicic acid precipitates forming phytoliths in the cell wall or in the cell lumen (Hodson, 2019). In the tissues with photosynthetic activity, like leaves and to a minor degree culms, Si polymerization due to supersaturation by transpiration-driven water loss seems to play a fundamental role in silica accumulation (Euliss et al., 2005; Schaller et al., 2013).

Sorghum bicolor (L.) Moench has been tested for Lsi1 protein sequence by Vatansever et al. (2017) who discovered two homologous genes codifying for the transporter channels (Vatansever et al., 2017). A recent phylogenetic analysis of Si transporters across the biological kingdoms shows a high level of conservation of Lsi2 in embryophytes (Coskun et al., 2019), indicating an early evolution which suggests that it should be present in all C_4 species. The study identified the presence of 5 Lsi2 homologous in *Sorghum bicolor* but highlighted the complexity and the diversity of Lsi2 transporters in the plant kingdom. To add to the uncertain picture of the genetics of Si absorption, mutations have also recently been discovered in sorghum by Markovich et al. (2019) who proved the existence of varieties unable to absorb high quantities of silica. In sorghum, a unique amino acid compositional protein involved in the precipitation of silica in the silica cells (Slp1) has also been discovered (Kumar et al., 2017a, 2020). These data suggest that Si plays a fundamental role in sorghum physiology since its deposition seems to be strictly regulated. While data exploring the genetics of Si content are available (Markovich et al. 2019; Vatansever et al., 2017), no information on the mechanisms for Si uptake in Pennisetum glaucum (L.) R. Br. and Eleusine coracana (L.) Gaertn. Is available so far.

4.1.1 Phytoliths classification and morphotypes functions

Researchers coming from different fields have for a long time tried to classify phytoliths based on their anatomical and physiological features. The morphological classification has led to the code of nomenclature published first by Madella and colleagues (2005), and then recently revised and expanded by Neumann et al. (2019). The code of nomenclature names specific morphotypes basing the classification on anatomical features. Most of the work related to nomenclature and anatomical classification of phytoliths involve species and genus recognition for paleoecological and archaeological purposes. Nevertheless, physiologists and micro archaeologists have long appreciated grouping classes of morphotypes for the mechanisms that led to their formation, to use phytolith assemblages as proxies for the reconstruction of the growth environment. The methodologies for classifying phytoliths according to their formation process can be divided into three macro categories: the one suggested primarily by Madella et al. (2009) that is based on the distinction between the mechanism governing the deposition process, and the two proposed by Hodson (2016) and Kumar et al. (2017). Hodson (2016) and Kumar et al. (2017) combine Madella's explanation with a more structural theory involving the distinction between cellular districts in which Si deposition occurs. Madella et al. in 2009, basing their hypothesis on the studies published previously by Hodson and colleagues in 2005 and Blackman and Parry in 1968, divided phytoliths into genetically determined shapes and environmentally controlled silicification mechanisms. Genetically controlled morphotypes derived from the silicification of cells devoted to active accumulation of silica, like the silica-cells in the leaf epidermis or the so-called short cells (dumbbell [bilobate for the ICPN 2.0], saddle, cross phytolith types). The silicification of these cells result in the production of phytoliths independently of the growing conditions. On the contrary environmentally controlled silicification mechanisms has been associated with all those cells that do not have a genetic control on silica deposition in their lumen, like the epidermal elongates, as also proposed by Piperno (1989). The mechanism of deposition of environmental morphotypes has been related to the water flow and the excess of monosilicic acid in the plant (Richmond and Sussman, 2003). In the study by Madella and colleagues (2009) a third category of morphotypes has been also mentioned, composed mostly by trichomes (acute bulbosus for the ICPN 2.0), whose silicification is not necessarily genetically controlled but stimulated by the specific function of the cells to improve pest resistance (Richmond and Sussman, 2003). Nonetheless, Madella et al. advocated for a better understanding of the complex relationship between Si, water uptake and water loss in different taxa and within the same taxon. In 2016 Hodson published a review where phytoliths development has been related to their chemistry, proposing that phytolith composition should be associated with the mechanism of silicification (Hodson, 2016). In this case phytoliths were divided into those where silicification started from the cell wall and those where Si first precipitated in the cell lumen. This classification was supported by previous studies by Sangster et al. (1983), Hodson (1985), Perry et al. (1987), Law and Exley (2011), Zhang et al. (2013) and Leroux et al. (2013) that had already focused on mechanisms of silica deposition in specific species. In 2019 Hodson expanded and elaborated on the same grouping proposed in 2016 (Hodson, 2019). Three main types of silica deposition in plants were described: one where silica is deposited onto a carbohydrate matrix such as the cell wall; one where silica deposition lacks an obvious matrix onto which it is deposited, and it happens mainly in the cell lumen; and one where the deposition occurs into the intercellular spaces, mechanism which does not lead to phytolith formation (Hodson, 2019). Therefore, only two main types of phytolith in plants existed, the cell wall types which are formed on a carbohydrate matrix, and the cell lumen types which are not. Cell wall phytoliths are composed by three different subgroups: (1) cells where the wall silicified while the protoplast remained intact; (2) cells where the silicification started from the secondary cell walls and developed by almost filling the lumen by forming a secondary wall layer; (3) cells where silica is deposited in the space between the primary cell wall and the protoplast. There is then a transition class where the silicification is first deposited in the primary cell wall, but later grows into the lumen. Cell wall morphotypes have been listed as: trichomes and papillae of the glumes, elongates and trichomes of the inflorescence bracts and lemma, papillae, trichomes, and elongates of the leaf and of the culm and endodermal cells of roots. Plant scientists (e.g., Coskun et al., 2019) have focused on the cell wall silicification process, recording many important mechanisms, including transport, detoxification of metals and defence against pathogens. Cell lumen deposition, particularly in the epidermis, is apparently more common in grasses than in the rest of the plant kingdom. Cell lumens phytoliths are mainly represented by silica short cells, produced both in leaves and inflorescence bracts, and bulliform cells and elongates of the leaves. It is interesting to note that this division takes into consideration that the same morphotype forming in different tissues can be classified into different categories (e.g., elongates can be both cell lumen or cell wall morphotypes depending on the species or the tissue where they are formed). The third classification has been proposed by Kumar and colleagues in 2017 (Kumar et al., 2017a, 2017b). Kumar and colleagues combined Madella's and Hodson's classification by suggesting three different classes of phytoliths, divided for the mechanism of silica deposition and simultaneously for the genetic or environmental control over it. The three groups of phytoliths are (1) passive cell wall silicification types- environmentally controlled: distinctive of mature and/or intensely transpiring organs, where the condensation of Si is driven by dehydration. In this case a continuous supply of Si infiltrates the cell walls, and its deposition occurs without being metabolically controlled by the cells. (2a) A controlled cell wall silicification where silica is deposited directly in the cell wall matrix, even before the organ is exposed to the transpiration. Silicification is possibly triggered by the cell wall polymers inducing the silicic acid polymerization (cell wall silicification morphotypes where the process is genetically controlled). (2b) In some of these cases, during the silicification of the cell wall the protoplast dies, allowing spontaneous silica deposition driven by transpiration in the cell lumen, without further organic primers. In this last instance there is a first genetically controlled silicification followed by dehydration driven by transpiration. (3) Silica cell silicification genetically controlled, where the Si is deposited on the external side of a functional plasma membrane, possibly in a volume that contains materials that enhance silica deposition, independent of transpiration (option 2 of the cell wall silicification suggested by Hodson (2019)). With this alternative classification of phytoliths Kumar and colleagues merged the two main theories about phytolith formations: the first based on a passive mode of silicification, relying on organ transpiration and implying a spontaneous process resulting from auto-condensation of Si molecules (Euliss et al., 2005; Miller Rosen and Weiner, 1994; Sangster and Parry, 1971; Yoshida et al., 1962); and the second suggesting that phytolith formation should be catalysed by molecules genetically controlled (Twiss et al., 1969). The hypothesis has already received support from various researchers who have hypothesised the simultaneous involvement of both mechanisms in the process of phytolith formation (Markovich et al., 2019; Motomura, 2004).

4.1.1.1 Phytolith classes

The innovative classification methods show that it is not always straightforward to attribute a phytolith morphotype to a specific group since the deposition process depends on (1) the genetics related to Si uptake (active accumulator/passive accumulator species); (2) and the deposition tissue (figure 4.1). For instance, elongates demonstrated to be lumen phytoliths in the leaf of rice and cell-wall type in the inflorescence bracts of

Avena sativa L. (Hodson 2019). Thus, the challenge is to understand which function each morphotype plays once formed in the plant shoots to link their morphology to the depositional process and use them as palaeoenvironmental proxies. This section will list the main categories of phytoliths in relation to their function as epidermal cells and their Si deposition process, attempting to unify information from different classification proposals.

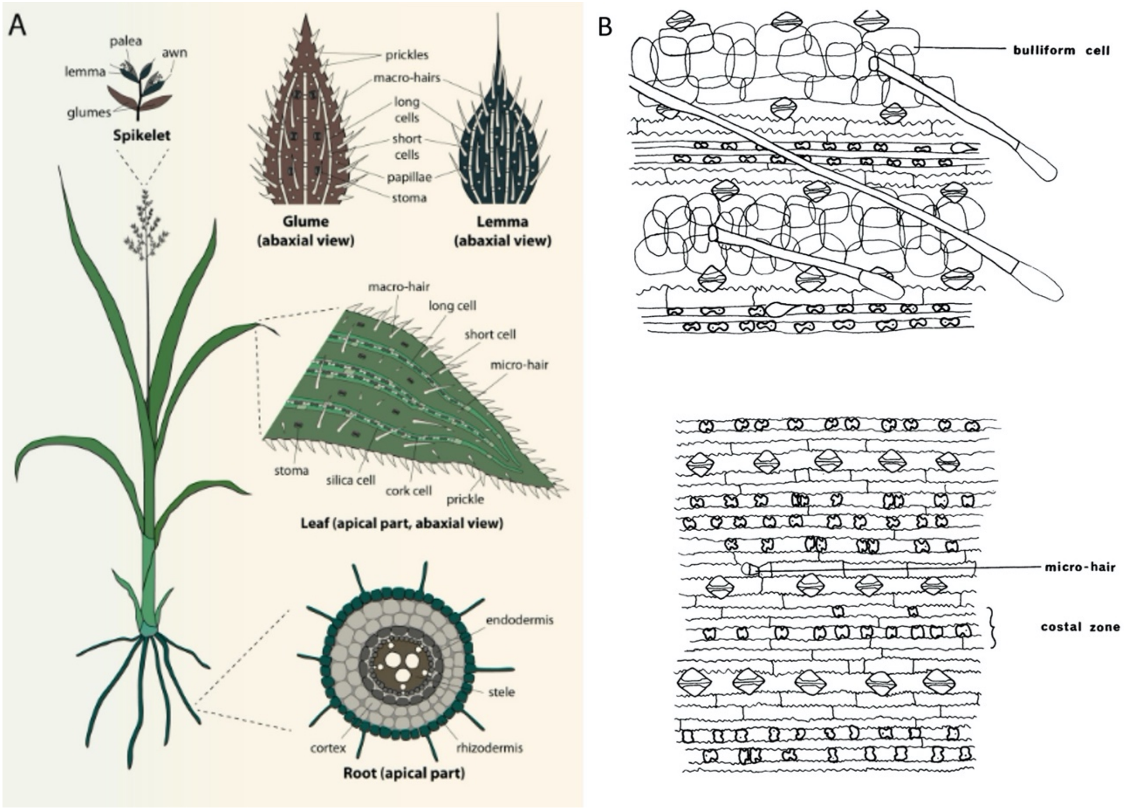

Figure 4.1 - A) Silica deposition diagram in grasses represented by Kumar et al., 2017. B) Leaf blade epidermis phytolith anatomy, drawn by Renvoize 1985. Hairs = trichomes; long cells = elongates for ICPN 2.0.

Bulliforms

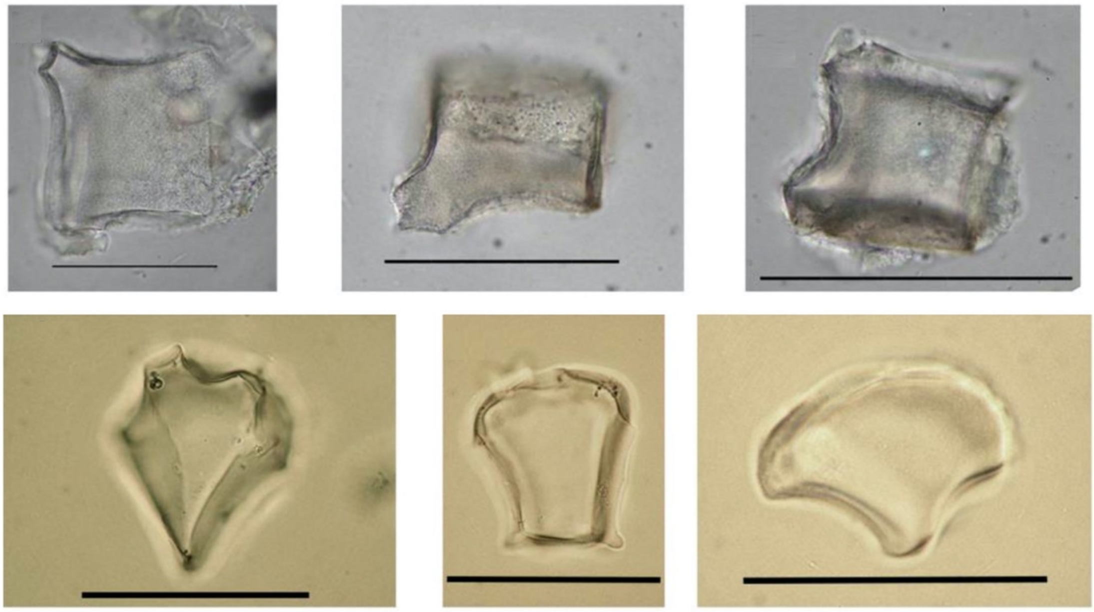

Figure 4.2 - Bulliforms parallel-blockies (first row) and bulliforms flabellate of *Poaceae* (second row). Scale bar: 50 µm. Pictures of the International Code for Phytolith Nomenclature (ICPN) 2.0. (2019).

Bulliforms are large, specialised epidermal or mesophilic cells that occur in longitudinal rows in the furrows of the leaf surface (Esau, 1965). Their role is to facilitate leaf rolling and folding by decreasing turgor pressure when plant water potential decreases, through the filling and emptying of the considerable vacuoles they are equipped with (Speranza and Calzoni, 2005). Bulliforms as phytoliths can be subdivided into parallel (blockies in the ICPN 2.0) and flabellate (Neumann et al., 2019) united, apart from the physiological role, by the bigger 3D size in respect to the other morphotypes. Bulliforms parallel are solid phytolith, resembling a parallelepiped with 6 or more faces that can be both slightly convex or concave (figure 4.2). Bulliforms flabellate are heavily built, fan-shaped phytolith, where the outline of the apical part is generally convex and may be facetate (Neumann et al., 2019). Bulliforms are very common in the leaves of *Cyperaceae* and *Poaceae* but they are also found in other monocotyledonae, as well as in dicotyledonae and conifers (Strömberg, 2004). In rice, larger bulliform have been proved to occur in locations with higher temperature, precipitation, and water levels (Wang et al., 2019), suggesting that their formation is somehow correlated to environmental factors. Bulliforms silicification is probably a lumen type (Hodson 2019) since granules of silica have been observed in the protoplast of the corresponding living cells (Motomura et al., 2004). However, silicification can probably happen spontaneously in the cell wall, resulting from Si auto-polymerization (Kumar et al., 2017b). Silicification in the bulliform cell walls is rather characteristic of mature, sometimes senescent leaves (Sangster and Parry, 1971). Also, Parry and Smithson (1958) suggested that bulliform cells accumulate silica at a later stage, after silicification occurred first in elongates epidermal cells, since silica depositions in bulliform may completely alter their water storage functions causing the desiccation of the developing leaf.

Elongates

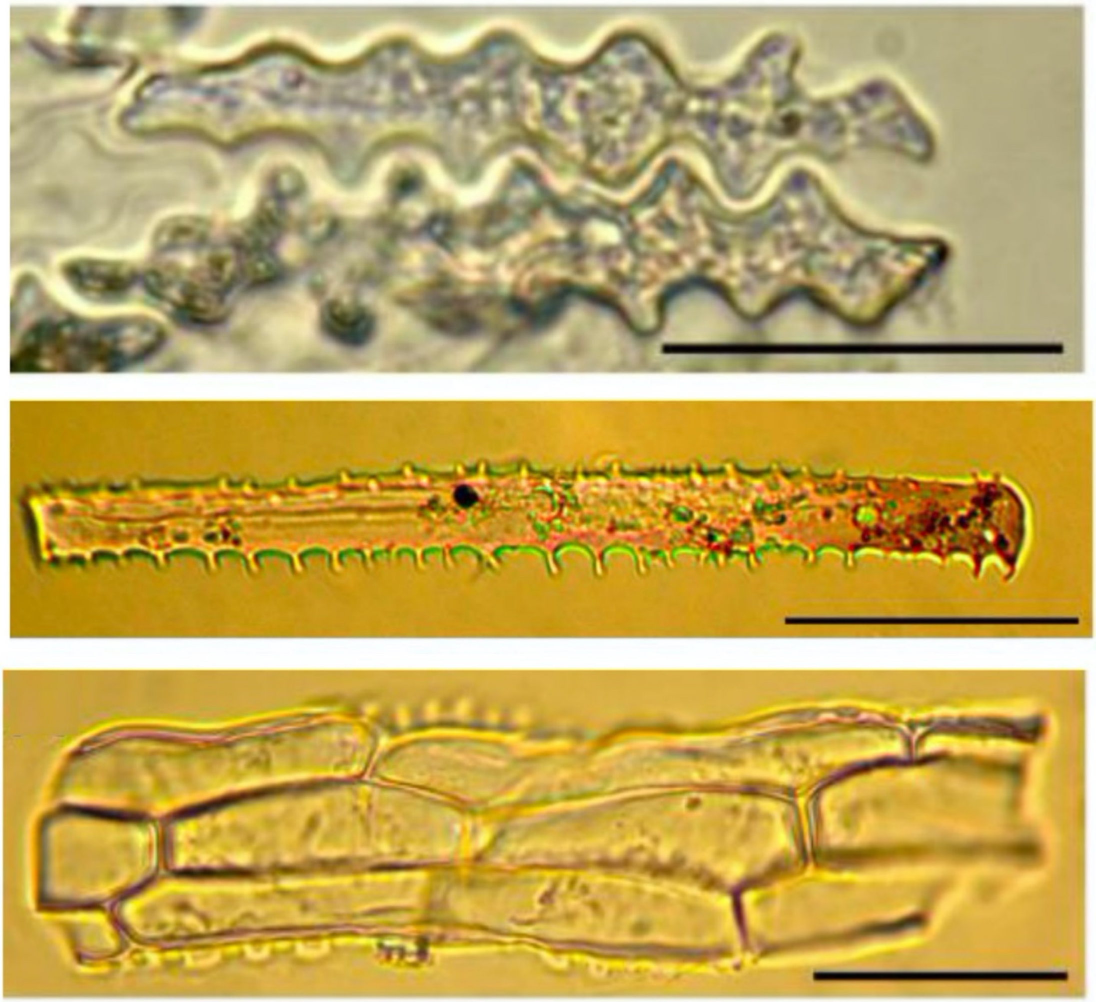

Figure 4.3 - Leaf elongate sinuate phytoliths (archaeological) (first row); Elongate dentate of *Poaceae* (second row); Leaf elongates entire in silica skeleton connection (third row). Scale bar: 20 µm. Pictures of the International Code for Phytolith Nomenclature (ICPN) 2.0. (2019).

Elongate phytoliths are defined as the fundamental epidermal cells, greatly elongated and parallel to the long axis of the tissue (Twiss et al., 1969), considered almost 2D shapes (Neumann et al., 2019). Elongates are commonly classified by the morphology of their margins that allow to distinguish among leaf and inflorescence types. Their margins can vary from smooth without projections of elongates entire to the formation of long wavy lobes with concave sides of elongates dentate and dendritic (Madella et al., 2005; Neumann et al., 2019) (figure 4.3). Inflorescence elongates, especially dentate and dendritic morphotypes, display patterns that may be diagnostic at some taxonomic levels (Ball et al., 2016a; Rosen, 1992). Elongates are also useful to distinguish domesticated cereals (Albert et al., 2008; Ishida et al., 2003; Madella et al., 2014; Portillo et al., 2012; Shillito, 2011; Zhang et al., 2013); and they are common in wild grasses (Novello and Barboni, 2015). Elongates, for their thin structure, are frequently found broken in soils and sediments. If they are not broken very often it is because they have been kept within articulated silica skeletons (Berlin et al., 2003; Helbaek, 1960; Pető, 2013; Rosen, 1992). Among other epidermal cell types, elongates accumulate silica in their walls as soon as the leaf starts to transpire (Sakai and Sanford, 1984). In the inflorescence, silica accumulation starts about one week after glume emergence in the outer wall and silica deposition seems to initiate two weeks after the glume emergence, together with cell death (Hodson et al., 1985). While the lumina of the long cells is infilled with silica, also the plasmodesmata, connecting adjacent cells, is filled with silica forming the characteristic extraflexed margins of decorated elongates (Neumann et al., 2019). Elongates silicification seems to depend on water evaporation (Kumar et al., 2017b) and correspond to the passive cell wall silicification types- environmentally controlled morphotypes.

Short cells

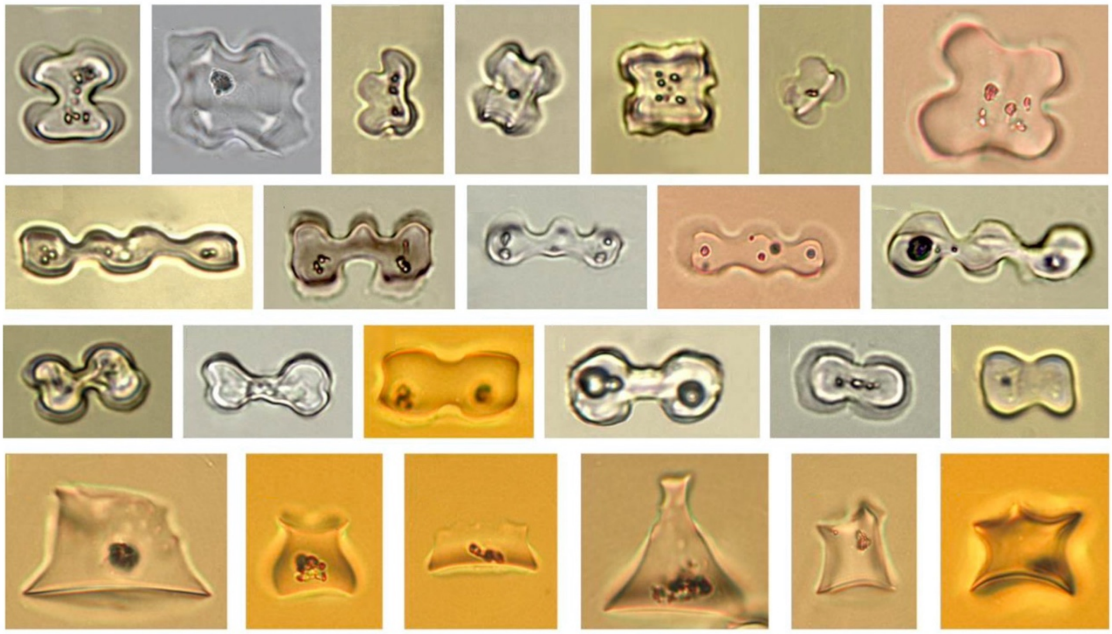

Figure 4.4 - Crosses of genus *Bracharia*, *Digitaria*, *Lithachne, Pennisetum* and *Sorghum* (first row); Polylobates of genus *Pennisetum* and *Sorghum* (second row); Bilobates of genus *Adropogom*, *Bracharia*, *Pennisetum* and *Sorghum* (third row); Rondels from *Poaceae* leaf. The length and the width of each cell is between 10 μm and 20 μm, apart from polylobates that measure up to 30 μm in length. Pictures of the International Code for Phytolith Nomenclature (ICPN) 2.0. (2019).

Short cells are defined as solitary, scattered, small (comparing to elongates, bulliforms and trichomes) cells with or without associated silica, present in the grass epidermis in between long cells (Neumann et al., 2019; Renvoize, 1985). These cells are well connected to their neighbouring short cells, but not to the neighbouring long cells (Lawton, 1980). Short cells are metabolically very active with large nucleus and high numbers of ribosomes and mitochondria (Kumar et al., 2017). Silica short cells, also called only “silica cells” are found as stretches of short cells in the epidermis of leaves (Sangster, 1970), stem internodes (Kaufman et al., 1981) and abaxial epidermis of glumes (Hodson et al., 1985). In sorghum they have been found in rows above and below the longitudinal secondary vasculature of the leaf (Kaufman et al., 1985). In contrast to the limited availability of silica cells along the large veins, silica cells along the small veins are abundant, leaving open the hypothesis that their formation can be somehow linked to the water supply and/or a structural support (Kumar et al., 2017a). Different morphotypes are included into the category of short cells: bilobates, crosses, polylobates, rondels, saddles and trapezoides (Neumann et al., 2019) (some examples are provided in figure 4.4). Depending on the photosynthetic pathway one morphotype is more abundant in respect to others: NAD-ME (*Chloridoideae* as finger millet) are rich in saddles, while NADP-ME (sorghum) are characterised by dumbbell-shaped in general (bilobates, crosses and polylobates) (Kumar et al., 2017b; Rossouw, 2009). Short cells Si composition is apparently distinct from that of elongates and trichomes: Zancajo et al. (2019) showed that bilobate silica cells have a different silica molecular structure compared with trichomes and elongates which indicates a variation in the atomic organisation of the mineral. They are among the first types of cells to be silicified (silicification occurs in hours), sometimes even before the tissue is exposed to the atmosphere (Kaufman et al., 1981; Kumar et al., 2017b; Motomura et al., 2006; Sangster, 1970), suggesting that the process is genetically controlled. However, because in silica cells the body of the cell is fully mineralized, it is assumed that their silicification occurs only in mature cells in the distal part of the leaf tissue, far from the basal cell multiplication space (Kumar et al., 2017a). In addition, tests made by Sangster and Parry (1971) and Kumar et al. (2017a) indicate that silicification does not occur in dead short-cells (even in live leaves) and testify that short cells die with or without a consequence of silica deposition. Both considerations demonstrate a metabolic control over the process. In addition, in rice, the walls of silica cells lignify before silica deposition (Zhang et al., 2013). Thus, given all these considerations, even if previous studies proposed that silica cells get silicified in a passive way (Gallagher et al., 2015; Sangster, 1970; Sangster and Parry, 1971), Hodson (2019) classifies them into the lumen, and Kumar et al. (2017a) suggest a genetic control over their formation.

Stomata

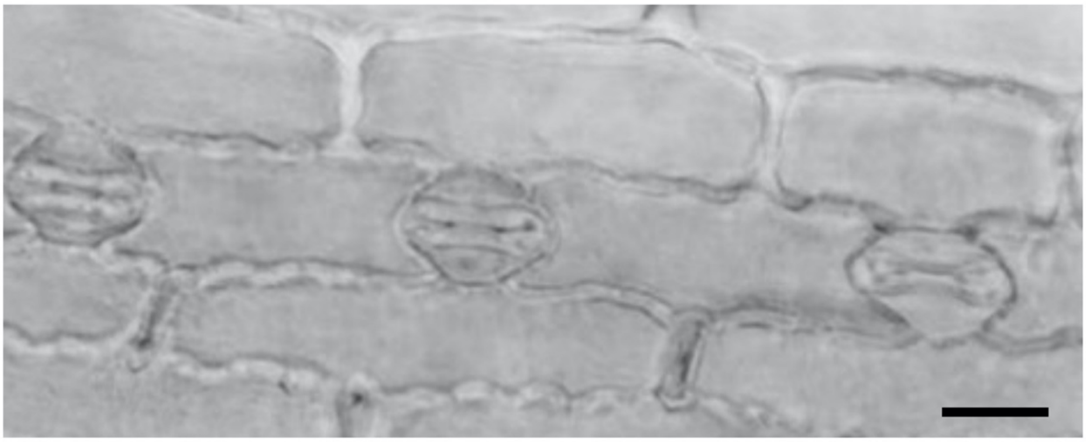

Figure 4.5 - Three stomata phytoliths in anatomical connection with elongates sinuate in a leaf of *Panicum miliaceum*. Scale bar: 20 µm. Picture by Out and Madella (2016).

All higher plants possess stomata, pores surrounded by two guard cells, found in the epidermal surface of chlorophyllous organs (Louguet et al., 1990). By adjusting guard cell turgor pressure, plants can alter stomatal pore aperture, moderating gas exchange rates between the leaf interior and the atmosphere (Kollist et al., 2014). The signals governing the opening and closing of stomata depends on many environmental factors, including changes in temperature, light intensity, atmospheric CO_2 concentration, air humidity, and soil moisture content (Assmann and Jegla, 2016; Chaves et al., 2016; Farquhar and Sharkey, 1982; Mott, 2009; Schroeder et al., 2001). Prabhakar (2004) described over 62 different stomata structures which differ for the anatomy of the guard cells and for the disposition of the subsidiary cells around them. Eudicots typically have kidney-shaped stomata, formed on the leaf epidermis without a predetermined location (Pillitteri and Dong, 2013). Grasses display characteristic dumbbell-shaped stomata with neighbouring subsidiary cells (Bertolino et al., 2019), collectively termed a stomatal complex (Rudall et al., 2017). In grasses, stomatal development is constrained to the leaf base, with stomatal pores forming in specific cell rows adjacent to veins (Hepworth et al., 2018; Rudall et al., 2013). A stoma phytolith is considered the complete silicified structure composed by guard cells and subsidiary structures (figure 4.5). Sometimes the external side of the stoma can present decorated structures (Neumann et al., 2019). Silicification of stomata is not largely discussed, probably because it is taken for granted that stomata phytoliths are formed by spontaneous precipitation of silica after water removal from the cells by transpiration. Nevertheless, even though most of the water that evaporates from the mesophyll passes through stomata, silicification of the guard cells occurs late in leaves (Motomura et al., 2004). Not all the stomata present on the leaf surface silicify (Motomura et al., 2004), contrary to what happens to trichomes and elongates that accumulate silica in the cell walls as soon as the leaf starts to transpire (Kumar et al., 2017b). The process of silica deposition in stromal cells has not been described yet but Hodson (2016) suggested a possible cell wall mechanism.

Trichomes

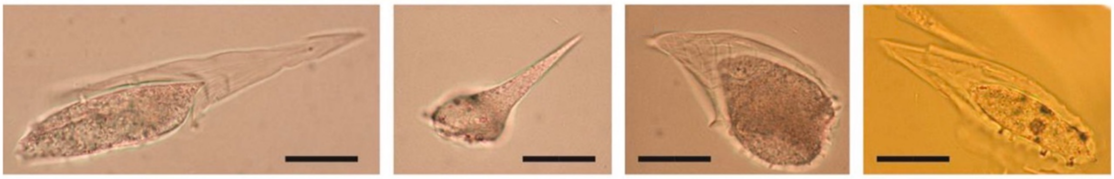

Figure 4.6 - Acute Bulbosus-trichome phytoliths of *Poaceae* spikelet. Scale bar: 20 µm. Pictures of the International Code for Phytolith Nomenclature (ICPN) 2.0. (2019).

This morphotype includes a wide morphological spectrum characterised by an acute apex and a bulbous base (Neumann et al., 2019). Trichomes are found in leaves and chaff of many grasses (Piperno, 2006, 1989; Runge, 1999), in monocotyledones (Piperno, 1983, 1988; Strömberg, 2003), and in low frequencies, in certain dicotyledons, and palms (Piperno, 1989; Strömberg et al., 2013). In grasses, trichome phytoliths are called acute bulbosus and represent the infilling of the interior of a hair cell (figure 4.6). In other taxa, it is not always clear whether the acute apex shape with a bulbosus base is the infilling of an epidermal appendage such as a trichome, or a cell type from another tissue (Neumann et al., 2019). In grass trichomes, silica is deposited during two stages that initiate with the panicle emergence in the inflorescence while it is before the leaf exposure to the outside emergence in the leaf (Kumar et al., 2017b; Sakai and Sanford, 1984). The process starts at the tip, induced by the outer wall primers and it is followed by a spontaneous Si precipitation inside the lumen driven by the degradation of the protoplast and evapotranspiration (Markovich et al., 2019; Motomura et al., 2006). In all those grasses where trichomes are bicellular (having a basal and a cap cell), silicification initiates from the basal cell, probably at the cell wall. The process is then followed by the passive lumen infilling when the cell dies and by the cap cells silicification, which happens only after leaf opening (Motomura, 2000). Kumar et al. (2017) and Hodson (2019) suggested that the lumen of trichomes is filled with silica without the in-growth of the cell wall enhancing the silicification. The process might be driven either by passive transpiration (Bennett, 1982), or facilitated by an organic matrix accessible only after cell death (Sangster and Parry, 1971). However, the transpiration-driven passive mode of silicification is supported by the fact that lumen silicification continues long after cell death (Kumar et al., 2017) and because the number of silicified hairs increases with age (Motomura, 2004), suggesting a dependency on transpiration.

4.1.2 Phytolith functions

Once the existence of a regulatory gene has been demonstrated for silica deposition in plant tissues, research has focused on trying to understand which function phytoliths play once formed in the plant shoots. Indeed, while in the past phytoliths have been considered to play a minor role, if any, in plant physiology, it has been recently suggested by Katz and colleagues (2019) that Si content is a plant functional trait which plays a role in improving the fitness of the plant. It has been proposed that grasses became phytolith accumulators long before they started occupying open habitats, at least 37 million years before grass-eating mammals evolved, suggesting that phytolith accumulation did not change because of selective pressure from mammalian herbivores (Strömberg et al., 2016). Essentially, while in the past phytoliths have been considered a secondary effect of the water uptake, where Si particles were absorbed unintentionally with no possibility for the plant to discard the inorganic compound, now several physiological mechanisms have been connected with phytolith formation. Nevertheless, much work remains to be done to demonstrate the genetics and physiology related to Si content. Hence, even though the precise mechanical properties of Si remain elusive, it has recently been argued that Si accumulation has little if any intracellular role (Coskun et al., 2019), while the biosilicification of phytoliths can act as a protection against numerous environmental stresses (Nawaz et al., 2019). It has been demonstrated that Si has a positive effect on mineral nutrient balance, improving resistance to heavy metal stresses and water storage by diluting salts in the cells (Daoud et al., 2018; Mateos-Naranjo et al., 2013). Opaline silica has also been proven to have a strong impact in preventing pathogens infection by making difficult to digest abrasive tissues covered by silicified hairs (Fauteux et al., 2005; Oliva et al., 2021). The same genes which regulate lignin deposition govern phytolith formation (Piperno et al., 2002). Since phytoliths are energetically inexpensive in comparison to the deposition of organic carbon-based polymers and, in addition, they stimulate light inceptions and so photosynthesis (Strömberg et al., 2016), it has been suggested that phytolith deposition in arboreal plants helps young tissues to stiffen before organic materials (e.g., lignin) are deposited (Schoelynck et al., 2010).

Recently it has been suggested that Si could play a fundamental role in response to water stress conditions as well. Indeed, Si could change the hydraulic conductivity and the osmotic adjustment (Hosseini et al., 2017). Opaline silica could form a thin layer in the epidermal cells that gives rigidity to the tissues, prevents water losses, and confers a beneficial support in water stressed environments (Rodrigues et al., 2003; Yoshida et al., 1962). Phytoliths can harden externally the walls of the veins to keep water supply running in limited water conditions, when vessels, due to dryness of the tissue, may lose turgidity (Meunier et al., 2017). Opaline silica can provide better light inception leading to improved assimilation rate and chlorophyll biosynthesis (Cornelis and Delvaux, 2016; Goto et al., 2003); Phytoliths formation may also impact water use efficiency acting directly on the stomata movement (Gao et al., 2020). These innovative studies, which connected the formation of phytoliths with the ability to withstand water stress conditions, form the scientific rationale for researching a relationship between phytolith assemblages and plant water availability.

4.2 Phytoliths as proxies for past agriculture

Major and minor domesticated crops have been analysed for their phytolith production to recognize their presence in the archaeological records. *Poaceae*, as for example maize (*Zea mays* L., Sp. Pl.), rice (*Oryza sativa* L.), barley (*Hordeum vulgare* L.), einkorn (*Triticum monococcum* L.), emmer (*Triticum dicoccum* L.), wheat (*Triticum aestivum* L.), sorghum (*Sorghum bicolor* L.Moench) and pearl millet (*Pennisetum glaucum* L.R.Br), as well as all monocotyledoneae, have been discovered to produce many diagnostic classes of phytoliths (Ball et al., 2016a; Iriarte, 2003; Piperno, 2006; Piperno and Pearsall, 1998). Phytoliths have been also recognized as potential tools for usage/crop-processing activities reconstruction (García-Granero et al., 2018; Portillo et al., 2017; Rashid et al., 2019), space organization (Ball et al., 2016b) and to research ancient plant domestication (e.g., Ball et al., 2016b; Cabanes et al., 2011; Harvey and Fuller, 2005; Jenkins et al., 2016, 2020; Madella et al., 2016; Miller Rosen and Weiner, 1994; Rudall et al., 2014). This is because some silica bodies, formed in the vegetative tissues, have taxonomically significant shapes that consent, up to certain level, the species/genus-specific identification (e.g maize, einkorn, emmer wheat or rice) (Piperno, 2006) and anatomical differentiation (Logan, 2012). In addition, due to their inorganic composition, phytoliths preserve better than other organic materials (as seeds or starches) in the archaeological record for long periods of time (Harvey and Fuller, 2005). Although also phytoliths are affected by pre- and post- depositional taphonomy, bias can be reduced at minimum thanks to stratigraphic control and calculation of representativeness (Madella and Lancelotti, 2012; Strömberg, 2009; Zurro, 2018). Fire-related problems, such as charring or cooking events, have been proved not to affect phytolith structure since they only melt at 900-1100ºC (Brochier, 2002; Elbaum et al., 2003). Several studies demonstrated that temperatures below 500ºC prevent a second water release that induces phytoliths to shrink or to warp (Jones and Milne, 1963; Parr et al., 2001; Pearsall, 2016). Elbaum et al. (2003) proved that below 700ºC phytoliths showed a very weak shift in their refractive index. Therefore, phytoliths can stand cooking procedures, fires and carbonization events. As a consequence, phytoliths can be retrieved from grinding stones, ceramics, stone tools, teeth, faeces and soils and sediments providing valuable information about diet and human subsistence practices (Ball et al., 2016b; Dal Corso et al., 2018, 2017; Lancelotti et al., 2021; Lombardo et al., 2020; Madella et al., 2002; Portillo et al., 2009; Shahack-Gross et al., 2014).

Apart from species presence and crop-processing detection, phytolith assemblage could also inform about field extensions and composition. Research on modern soils phytolith composition compared to the existent vegetation demonstrated an accurate representation of the plant environment with a range of hundreds of square metres (Blinnikov et al., 2013). Moreover, indices based on the relative abundance of species specific morphotypes retrieved from soils, have been proven to give trustable results in the representation of the general vegetation (Bremond et al., 2005), the physiognomy of the plant communities and the transition between them (Aleman et al., 2012; Alexandre et al., 1997). Therefore, phytoliths have been applied for reconstructing past environments and changings in their vegetational compositions (e.g., Bremond et al., 2005; Ermish and Boomgarden, 2022; Leng et al., 2009; Lombardo et al., 2019; Stamm et al., 2020; Webb and Longstaffe, 2002).

4.3 Can phytoliths be proxies of plant water availability?

Given the great applicability of phytoliths studies to different fields of archaeobotany and paleoecology, they have been also tested as indicators of past water availability with the idea of reconstructing agricultural management. Phytoliths are associated with water availability to plants since the translocation of Si in the plant aerial structures is mediated by the water flux and it happens by diffusion, with the transpiration stream acting as the main motive force (Nawaz et al., 2019). In the tissues with photosynthetic activity, such as leaves, culms and to a minor degree inflorescences, the formation of specific morphotypes is most likely due to supersaturation by transpiration-driven water loss (Schaller et al., 2013). However, as mentioned above, the role and function of Si in plants is not yet fully understood and this impairs the comprehension of the mechanisms that drive phytolith formation, which have been described so far as a mutual contribution of genetics and environmental factors. Although transpiration has been investigated as one of the main factors, producing high concentrations of silicic acid in plant shoots (Euliss et al., 2005), it is clear that it is not the only parameter that accounts for biosilica precipitation. So far, many scientists tested phytoliths indicators to assess water availability. Being agricultural management the main subject of this research (and not palaeoclimatic reconstruction), all the indexes or ratios based on C_3/C_4 phytolith abundance have been excluded for the purpose, even if they have been found as good indicators of rainfall zones (e.g., Cordova, 2013). These ratios, based on the relative abundance of wild C_4 species in respect to C_3 (which are better adapted to higher rainfall levels) do not serve when assemblages coming from agricultural scenarios are tested. For the purpose of analysing agricultural contexts, the relative production, morphometry and composition of genetically controlled morphotypes *versus* morphotypes that are supposed to be environmentally controlled have been considered. Rose and Weiner (1992) first suggested using the dimension of silica skeletons, under the assumption that a greater water absorption by the plant led to a greater silica uptake, which induced the formation of larger silica conjoined structures. The methodology has been applied to the archaeological context by Katz et al. (2007) who hypothesised the presence of dry farming in the Chalcolithic of the South Levant through observation and counting the elongates included in the silica skeletons found in the archaeological record. Nevertheless, taphonomic mechanical breakage of silica skeletons (Madella and Lancelotti, 2012) or even phytolith extraction method (Jenkins, 2009) can cause cell disarticulation and compromise the effectiveness of silica skeletons as proxy. The paleoenvironmental study of Bremond et al. (2005) suggested a humidity-aridity index of phytoliths, based on the abundance in the assemblage of fan-shaped bulliforms, hypothesising that the more the plant transpire and/or suffer water stress, the more silicified bulliform cells they would produce. This research is noteworthy, even if it is not directed at archaeology, because it places for the first time the bulliforms flabellate of the *Chloridoideae* in the shortlist of possible morphotypes whose production is influenced by transpiration. Madella et al. (2009) suggested the use of a ratio of fixed-genetically determined (short cells) - to sensitive-environmentally controlled (elongates and stomata) morphotypes. The ratio was tested in bread wheat, emmer wheat and barley. Madella and colleagues categorised elongates and stomata as water sensitive shapes and excluded trichomes whose formation should be mostly connected to pest resistance (Richmond and Sussman, 2003). The methodology was applied by Weisskopf et al. (2015) to detect water availability of cultivated rice fields, and by Jenkins et al. (2016) who built an experimental cultivation of barley and durum wheat to test the effectiveness of the ratio in these additional cereals. Both studies showed distinctive phytolith patterns between wet and dry plants. Jenkins et al. (2020) proposed for the first time to apply the same ratio to sorghum. The same ratio has been applied by Ermish and Boomgarden (2022), who tested how sensitive to fixed phytoliths ratio and elongates proportion of maize respond to wet-dry conditions. The results of both Jenkins et al. (2020) and Ermish and Boomgarden (2022) highlighted strong differences between well irrigated and less-irrigated crops, indicating that the methodology is effective even in crops with reduced transpiration. These last studies, concluded with promising results, were the basis on which this thesis research was developed, leaving open the possibility that this ratio of sensitive to fixed morphotypes could also work for indicating water availability in sorghum, pearl millet and finger millet. On the other hand, it must be mentioned that not all the research conducted on the effect of water levels on phytoliths production have resulted in positive correlations between the two variables. Katz et al. (2013) tested phytolith accumulation in eight different Asteraceae species collected in five different sites in Israel, characterised by different rain-fall levels which ranged from 900 mm to 120 mm per year. The results of the research highlighted no difference among sites but showed more substantial discrepancy among species.

4.3.1 Silicon isotopes in phytoliths

The uptake of silicic acid by plants is associated with a biochemical Si fractionation which turns out to be equal to plant-water = 0.9988, meaning that phytoliths in plants have lower δ^{30}Si values than silicic acid in soils (Ding et al., 2008). Phytoliths, which are the only source of silica deposition in plants, have been proved to become increasingly enriched in ^{30}Si by up to +3.3‰ as the pool of available silicic acid becomes depleted in ^{28}Si (Opfergelt et al., 2006). The cause of this phenomenon is, so far, attributed to both equilibrium effects (Stamm et al., 2020) and kinetic effects (Rayleigh fractionation) (Geilert et al., 2014; Poitrasson and d'Abzac, 2017), which explain the accumulation of heavy isotopes in the upper parts of the plant. Essentially, this would involve the lighter ^{28}Si isotope being more reactive, and so more likely to be deposited earlier in the plant tissues. Thus, proportionately more ^{28}Si isotope would be deposited in the culm phytoliths, and a greater proportion of ^{30}Si and ^{29}Si would continue in the transpiration stream to the leaf sheath or the inflorescence bracts. In the sheath the same fractionation occurs, leading to an even greater concentration of heavier isotopes in the leaf blade (Hodson et al., 2008). Even though it is known that heavier isotopes of Si accumulate in the upper parts of plants in a variety of taxa, there is much that remains to be investigated about the process of fractionation along the plant transpiration stream, including whether it is affected by environmental factors. Nevertheless, once the existence of flow-dependent fractionation was proven, it has been proposed to study whether or not δ^{30}Si or δ^{29}Si are related to the water level as well as to the Si availability during plant growth (Ding et al., 2008). Theoretically, if Si is absorbed along with water, when the hydric supply is consistent, there will also be a higher absorption of Si. Thus, if the pool of ^{28}Si, ^{29}Si and ^{30}Si increases, discrimination due to the Rayleigh fractionation should increase, as more ^{28}Si and ^{29}Si is available and precipitates more rapidly. The assumption has been sustained by the results presented by Opfergelt et al. (2006) regarding the Si absorption and distribution of *Musa acuminata* (Colla). The study concluded by theorising that preferential passage of light Si isotopes contributes to a progressive isotopic fractionation of the solution moving from the uptake sites in roots to the transpiration termini in lamina. Geilert et al. (2014) who conducted a series of flow-through experiments to investigate Si isotope fractionation during abiotic precipitation, sustained the theory too by concluding that the flow-through system could be an approximate model for a transpiring plant where water is continually flowing through the xylem when temperature ranges between 10°C and 20°C.

With time, however, it was realised that the application of δ^{30}Si study is complicated by the isotopic heterogeneity of the phytoliths within a plant (Prentice and Webb, 2016). It has been demonstrated that two Si transporters, Lsi1 and Lsi2 that are involved in the uptake of Si through plant roots, but it still remains to be tested whether or not they display any selectivity with regards to the different silicon isotopes (Köster et al., 2009) and if some of these have yet to be discovered along with their mutants. To further complicate the scenario, recently Frick et al. (2020) have presented the hypothesis that brings together all previous theories and somehow "negated" the importance of discrimination of each individual Si channel. They argued that whether Si is taken up through Si permeable channels (orthologues of Lsi1) or distributed passively with the water flow, both pathways favour the light isotopologue because of its greater diffusion coefficient (Sun et al., 2008). Furthermore, when evaluating δ^{30}Si, the phytolith deposition process needs also to be considered (Hodson et al., 2008). Isotopes are directly evaluated in phytoliths that, as described above, can be formed in the cell lumen or alternatively, in different districts of the cell membrane. This may imply that Si undergoes different selective processes before the deposition and as a consequence its δ^{30}Si could change depending on the morphotype. In conclusion, before using δ^{30}Si as an environmental proxy, we need to understand how much Si channels affect discrimination and how much the process of Si precipitation in phytoliths affects different plant species fractionantion.

4.3.1.1 δ^{30}Si and δ^{29}Si issues related to the archaeological investigation

The recent findings on δ^{30}Si preempts the direct application of this analysis to archaeological phytolith assemblages. Apart from the above-listed problem, there are issues that arise when analysing archaeological phytoliths, not deriving from modern experimental cultivation. Ding et al. (2005) in rice and Hodson et al. (2008) in wheat demonstrated that culm and rachis display negative δ^{30}Si values, while the leaf sheaths and inflorescence bracts all have positive values, with leaf blades having the highest. Whether this is due to a biochemical issue related to the abundant presence of Si transporters; or whether it results only from a kinetic phenomenon consequent to the movement of Si along the transpiration stream, the fact remains that different tissues present different levels of δ^{30}Si discrimination. Thus, a bulk archaeological sample, which includes phytoliths coming from all these organs, will have a δ^{30}Si value that is approximately the mean of its component parts. In addition, Prentice and Webb (2016) demonstrated that $\delta^{30}Si$ values of phytoliths are modified when phytoliths partially dissolve, warning of the possibility that the taphonomic process could change the outcome. Therefore, to solve these archaeological related issues, it is essential to start with experiments on the modern (as in this study) to clarify, for example, what the differences are between tissues and between morphotypes of the same tissue and between species. Working in an analytical manner, starting with individual differences between phytoliths, will make it possible in the future to read the result of archaeological bulk analyses (means of the phytoliths coming from different tissues and species). Working with several modern replicas of plants subjected to different phytological stresses should enable to read whether there are differences in the composition of $\delta^{30}Si$ and $\delta^{29}Si$ of phytoliths depending on plant management and environmental conditions.

4.4 Conclusions

Phytoliths are microscopic plant silica bodies preserved in modern and fossil soils and sediment, as well as in archaeological contexts. They record unique characteristics of past vegetation and, unlike palynomorphs and macrofossils, do not require specific preservation conditions (Strömberg et al., 2018) such as waterlogging or charring, although they can be subject to mechanical breakage (Jenkins, 2009) and variable dissolution in alkaline environments (Albert et al., 2006; Cabanes et al., 2011; Fraysse et al., 2009). Phytoliths are often more ubiquitous in archaeological sites than macrobotanical remains, particularly in prehistoric sites, and they have been long used for understanding early agricultural practices (Ball et al., 2016b). All these characteristics make phytoliths good proxies for archaeological research and also allow them to be used as indicators for C_4 plants, which, as described in chapter 3, present certain issues. However, many methodological aspects of phytolith formation and classification are not yet fully resolved and/or standardised. Although their morphology has been widely discussed, their formation process remains uncertain. Great variability among species and tissues, both for composition and morphology of phytoliths, has been evidenced in the last decades of research on the subject. The major objective is to try to understand the function performed by phytoliths in order to understand the regulation of their formation process, and the possible response to water levels. Hence, it is of paramount importance to focus on the study of modern plants, in order to gain a deeper understanding of the mechanisms that drive phytolith deposition and their dependence on watering.

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Chapter 5 - Methodology

5.1 Experimental cultivations

The experimental cultivation is the first and fundamental building block of this research and the starting point for answering the scientific questions on which this investigation is based. The aim was to cultivate crops in monitored conditions where the environmental variable "water" was under strict control while the plants grew in a field-like context. In fact, the experimental cultivation endeavoured to simulate real field conditions subjecting plants to climatic fluctuations, such as temperature, light intensity, wind speed etc., that could be compared to those of an open-air field. Considering that there is an open debate on whether or not the concentration of phytoliths can be directly related to the amount of rainfall/irrigation [total water added] (see Katz et al., 2013), the experimental cultivation system was set up to try to explore this relationship in more detail, separating the concept of available water (either rainfall, irrigation or total water added) and that of total water used (such as transpiration). In addition to total water added, efforts have been made to measure the transpiration rate for each plant. Indeed, the quantity of water provided to the plant, either in the form of rainfall or of controlled irrigation, does not necessarily mean that the same amount of water has been absorbed by the plant. Conversely, transpiration values give a certain indication of the amount of water that the plants actually used for growing and, as a consequence, to produce phytoliths. Transpiration additionally takes into account the inherent ability of species and varieties to absorb and utilise water and to respond to soil water availability, light, and atmospheric conditions. In this study transpiration and rainfall were two positively correlated variables and both of them gave an indication of the environmental growing conditions. In order to measure transpiration, while keeping the plant in growing conditions similar to those in the open field, cultivation took place inside lysimeters, long PVC tubes filled with soil and arranged in rows inside an open-air pit. The lysimeters allowed us to simulate natural growth conditions while keeping the watering level under strict control. Moreover lysimeters allowed the calculation of the transpiration rate on a weekly basis. Experiments were conducted at the ICRISAT facilities (Patancheru, India), in a semi-tropical environment during the dry season, to more accurately approximate the environmental condition RAINDROPS was interested in studying, i.e., the one in drylands. The aim of the experimental cultivation was to grow crops that would respect plant development, and to produce material that could then be used to build a phytolith comparison collection for correlation with archaeological samples.

5.1.1 Selection of landraces

Traditional landraces were preferred to observe the variability of phytolith production in un-improved crops, which should exclude any possible recent modern change in the genetics of biosilica accumulation. The process to chose the landraces used the Climatic Research Unit TS3.10 Dataset, a Google Earth Pro application that divides the entire world into high-resolution climate grids (Harris et al., 2013) combined with climate data from Climate-Data.org (https://en.climate-data.org accessed on 15 October 2018) and Climate North West Knowledge, (https://climate.northwestknowledge.net accessed on 22 August 2022) which provide high resolution data on world temperature, precipitation and relative humidity. Thus, the landraces selected originate either from the area of interest (Ethiopia, Pakistan, Sudan), or -for the African crops- from areas assumed to be domestication centres and where the climatic parameters are comparable to those of interest (Kenya and Tanzania) (Fuller and Boivin, 2009). Landrace seeds of pearl millet (*Pennisetum glaucum* L.R.Br), finger millet (*Eleusine coracana* L.Gaertn) and sorghum (*Sorghum bicolor* L.Moench) were obtained from the collection of ICRISAT (Patancheru, India) genebank (tables 5.1-5.2) and they are all FAO in trust varieties (varieties equipped with crop passport descriptors, collected and recorded in agreement with the country of origin and local communities).

	Sudan	**Ethiopia**	**Pakistan**	**Kenya**	**Tanzania**
Climatic data ***-Mean temperature*** ***-Average sun hours*** ***-Precipitation*** ***-Rainy days*** ***-Humidity***	32.79 ºC 10.9 h 70 mm 13 days 25.16%	27.63 ºC 10.5 h 519 mm 60 days 37.16%	27.34 ºC 10.7 h 152 mm 15 days 44.66%	30.03 ºC 10.5 h 213 mm 25 days 47.91%	24.89 ºC 10.3 h 602 mm 63 days 49.33%
Sorghum	S1: IS23074 S2: IS23075 S3: IS3450	S4: IS11060 S5: IS11061 S6: IS38025 S7: IS17415	S8: IS35215 S9: IS35216 S10: IS35217		
Pearl millet	PM1:IP13327 PM2: IP9859 PM3: IP9888 PM4: IP13329	PM5: IP2367	PM6: IP18119 PM7: IP18019 PM8: IP18020 PM9: IP18021 PM10: IP18022		
Finger millet				FM1: IE2511 FM2: IE3476 FM3: IE5335 FM4: IE6717 FM5: IE7509	FM6: IE4450 FM7: IE4456 FM8: IE4458 FM9: IE3193 FM10: IE2822

Table 5.1 - Selected landraces from ICRISAT genebank used for the experimental cultivation with a. the acronym used to identify them in this study (e.g., PM1) and b. their accession number. Climatic data are expressed as the annual mean. Precipitation and rainy days represent the total annual condition. Climatic indices are specific to the region of interest from which the samples come. Weather data was collected between 1991-2021 for the variables temperature, precipitation, humidity, rainy days. Sunhours use the timeframe 1999-2019. The only area where there should be remarkable changes in respect to the past climatic conditions is Sudan. The Sudanese archaeological samples of RAINDROPS come from the African Humid period, where conditions were supposedly wetter than at present (Iacumin et al., 2016).

Table 5.2 Species	**Landraces**	**Local name**	**Collection site**	**Latitude**	**Longitude**	**Elevation**	**Country-Province**
Sorghum	***S1: IS23074***		Sharom	15.2899	32.459999	399	Sudan
	S2: IS23075		Sharom	15.2899	32.459999	399	Sudan
	S3: IS3450	*Ankolib*	Jabal Al Awliya	15.1400	32.290001		Sudan
	S4: IS11060		Fadis valley outside of Dire Dawa	14.3999	39.380001	1600	Ethiopia
	S5: IS11061		Fadis valley outside of Dire Dawa	14.3999	39.380001	1600	Ethiopia

	S6: IS38025	*Mashila azebo*	Adigrat; Agame	14.2739	39.422016	2450	Ethiopia-Tigray
	S7: IS17415		Adigrat; Agame	14.2739	39.422016	2450	Ethiopia
	S8: IS35215		Arifwala; Sahiwal district	30.2800	73.059997	195	Pakistan-Punjab
	S9: IS35216		Arifwala; Sahiwal district	30.2800	73.059997	195	Pakistan-Punjab
	S10: IS35217		Arifwala; Sahiwal district	30.2800	73.059997	195	Pakistan-Punjab
Pearl millet	***PM1: IP13327***			15.42	32.419998		Sudan-Khartoum
	PM2: IP9859			16.2299	32.360001		Sudan-Khartoum
	PM3: IP9888			13.1700	34.779998		Sudan-Eastern
	PM4: IP13329			13.1300	35.189998		Sudan-Eastern
	PM5: IP2367	*Gelacha mill*	Gelalcha	8.85999	42.689998		Ethiopia-Hararge
	PM6: IP18119		Dullewali Bhakkar	31.8299	71.430001	140	Pakistan-Punjab
	PM7: IP18019		Arifwala; Sahiwal district	30.2900	73.069999	195	Pakistan-Punjab
	PM8: IP18020		Arifwala; Sahiwal district	30.2900	73.069999	195	Pakistan-Punjab
	PM9: IP18021		Arifwala; Sahiwal district	30.2900	73.069999	195	Pakistan-Punjab
	PM10: IP18022		Arifwala; Sahiwal district	30.2900	3.069999	195	Pakistan-Punjab
Finger millet	***FM1: IE2511***						Kenya
	FM2: IE3476						Kenya
	FM3: IE5335						Kenya
	FM4: IE6717						Kenya
	FM5: IE7509						Kenya
	FM6: IE4450		Makutopola	-5.98000	35.729999	1140	Tanzania-Dodoma

	FM7: IE4456		Siuyu	-4.82000	34.740000	1720	Tanzania-Singida
	FM8: IE4458		Kisaki	-4.9	34.729999	1600	Tanzania-Singida
	FM9: IE3193		Ikokoto	-7.78000	35.689998	1400	Tanzania-Iringa
	FM10: IE2822		5N of Iringa	-7.73999	35.709999		Tanzania-Iringa

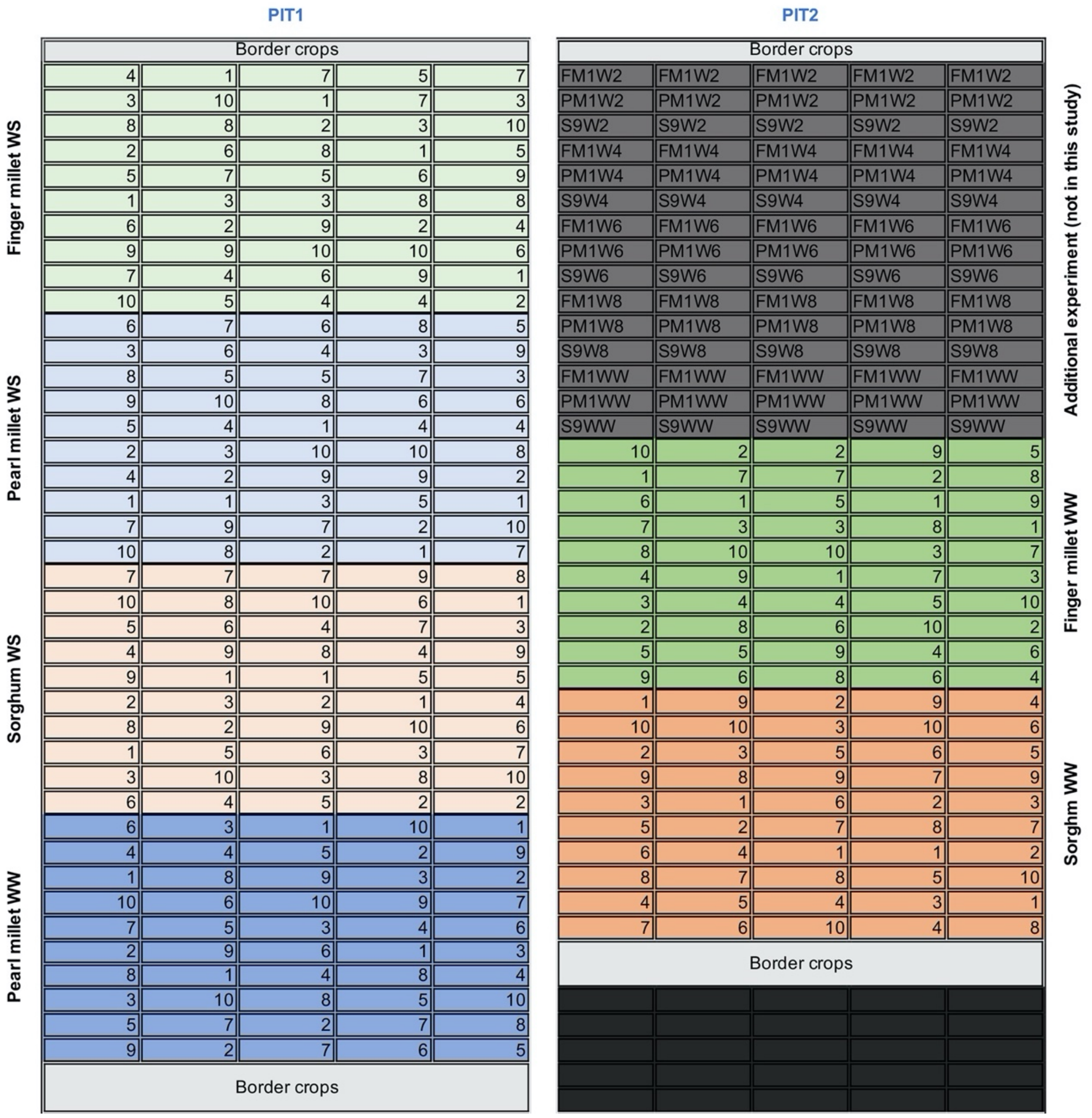
Table 5.2 - Landrace passport information from ICRISAT genebank dataset.

5.1.2 Experimental cultivation in lysimeters

Two experimental cultivation seasons were conducted in ICRISAT, Patancheru, India (17°31' N 78°16' E) in the period between February and May 2019 and 2020. Both seasons were conducted using the same parameters and experimental design. To recreate field conditions while keeping a tight control on water-related parameters, the plants were cultivated in lysimeters (PVC tubes of 200 cm in length and 25 cm in diameter). These were positioned in two parallel pits about half a metre apart (figures 5.1-5.2) to simulate real field conditions regarding plant spacing (11 plants m^2), soil availability for ground water exploration (2 m of soil available for each plant in tubes of 25 cm diameter), and general growing conditions as the tubes are placed outdoors (but covered by a rain-out shelter in case of rain). The tubes were filled with a mixture of 1:1 Alfisol-Vertisol and several crops had been previously cultivated in the same lysimeters with the same soil mixture. At the time of the first experiment, the lysimeters had been fallowed for about 8 months. Lysimeters did not receive fertilisers in the near past. Lysimeters provide control on water availability and transpiration rates thanks to their regular weighing, which allows transpiration assessment and possible re-watering to desired soil water content levels (Vadez et al., 2011b). The technique of cultivation through lysimeters involved three stages: (1) while the plants were in the early stages of development, the cylinders were watered regularly to gradually reach the 100% of field capacity; (2) when the plants grew to c. 20 cm, about three weeks after planting, and field capacity was reached, then the soil surface was covered with a plastic sheet and 2 cm of low-density polyethylene granules, in order to block about 90% of soil evaporation (Vadez et al., 2011a); (3) the lysimeters were weighed weekly to evaluate plant water loss from transpiration only (Vadez et al., 2011b). In 2020 cylinders were weighed every two weeks, due to an overload of work for the ICRISAT staff during the lockdown provoked by Covid-2019 emergency. The results are not expected to be altered, except for a lower resolution of the data as the measurements were taken every fortnight and not every week.

Figure 5.1 - Lysimeters in ICRISAT (Patancheru, India) during the experiment in 2019. Picture a) 15 days after the sowing; b) 56 days after the sowing.

Two different water managements were tested to simulate water availability in (a) irrigated conditions which also acted as control (well watered, WW hereafter) and (b) rainfed conditions in arid environments (water stressed, WS hereafter). WW plants were watered weekly to maintain 80% of soil field capacity, which is the optimum for crops well adapted to dry climates (Zaman-Allah et al., 2011). With WS replicas, the intention was to imitate a real rainfed scenario where water is available at the beginning of the plant life cycle but then scarce/absent during the reproductive stage (Portmann et al., 2010). Therefore, WS plants were watered like WW plants until flowering, and then irrigation was then stopped until maturity. The average of the total water added for WW plants is given in table 5.3 and the weekly oscillation of transpiration is shown in figure 5.3. The values of water added and water transpired weekly for each replica in both cultivations are available in the appendices (file A5.1).



PIT1

	Border crops				
Finger millet WS	4	1	7	5	7
	3	10	1	7	3
	8	8	2	3	10
	2	6	8	1	5
	5	7	5	6	9
	1	3	3	8	8
	6	2	9	2	4
	9	9	10	10	6
	7	4	6	9	1
	10	5	4	4	2
Pearl millet WS	6	7	6	8	5
	3	6	4	3	9
	8	5	5	7	3
	9	10	8	6	6
	5	4	1	4	4
	2	3	10	10	8
	4	2	9	9	2
	1	1	3	5	1
	7	9	7	2	10
	10	8	2	1	7
Sorghum WS	7	7	7	9	8
	10	8	10	6	1
	5	6	4	7	3
	4	9	8	4	9
	9	1	1	5	5
	2	3	2	1	4
	8	2	9	10	6
	1	5	6	3	7
	3	10	3	8	10
	6	4	5	2	2
Pearl millet WW	6	3	1	10	1
	4	4	5	2	9
	1	8	9	3	2
	10	6	10	9	7
	7	5	3	4	6
	2	9	6	1	3
	8	1	4	8	4
	3	10	8	5	10
	5	7	2	7	8
	9	2	7	6	5
	Border crops				

PIT2

	Border crops				
Additional experiment (not in this study)	FM1W2	FM1W2	FM1W2	FM1W2	FM1W2
	PM1W2	PM1W2	PM1W2	PM1W2	PM1W2
	S9W2	S9W2	S9W2	S9W2	S9W2
	FM1W4	FM1W4	FM1W4	FM1W4	FM1W4
	PM1W4	PM1W4	PM1W4	PM1W4	PM1W4
	S9W4	S9W4	S9W4	S9W4	S9W4
	FM1W6	FM1W6	FM1W6	FM1W6	FM1W6
	PM1W6	PM1W6	PM1W6	PM1W6	PM1W6
	S9W6	S9W6	S9W6	S9W6	S9W6
	FM1W8	FM1W8	FM1W8	FM1W8	FM1W8
	PM1W8	PM1W8	PM1W8	PM1W8	PM1W8
	S9W8	S9W8	S9W8	S9W8	S9W8
	FM1WW	FM1WW	FM1WW	FM1WW	FM1WW
	PM1WW	PM1WW	PM1WW	PM1WW	PM1WW
	S9WW	S9WW	S9WW	S9WW	S9WW
Finger millet WW	10	2	2	9	5
	1	7	7	2	8
	6	1	5	1	9
	7	3	3	8	1
	8	10	10	3	7
	4	9	1	7	3
	3	4	4	5	10
	2	8	6	10	2
	5	5	9	4	6
	9	6	8	6	4
Sorghm WW	1	9	2	9	4
	10	10	3	10	6
	2	3	5	6	5
	9	8	9	7	9
	3	1	6	2	3
	5	2	7	8	7
	6	4	1	1	2
	8	7	8	5	10
	4	5	4	3	1
	7	6	10	4	8
	Border crops				

Figure 5.2 - Experimental design of 2019 cultivation. Each rectangle corresponds to a cylinder marked with the genotype corresponding number. Blue is for pearl millet, green for finger millet, orange for sorghum, black for empty cylinders and grey for border crops of pearl millet. Border corps were distributed around the entire perimeter of the pits with the aim of protecting the experimental plants, increasing the chances of pollination and making the plants in the marginal rows grow as if they were competing for environmental resources in the middle rows. Bright colours are for WW while dull colours are for WS.

	2019	2020
	WW	**WW**
Sorghum	34.99 ± 0.93 L	20.98 ± 0.54 L
Pearl Millet	35.80 ± 1.16 L	15.48 ± 0.57 L
Finger Millet	48.93 ± 0.82 L	15.92 ± 0.62 L

Table 5.3 - Total water added on average with ± the standard error for each cylinder in the two experimental replicas after cylinders weighing started. Water is expressed in litres (L).

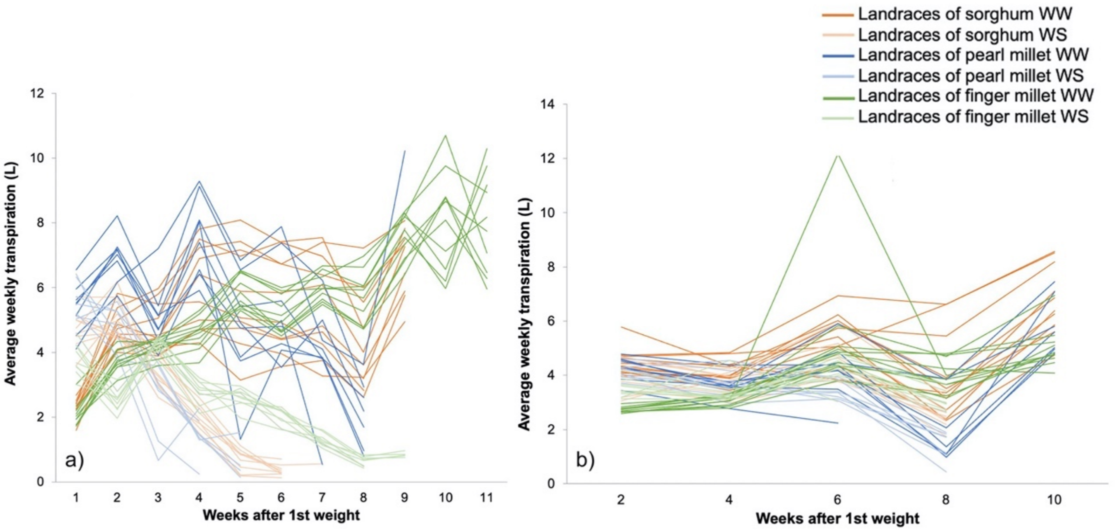


Figure 5.3 - Weekly average transpiration in the year 2019 (a) and in 2020 (b). Water has been administered on the basis of the oscillation of the weekly transpiration. Blue lines are for pearl millet, green for finger millet, and orange for sorghum.

Five replications for each treatment per landrace have been cultivated (n = 100 cylinders per species, total = 300 cylinders per year). Genotype replications were randomised in the two pits in order to prevent unintended environmental effects (e.g., heat gradient from the pit walls) on only some genotypes. To simulate real plant spacing in the field, two plants of the same genotype (same replication) were planted in each cylinder. The experiment followed a complete randomised block design with species-water treatment as the main block and genotypes as sub-factor randomised within each block (figure 5.2). Data on temperature and relative humidity were collected every 30 minutes by 2 Gemini Tinytag Ultra 2 TGU-4500 Datalogger placed on top of the crop canopy. The average temperature across the season in 2019 was 32.28 ± 0.10 ºC (max: 50.5 ºC, min: 19 ºC) and relative humidity at 42.57 ± 0.23 % RH (max: 87%, min: 13.5%). In 2020 the average temperature across the season was 28.64 ± 0.52 ºC (max: 42.8 ºC, min: 11.6 ºC) and relative humidity at 61.70 ± 1.47 % RH (max: 98%, min: 18%). Flowering dates were also recorded, and biomass measured after harvest. The physiological parameters measured (data available in the appendices - file A5.1) are described in table 5.4. We harvested WW plants when the panicles were mature (when at least ⅗ replications reached maturity) and the WS plants when their transpiration rate dropped below 10% of the WW value for the same period, indicating stomatal closure (cuticular water loss only) (Schuster et al., 2016). Panicles, leaves and stems were first dried in ICRISAT at 60-70 ºC for one week and then transported to the Laboratory for Environmental Archaeology of the University Pompeu Fabra (Barcelona, Spain) where samples were processed and phytoliths extracted.

Variable measured	Definition
Total water used (TWU)	TWU= total water transpired by the plant throughout its life cycle (sum of the water transpired every week in L), also found in literature as Total Water Transpired (TWT) (Zaman-Allah et al., 2011). It was calculated according to GEMS team guidelines (http://gems.icrisat.org accessed on 3 February 2019) indicating as weekly transpiration the difference between the weight of the cylinder one week and the weight of the same cylinder the following week (considering the water evaporation of the soil interrupted by the polyethylene beads and the negligible increase in weight of the plant) + the litres of water added to the cylinder during that week.
Total water added (TWA)	Water directly given to the plant during irrigation (L) to maintain the 80% of the soil field capacity. It was calculated according to GEMS team guidelines (http://gems.icrisat.org accessed on 3 February 2019) as the difference between the weight of the cylinder at the soil field capacity and the weight of the same cylinder the considered week, minus the 20% of the litres of water representing the soil field capacity.
Transpiration efficiency (TE)	TE = biomass (g)/total water transpired (L) (Vadez et al., 2014).
Biomass	Sum of the dried components (g) (leaves + culms + panicles).
Leaves	Total dry leaf weight (g).
Leaves Percentage %	Percentage of leaves weight in respect to the total weight of the plant (g).
Stem	Dry stem weight (g).
Panicle	Panicles (seeds + chaff) dry weight (g).
Flowering time	Flowering time coincides with the number of days until blooming.
Harvest Index (HI)	HI= panicle yield (g) /biomass (g) (Hay, 1995).

Table 5.4 - Summary of the physiological parameters measured and how they were calculated.

5.2 Phytolith analysis

5.2.1 Samples selection

It was decided to reduce the analysed dataset of genotypes by subsampling (table 5.5). The genotypes at a physiological level proved to be inhomogeneous within the same species, so it was decided to analyse the most diverse ones, obtaining a representative selection of the various physiological responses to watering. To choose those landraces that showed the greatest differences, capturing variability within and among the species, all measured physiological parameters have been taken into account, with a focus on transpiration efficiency, which is a parameter that simultaneously considers biomass production, water consumption, transpiration capacity and thus drought adaptation (figure 5.4). For each genotype two replications were selected and one plant for each replication. For one of the selected genotypes grown under WW conditions, both plants corresponding to one replication have decided to be analysed, to also test for possible intra replication variability. Selected landraces are listed in tables 5.5.

	Sudan	**Ethiopia**	**Pakistan**	**Kenya**	**Tanzania**
Sorghum	S2: IS23075	S5: IS11061 S6: IS38025	S8: IS35215 S9: IS35216		
Pearl Millet	PM1:IP13327 PM2: IP9859	PM5: IP2367	PM7: IP18019 PM9: IP18021		
Finger Millet				FM1: IE2511 FM2: IE3476	FM6: IE4450 FM7: IE4456

Table 5.5 - Selected landraces for phytolith extraction.

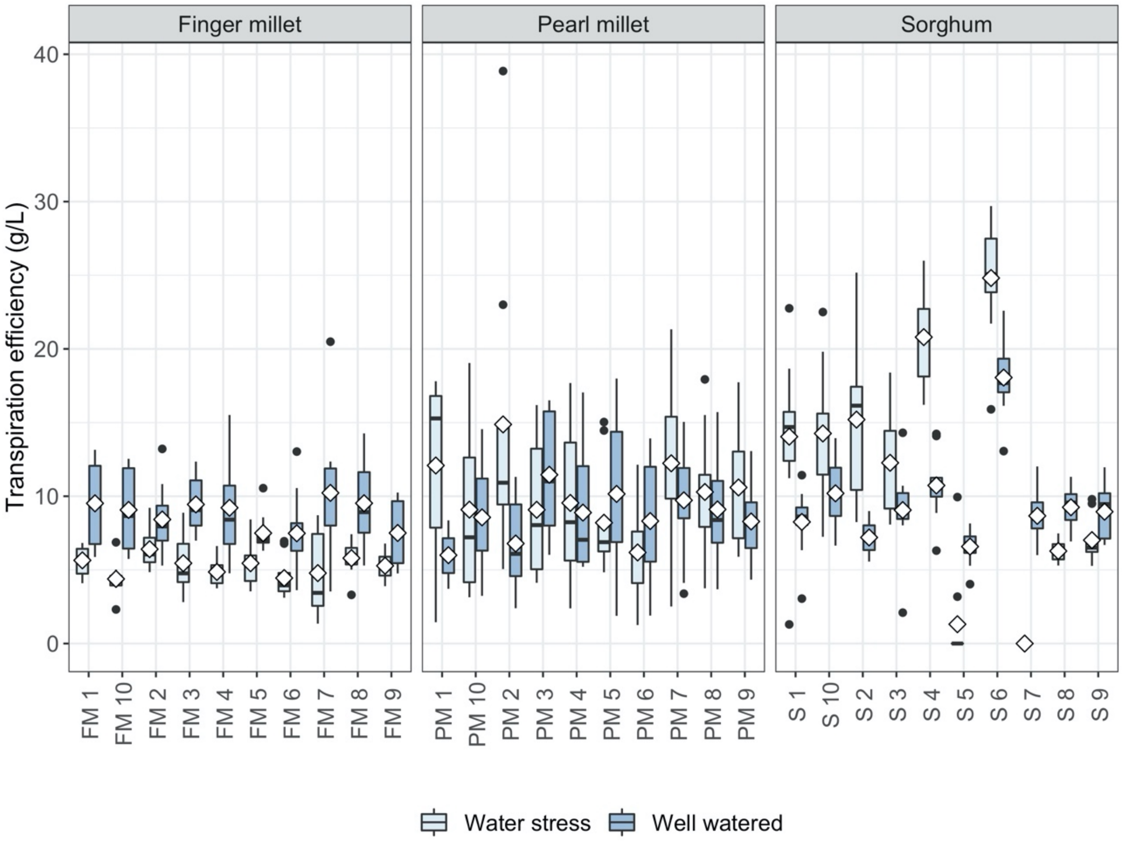


Figure 5.4 - Boxplots of transpiration efficiency by water treatments sorted for species. Horizontal bar: median, white diamond: mean, black spot: outliers.

Leaves are the organs where transpiration rate is highest and therefore where the maximum production of phytoliths unfolds, including most of those supposedly related to hydration mechanisms, such as stomata and bulliforms, that are produced neither in the chaff nor in the stem. For this reason mainly leaf samples were analysed, first bulk leaf samples (samples 2019 - chapter 7) and then young and old leaves separately (samples 2020 - chapter 8) (table 5.6), to try to approximate whether there was a difference in silicon accumulation depending on the stage of leaf development as well. “New leaves” correspond to the 5 youngest leaves from the upper apex of the stem, “old leaves” are the mature leaves at the base of the plant. The chaff tissues were analysed in the knowledge that a large proportion of the phytoliths essential for genus/species recognition in cereals accumulate in these organs and are, therefore, recognisable in the archaeological record. In addition, some morphotypes are ubiquitous (e.g., trichomes or elongates entire) and morphologically identical in leaves and chaff, making it impossible to discriminate in the archaeological record which come from inflorescence and which from the leaves. As a consequence chaff samples were included in the analysis. Chaff samples have been processed for phytolith production both for sorghum and pearl millet, while finger millet did not produce any panicles neither in WS nor in WW conditions, neither in 2019 nor in 2020.

	2019			**2020**		
	WW	**WS**	**Total**	**WW**	**WS**	**Total**
Sorghum	12 leaves	10 leaves	22	21 new leaves 21 old leaves 17 chaff	20 new leaves 20 old leaves 14 chaff	113
Pearl Millet	12 leaves	10 leaves	22	20 new leaves 20 old leaves 20 chaff	20 new leaves 20 old leaves 15 chaff	115
Finger Millet	10 leaves	8 leaves	18	18 new Leaves 18 old leaves	18 new leaves 17 old leaves	71

Table 5.6 - Summary of the analysed samples.

5.2.2 Phytolith extraction and classification

5.2.2.1 Phytolith recovery

Phytolith extractions from leaf fresh tissues have been performed at the laboratory of environmental archaeology at the University Pompeu Fabra. The extraction method was developed specifically for this thesis and based on a comprehensive review of the available methodologies. Although the study of Si in plants has become a subject of interest in different disciplines, there is still no consensus on standardised techniques for phytolith recovery and quantification, mainly due to the fact that different types of samples require specific extraction processes. Phytoliths are found and can be extracted from various materials (i.e., modern plant tissues, soils/sediments, dental calculus, faeces, or human artefacts), that require specific chemicals, and equipment to be processed. For the extraction of phytoliths from fresh plant material different methods are in use but no uniform technique for the recovery, identification and counting of phytoliths exists. The conditions for extracting phytoliths may differ depending on the type of tissue, the hydration level of the sample and the species of origin. For this study, different methodologies were tested but none resulted in a clean extraction, free of residual organic material and with the morphotypes preserved in their form and composition. For this reason, following various tests, a new hybrid technique to extract phytoliths from fresh C_4 grass material have been derived. Finding a balanced methodology for extracting phytoliths from fresh/dried plant tissue, that combines the elimination of organic matter and high preservation of silica skeletons is extremely important for several reasons. First because modern plants are the main source of material for the creation of reference collections and to estimate phytolith production, assemblage compositions and plant/soil treatments (Fraysse et al., 2009; Gallaher et al., 2020; Ge et al., 2020; Gu et al., 2016; Guerriero et al., 2016; Hodson, 2019; Katz et al., 2013; Zancajo et al., 2019). Second, several studies utilised phytoliths from modern plant tissues for assessing occluded carbon ($\delta^{13}C$), oxygen ($\delta^{18}O$) and silicon ($\delta^{30}Si$) isotopes (Frick et al., 2019, 2020; Hodson et al., 2008; Prentice and Webb, 2016; Roy et al., 2020; Shahack-Gross et al., 1996; Yang et al., 2020). Last, the issue of the abundance of silica skeletons in tested samples (i.e., how to extract samples with preserved silica skeletons, how to handle phytolith counting in case most are included in multi-celled structures) is not widely discussed.

Hence, a specific extraction and counting protocol was developed for this study, coupling a dry ashing technique with a subsequent wet oxidation and an alternative way of counting phytoliths in a slide. The procedure has been published open access in protocols.io (dx.doi.org/10.17504/protocols.io.q26g74mb8gwz/v2 version 2 uploaded on 30 September 2022).

Protocol background

When approaching biosilica extraction from modern plant material, both the heterogeneous, anisotropic, and non-crystalline nature of phytoliths must be taken into account so as not to subject biosilica bodies to procedures that could change their chemical-physical characteristics. The mechanism of phytolith formation is considered heterogeneous, due to a gradual centripetal growth along undifferentiated fibrillar elements (Schulz-Kornas et al., 2017) and for the proportion of water molecules incorporated in the opal during phytolith formation, which is influenced by the growth microenvironment (Cabanes et al., 2011; Elbaum et al., 2003). The growth microenvironment where phytoliths form is determined by the plant tissues, the conditions of growth and the phylogenesis of the species (Andriopoulou and Christidis, 2020; Cabanes et al., 2011). Thus, phytoliths may consist of different mineralogical phases. These differences may result in a variation in the refractive index, the mineralogy of phytoliths and their solubility. As a consequence, it is difficult to produce a universal protocol that preserves the values of the refractive index, solubility and chemical composition of phytoliths for all the groups of plant species (e.g., monocotyledoneae, eudicotidae) and probably even for the same species grown in different environmental settings.

Another issue to be taken into account when extracting phytoliths from modern material concerns silica skeleton content and their preservation. The conditions of preservation of silica skeletons in the extracted residue can be related in part to physiological and in part to methodological concerns. The three main topics to consider are:

1) Protocols involving the dry ashing step can provide a good preservation of the silica skeletons (as observed by Jenkins, 2009 and during this study);
2) Phytoliths recovered from modern plant tissues are mostly embedded in silica skeletons since there was no post depositional taphonomic activity disarticulating the cells (Madella and Lancelotti, 2012);
3) Monocots plants form much more silica skeletons in respect to other species (Hodson et al., 2005; Piperno, 2006). As a consequence, it is much more likely for biosilica to precipitate forming silica skeletons in grasses since more biosilica is stored in their tissues.

Two strategies have been proposed in the past to recover phytolith from modern plant tissues: wet oxidation, which involves the use of chemicals to digest organic matter and carbonates (Carter, 2009; Madella et al., 1998; Piperno, 2006; Roy et al., 2020; Watling et al., 2011; Webb and Longstaffe, 2010) or, alternatively, an ashing procedure in which plant tissues are charred in an oven furnace (Andriopoulou and Christidis, 2020; Braune et al., 2012; Corbineau et al., 2013; Elbaum et al., 2003; Jenkins, 2009; Kameník et al., 2013; Schulz-Kornas et al., 2017; Twiss et al., 1969). In the first case, an oxidising agent (e.g., hydrogen peroxide or nitric acid) is used to digest the organic compounds of the sample while the inorganic silica is left unaltered. The ashing procedure eliminates the organic component of the tissues by incineration. Both methods have been shown to preserve phytolith morphology (Piperno 2006), although in both some issues have been observed. Such issues can be grouped into two macro-categories: a) use of strong chemicals/reagents; and b) furnace heating.

a) Several different chemical solutions have been suggested for digesting both the organic matter and the carbonates. The most used combinations of reagents (to be added separately) are: hydrochloridric acid (HCl) + nitric acid (HNO_3) (Pearsall, 2016), hydrochloridric acid (HCl) + hydrogen peroxide (H_2O_2) (Hodson et al., 2008; Parr et al., 2001), hydrogen peroxide (H_2O_2)+ hydrochloridric acid (HCl) + sulfuric acid (H_2SO_4) (Carter, 2009; Parr, 2006; Santos et al., 2010; Webb and Longstaffe, 2010), hydrochloridric acid (HCl) + nitric acid (HNO_3) + hydrogen peroxide (H_2O_2) for microwave extraction (Elbaum et al., 2009; Parr et al., 2001; Pearsall, 2016; Watling et al., 2011), potassium hydroxide (KOH) + hydrochloridric acid (HCl) + Schultze solution (nitric acid HNO_3 + potassium chlorate $KClO_3$) (Corbineau et al., 2013; Pearsall, 2016; Piperno, 2006). Concentrations of chemical solutions could vary from 5% (v/v) up to 30% (v/v).

H_2O_2 is known as a mild oxidation agent; HCl removes carbonates; HNO_3 and Schultze solution are known as strong and more efficient organic digesters; KOH contributes to remove organics and humic colloids/acids. Although HNO_3 and Schultze solution have been recognized as the most efficient agents for the removal of organic material, they are also very costly and more toxic than the other chemicals, requiring stricter safety measures. At the same time, HNO_3 has been found as a source of possible nitrogen residual contamination that can alter isotopical measurements, and it has also been proven to cause some minor effects on the $\delta^{18}O$ content (Hodson et al., 2008; Watling et al., 2011). As a consequence, HNO_3 digestion is not suitable for material that is used for isotope analysis (Tyler et al., 2007). Furthermore, strong chemical solutions composed by KOH, HNO_3 or H_2SO_4 have been suggested as a possible cause of phytoliths dissolution connected to pH acidification (Corbineau et al., 2013; Katz et al., 2010; Parr et al., 2001; Parr and Sullivan, 2014) and silica skeleton disarticulation due to mechanical breakdown (Watling et al., 2011). Occasionally strong acids such as Schultze solution, have been proposed for wet ashing the samples, accelerating the process of incineration while burning the samples in a wet environment (wet ashing procedure). This strategy has sometimes resulted in an incomplete oxidation of the tissues (Parr et al., 2001; Pearsall 2016) and thus prevented a good identification of phytoliths trapped into the residual organic matter. Phytoliths, in addition, are highly damaged by hydrofluoric acid and strong base solutions (Piperno 2006). Also, the combination of H_2O_2 + H_2SO_4 for the digestion of the organic material has been pointed out by Watling et al. (2011) and Parr and Sullivan (2014) as a source of chemical restructuration of both silica matrix and phytolith-occluded carbon. Moreover, phytolith extraction using uniquely acid digestion has been found not to be fully efficient in removing organic matter (Jenkins, 2009; Kameník et al., 2013; Parr, 2006), as well as in isolating a good quantity of well-preserved silica skeletons (Andriopoulou and Christidis, 2020; Jenkins, 2009). Notwithstanding the issues related to the use of strong oxidising agents, several combinations of chemicals have been proved to be efficient in extracting phytoliths to undergo isotopical analysis, while maintaining their opaline structure. The combination of HCl and H_2O_2 has been shown previously not to affect oxygen ($\delta^{18}O$) and silicon ($\delta^{30}Si$) isotope evaluation (Hodson et al., 2008; Tyler et al., 2007). The use of H_2O_2 to dissolve the organic matter reported trustable results also for $\delta^{13}C$ evaluation in phytoliths (Carter, 2009; Webb and Longstaffe, 2010).

b) Heating-related problems are mainly connected with phytoliths physical-chemical properties during the burning process: albeit phytoliths melt at 900-1100ºC (Brochier, 2002; Elbaum et al., 2003) lower temperatures can already cause body shrinkage and warping as well as changes in the refractive index (Elbaum et al., 2003; Parr et al., 2001; Pearsall, 2016) because of phytolith anisotropic nature (Schulz-Knornas et al., 2017). Exposure to heat (especially exceeding 24 hours) can imply the dissolution of phytolith margins and the charring of the silica surface, affecting the solubility and the refractive index (Cabanes et al., 2011) even when the temperature is below 900ºC (Elbaum et al., 2003). Also, repeated ashing up to 500ºC may lead to carbon leakage, which forces the extraction of a larger amount of phytoliths to obtain enough material to undergo carbon isotopic analysis (Corbineau et al., 2013). Furthermore, processing the plant tissues in a furnace may result in contamination because phytoliths could absorb material from the micro ambient (sodium, potassium, and calcium), which would change their chemical composition (this is known as secondary mineral formation) (Andriopoulou and Christidis, 2020; Pearsall, 2016). Despite the heating process has been long disputed by phytolith experts, it has also been regarded as a useful tool for extracting very well-preserved silica skeletons from plant tissues. Jones and Mile (1963) hypothesised that fast dehydration of the phytoliths in the furnace could cause a fusion between the silica cells, resulting in a stronger structure, which can then better survive the wet oxidation. More recently, Jenkins (2009) suggested the use of a dry ashing step to obtain pure phytolith powder while minimising silica skeleton breakage. Further studies demonstrated that keeping temperatures below 500ºC avoids a second water release that induces phytoliths to shrink (Jones and Minle 1963; Peasall 2016). In addition, Elbaum et al. (2003) proved that below 700ºC phytoliths showed a very weak shift in their refractive index.

The extraction method used in this study is based on a combination of dry ashing and wet oxidation. In the first step, samples were heated at 500ºC for 12 hours, and in a second step a bland solution of HCl and H_2O_2 was used. Furnace heating by maintaining temperatures below 500ºC did not compromise the composition and morphology of phytoliths and helped to remove organic matter quickly and made silica skeletons more robust. The use of HCl and H_2O_2 in mild concentrations allowed the removal of the last residual organic matter and carbonates without compromising the composition of the phytoliths and without dissolving the silica bodies outer surface. A cover lid on the crucibles prevented cross contamination in the oven. Jenkins (2009) suggested a very similar methodology for dry ashing extraction, which includes a first ashing step for 3 hours at 500ºC and a second wet oxidation with HCl 10%. Although the protocol achieved good results for *Triticum durum*, tests conducted during this research on other Poaceae species (*Pennisetum glaucum*, *Eleusine coracana* and *Sorghum bicolor*) were not satisfactory, and the extracts presented abundant organic matter which obscured phytoliths. Hence, I argue that the use of HCl alone is not sufficient to remove the organic matter left from the ashing procedure, and a longer ashing step combined with the addition of a second step using H_2O_2 can provide more pure samples.

The proposed extraction procedure

Samples were washed in an ultrasound bath (Ulsonix Proclean 3.0) at room temperature for 5 minutes to remove surface contaminants (such as soil particles) and then left to air-dry on aluminium foil in a ventilated but draught-free space. Once completely dried, 0.1 g was weighed to be analysed. Each sample was ashed in a furnace at 500ºC for 12 hours in a ceramic crucible covered by a lid. The 12 hours included the time the oven took to reach the temperature of 500ºC (in this case 1 hours on average). To digest carbonates and oxidise organic material that survived the ashing procedure the ashes were placed in an Eppendorf tube and then 900 µl of 10% v/v HCl was added, vortexing the tube to stir the solution. HCl was left reacting till the reaction stopped, which for these extractions was around 5 hours. Samples were washed by adding 450 µl of distilled water three times and centrifuging at 6000 rpm for 5 minutes each time to separate and discard the supernatant from the silica extract. Subsequently 900 µl of H_2O_2 (10% v/v) were added and the tubes were placed in a drying cabinet at 40ºC until the reaction stopped. Every 2 hours samples were rinsed with distilled water and 900 µl H_2O_2 were added again. The process took approximately 9 hours for each sample. After that, the sample cleaning process was repeated by adding 450 µl of distilled water, centrifuging and then discarding the supernatant three times. The final residue was left to dry in a drying cabinet at 60ºC. When samples appeared dry, they were removed from the drier, left to cool down and then weighed. To take the weight of samples completely dry they were weighed until no weight loss was observed.

To ensure that the extractions were successful, all the samples underwent microscope checks for assessing silica skeleton presence and the residue's purity. Samples were scanned with polarised light at 400x and checked for the absence of crystalline particles or starch grains. The high number of silica skeletons observed after the extraction (93% of the phytoliths counted was observed in silica skeletons) confirmed the efficiency of the protocol in not damaging the silica skeletons.

5.2.2.2 Phytolith count and classification

Procedure background

Two different approaches characterise the identification and counting of phytoliths from modern material: the morphological approach, which relies on the observation and description of the morphology of single, disarticulated phytoliths; and the botanical/physiological approach, which gives prominence to the orientation and location of phytoliths in the silica skeletons (former plant tissues such as epidermis or meristem) from an anatomical perspective (Pearsall 2016). The counting based on the morphological approach relies on absolute counts (Zurro, 2018) or relative abundances (ratios) (Strömberg, 2009) of morphotypes where the count size -the number of phytolith cells tallied on a slide- must be representative of the population/assemblage. In the case of quantifying and qualifying a phytolith assemblage, the minimum total number of phytoliths to be counted depends on how many different morphological variants (i.e., morphotypes) are present in a sample (Zurro, 2018). Otherwise, if the aim is to develop ratios, confidence intervals should be calculated on a case-by-case basis (Strömberg, 2009). Usually, a range between 200 and 300 phytoliths is considered statistically significant for both counting methods (Piperno, 2006; Strömberg, 2009; Zurro et al., 2016). However, the variety of strategies used by different specialists, which often depend on the specific application of phytolith analysis (e.g., palaeoecology and palaeovegentation studies or domesticates identification in archaeology) includes counts ranging between 100 and 5000 phytoliths (Ball et al., 1996; Piperno, 2006; Strömberg, 2009; Zurro, 2018; Zurro et al., 2016).

A variety of strategies are currently in use to count phytoliths under the microscope. In many cases, however, the full method is not properly explained or referenced (Karoune, 2021) and it is very likely that each phytolith researcher has developed unique methods of extraction as well as counting, based on the type of sample, research questions and laboratory equipment available. In this research, the counting strategy adopted was designed to effectively represent modern samples dominated by silica skeletons as well as to make those samples comparable with archaeological assemblages where silica skeletons are scarcer. Silica skeletons or articulated phytoliths cannot be counted as a single cell morphotype. Therefore, when counting silica skeletons from fresh material to be compared with archaeological samples, two aspects have to be taken into consideration: the assemblage richness (i.e., the number of distinct morphotypes) and the relative abundance distribution (i.e., evenness) must be representative; and the sample size of each variant (i.e., of each morphotype) must be comparable to those of the archaeological assemblages to compare. In modern samples of grasses, if the first 200-250 phytoliths encountered during a slide scanning are counted, as in archaeology is normally done, probably these phytoliths would be part of a single (or very few) silica skeleton, which are generally composed mostly of elongate cells. Alternatively, performing the counting only on disarticulated morphotypes, considering silica skeletons as separate structures, would lead to a misrepresentation of the evenness, because a good percentage of elongates would be ignored. In this study, this issue was noticed when sorghum slides were processed, but the same happened to the remaining two species of millets. The leaf phytoliths of sorghum occured in large silica structures, on average consisting of 50 or more phytoliths, occupying more than one field of view each, and being primarily composed of elongates. Thus, a sample size of 250 would be reached by counting three or four skeletons with very high percentages of elongates but few short cells or other morphotypes such as stomata and bulliforms. In the archaeological record, however, the situation is usually different, as silica skeletons tend to disarticulate and elongates can be highly affected by taphonomy (Cabanes and

Shahack-Gross, 2015). Short cells, on the other hand, heavily built and with lower surface area/volume ratio, respond better to taphonomy, and normally represent a good portion of the archaeological assemblage. Thus, a phytolith sample from fresh tissue is normally characterised by the abundance of elongates whereas an archaeological sample normally consists of many short cells. In this study, these two types of samples had to be compared in terms of morphotype composition, and a solution had to be found for phytolith counting that would be suitable for comparison, but which maintained the richness and the evenness of both types of samples. The problem of how to count samples with many, large silica skeletons is not widely discussed in the literature but there are few examples that mention the issue and offer solutions. Therefore, in the protocol published in protocols.io, a section dedicated to a new counting strategy was also added, which is the one used to carry out the phytolith analysis for this thesis.

Slide preparation and counting procedure

A dry mounting strategy was used: 0.0001 g of silica residue was placed on a microscope slide and then 4 drops of Entellan New® were added on the top. Entellan and the silica residue were mixed and stirred with the use of a clean spatula/micro spoon, making sure to distribute the powder as evenly as possible. Slides were covered with coverslips of 24 x 32 mm. About 12 hours were waited before observing the slides under the microscope, for the phytoliths to precipitate and so that Entellan New® reached the right density that allowed phytoliths rotation without too much displacement on the slide. This guaranteed a correct three-dimensional observation of the morphotypes and more accurate descriptions and identifications. Counting always started at the centre of the cover slip, to avoid distinct phytolith distribution patterns between the centre and the edges of the slide leading to different results. For each slide up to 50 silica skeletons were counted and phytolith included in the structure were classified. For each skeleton, the total number of cells for each morphotype recognized was counted. All disarticulated phytoliths encountered during the scanning were described and counted separately. At the end of the counting, the disarticulated phytoliths and those encountered inside the silica skeletons were summed together to obtain the total sum for each morphotype, and to calculate the concentration and relative percentage of each one. This procedure ensures reaching the minimum number of silica cells required for statistical representativeness of richness and evenness (Strömberg, 2009; Zurro, 2018). At the same time, it ensures that the number of short cells counted is comparable with that typically found in archaeological samples.

Classification of phytoliths

Morphotypes were classified according to the ICPN 2.0 (International Committee for Phytolith Taxonomy (ICPT) et al., 2019), which provides a detailed nomenclature system and an in-depth description of each morphotype. Table 5.7 provides a list of the identified morphotypes from the three different species of grasses, two of which are *Poaceae* (sorghum and pearl millet) and one *Chloroideae* (finger millet), and figure 5.5 shows some illustrative images of the observed phytoliths. In file A5.2, all counts (row data) for all samples are available.

Phytolith name following the ICPN 2.0 (2019)	**Description**	**Figure 5 corresponding images**
Acute bulbosus	Phytoliths with acute apex and a wider bulbous base, normally hemispheroidal. The apex can be straight or curved with a sharp or rounded tip. Dimension can vary substantially (10-30 μm length). The bulbosus base may be missing or alternatively only the base hair, globular, is evident.	a), b), u)
Bilobate	Phytoliths composed by two distinct end-lobes separated by a castula. The lobes can be completely convex or slightly concave. The length is larger than the width.	c)
Blocky	Square-shape and rectangle-shape parallelepipedal, heavily built phytoliths. Edges range from smooth to moderately sinuous.	d), e)
Bulliform flabellate	Fan-shaped heavily built phytoliths narrow on the apical part. The apical part is convex while the base is convex and may be facetate with sometimes echinates margins. Usually symmetrical.	f)
Cross	Silica bodies composed of four lobes. Length and width are approximately equal.	c)
Elongate clavate	Rectilinear bidimensional phytoliths with club shaped projections constricted proximally and enlarged in the distal end.	g)
Elongate crenate	Rectilinear bidimensional long phytoliths with crenate margins.	h)

Elongate dentate	Rectilinear bidimensional long phytoliths characterised by narrow and acute projections with concave sides.	i)
Elongate entire	Rectangular bidimensional long phytoliths having smooth margins without projections.	j)
Elongate sinuate	Rectilinear bidimensional long phytoliths with sinuous margins which alternates concavities with convexities: projections are enlarged proximally.	k), t)
Polylobate	Phytoliths consisting of two end-lobes separated by additional (more than one) distinctly separated lobes of similar size, either paired on both sides or not.	l)
Rondel	Silica bodis approximately circular or oval in the planar view, corresponding to a truncated cone in the lateral view. Either narrowed on the apical part, either hemispherical with spikes in the apex. Trapeziform rondels have a more accentuated polygonal shape in lateral view.	m)
Saddle	Symmetrical morphotype resembling a saddle, consisting of two convex faces connected by a concave structure. In side view they are concave. Length and width can be similar in size (collapsed or short saddle) or length can be longer than width (saddle bilobate).	n), o), p)
Stoma	Silicified stromal cells with guard cells and subsidiary structures heavily built. Sometimes the external side can be enriched with decorated structures.	q)
Tracheary anulate	Cylindric sulcate silicified tracheids normally articulated in multiple bodies.	r)

Table 5.7 - Phytoliths types identified in the three grass species: sorghum, pearl millet and finger millet. Classification has been based on the International Code for phytolith nomenclature (ICPN) 2.0 (International Committee for Phytolith Taxonomy (ICPT) et al., 2019).

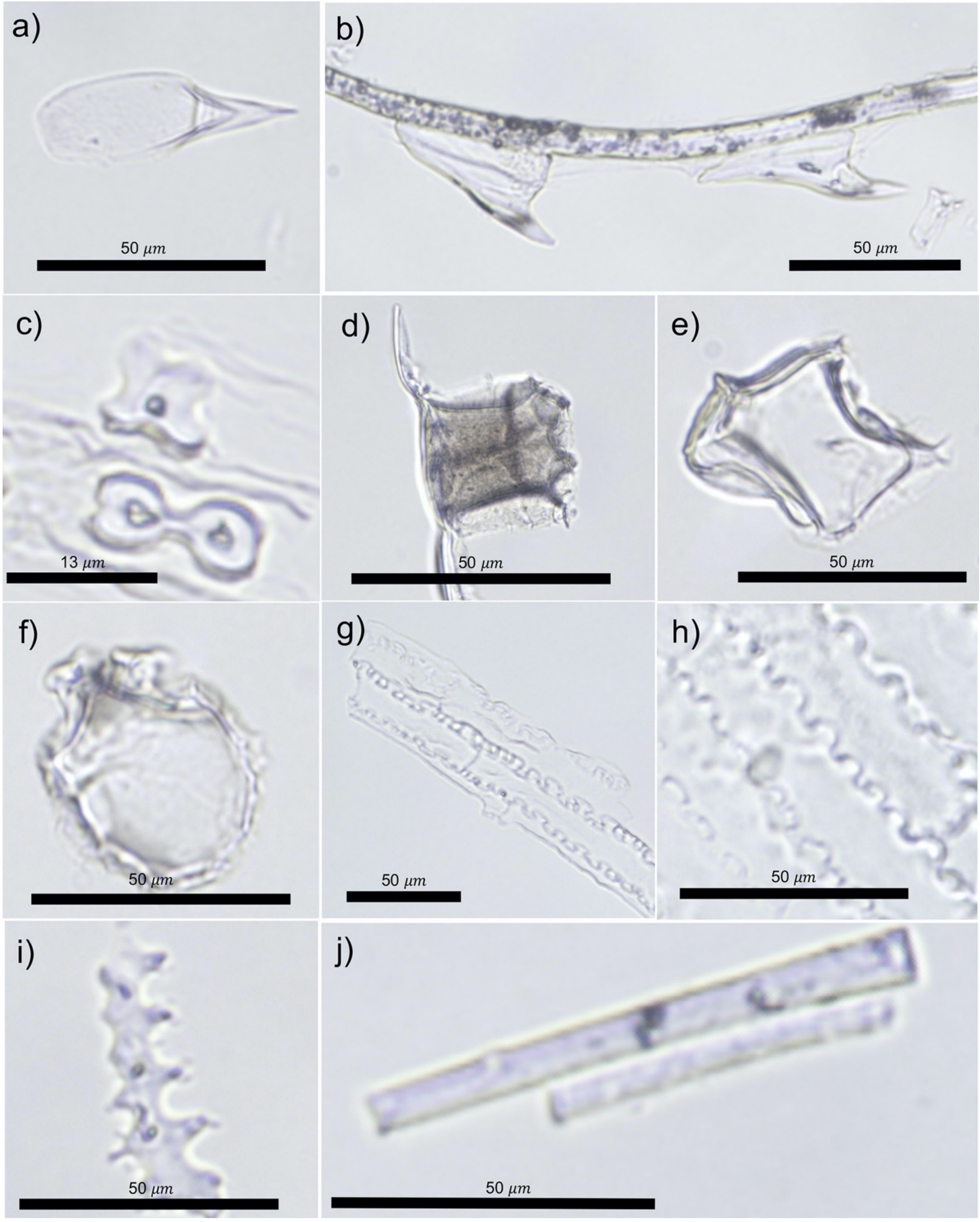

a)
50 μm
b)
50 μm
c)
13 μm
d)
50 μm
e)
50 μm
f)
50 μm
g)
50 μm
h)
50 μm
i)
50 μm
j)
50 μm

Figure 5.5

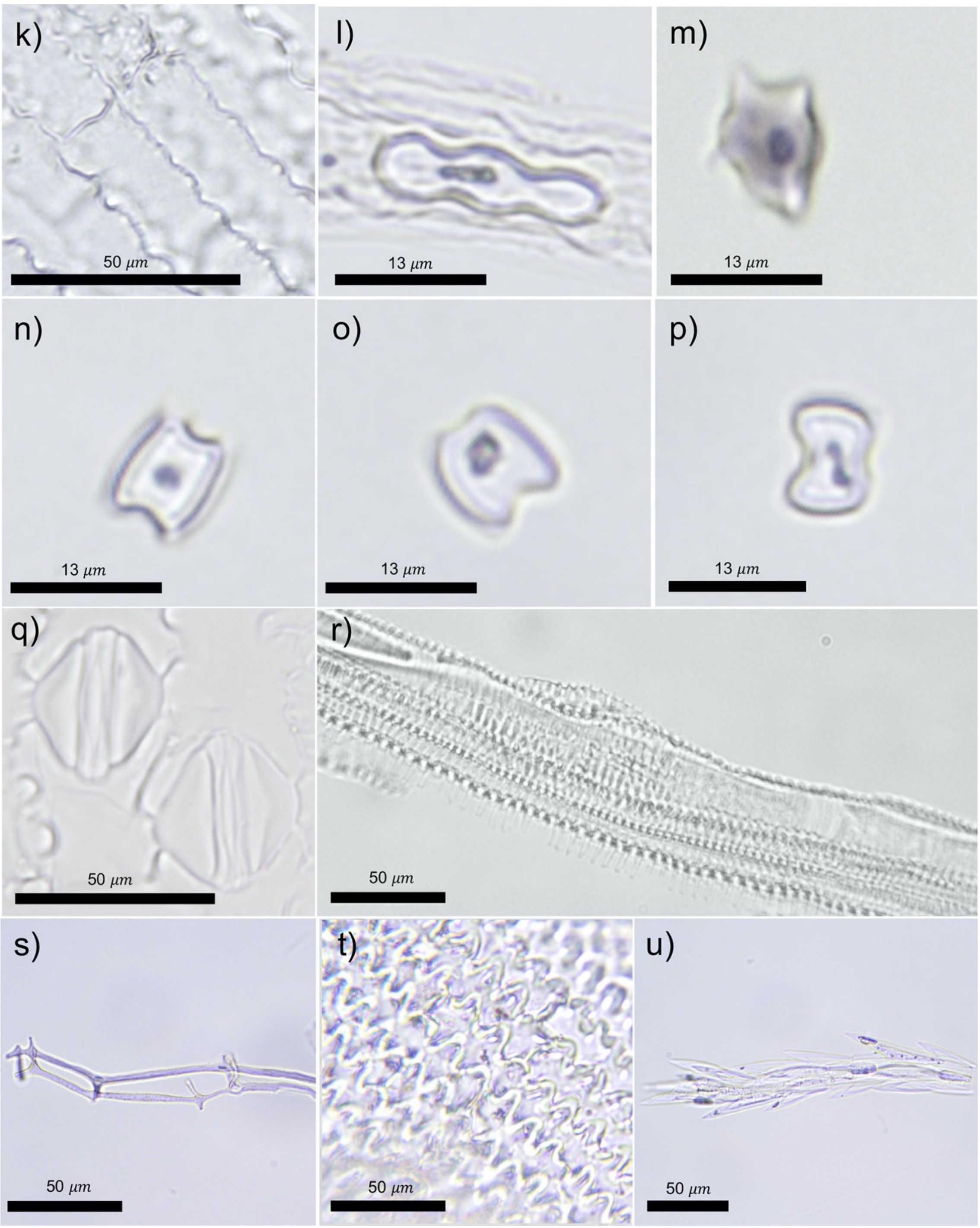

k)
50 μm
l)
13 μm
m)
13 μm
n)
13 μm
o)
13 μm
p)
13 μm
q)
50 μm
r)
50 μm
s)
50 μm
t)
50 μm
u)
50 μm

Figure 5.5

Figure 5.5. - Main phytolith morphotypes recovered from the leaf tissue of Finger millet, pearl millet and sorghum. Magnitude x400 and x600. IPS: Inner Pericranial Surface. a) Acute bulbosus of finger millet leaf (IPS view); b) silica skeleton of acute bulbosus connected by an elongate entire in pearl millet leaf (side view); c) one bilobate and one cross in a silica skeleton in pearl millet leaf (IPS view); d) silica skeleton with a blocky in sorghum leaf (side view); e) blocky in finger millet leaf (side view); f) bulliform flabellate of finger millet leaf (side view); g) silica skeleton of elongates clavate in pearl millet leaf (IPS view); h) silica skeleton of elongates crenate in sorghum leaf (IPS view); i) elongate dentate in sorghum leaf (IPS view); j) silica skeleton of two elongates entire in pearl millet leaf (IPS view); k) silica skeletons of elongates sinuates in pearl millet leaf (IPS view); l) polylobate in a silica skeleton in pearl millet leaf (IPS view); m) rondel with two spikes in the apex of pearl millet leaf (lateral view); n) saddle (short form) in finger millet leaf (side view); o) saddle (collapsed form) in finger millet leaf (side view); p) saddle (bilobed form) in finger millet leaf (IPS view); the various saddle variants have all been included in the general saddle category; q) two stoma in a silica skeleton in sorghum leaf (IPS view); r) tracheary anulate structures in finger millet leaf (IPS view); s) silicon structure normally identified as infilling (no phytoliths) very common in pearl millet to sorghum leaves (this comes from pearl millet leaves and reproduces the outward form of epidermal cells); t) silica skeletons of elongates sinuates in sorghum chaff (IPS view); u) silica skeleton of acute bulbosus connected by an elongate entire in pearl millet chaff (side view) (these are the most abundant structures encountered in pearl millet chaff).

5.2.3 Ratios and measurements taken on phytoliths

5.2.3.1 Phytolith ratios

Phytolith concentration was calculated for each morphotype identified as well as for the total silica accumulated by using the following formula:

$$\text{Phytoliths extracted} = \frac{(\text{total silica extracted (g)} \times \text{total phytoliths per slide})}{\text{total silica mounted (g)}}$$

$$\text{Concentration} = \frac{\left(\frac{\text{phytoliths extracted}}{\text{dry leaf weight (g)}}\right)}{1000000}$$

Along with the concentration, the percentage corresponding to each morphotype was also evaluated to make the analysis comparable to existing publications, especially archaeological ones where calculating the concentration of phytoliths produced per biomass unit is not possible (data available in the appendices, file A5.2). The concentration was preferred for further study of the assimilative biosilica rate (chapter 6), because it provided an absolute data (number of phytoliths extracted per gram of dry material), which better shows even the smallest changes in the abundance of a morphotype.

Since a large quantity of silica skeletons was available and because previous studies indicated a possible relationship between silica skeleton size (the number of phytoliths in skeletons) and irrigation levels (Miller Rosen, 1992), it was decided to also calculate the silica skeleton size ratio using the following formula:

$$\text{Silica skeleton size ratio} = \frac{\text{number of cells in a silica skeleton}}{\text{total number of phytoliths}}$$

With regard to the analysis of the ratios of sensitive to fixed morphotype, three indices have been proposed to date (Jenkins et al., 2016, 2020; Madella et al., 2009 - formulas 1 and 2), which differ in the total number, the types and the grouping of morphotypes involved in the equation. In addition to the published indices, a specific one was designed during this work in order to further research on those morphotypes that could in some way be indicative of watering (formula 3) according to recently published literature (Hodson 2019; Kumar et al., 2017, see chapter 4.1). Each formula was tested using as term of the equation both raw data, concentration data and percentages (all the results are available in the appendices, file A5.2):

$$\text{Formula 1: Madella et al. (2009) ratio} = \left(\frac{\text{elongates + stomata}}{\text{total phytoliths}}\right)\%$$

$$\text{Formula 2: Jenkins et al. (2016) ratio} = \frac{(\text{elongates + stomata})}{(\text{crosses + bilobates+ polylobates + rondels + saddles})}$$

$$\text{Formula 3: Sensitive morphotypes ratio} = \frac{\text{trichomes}}{(\text{bulliforms + stomata})}$$

$$\text{Formula 4: Elongates ratio} = \left(\frac{\text{sum of all the long cells}}{\text{total phytoliths}}\right)\%$$

Of the four indices tested, the ratio proposed by Jenkins et al. (2016) explained the highest percent of the variability of the dataset evaluated by the adjusted r-squared, proving to be the formula with the highest predictive capacity, and at the same time it relies on a significant number of phytoliths (table 5.8). In the following chapters, when reference is made to the sensitive/fixed ratio, this means the formula proposed by Jenkins et al. (2016).

Indices	**Adjusted r Squared ↓**	**Average of total phytolith counted to apply the formula**
Formula 3 (raw data)	0.05517	46
Formula 3 (concentration)	0.05517	46
Formula 3 (percentage)	0.0502	46
Formula 2 (percentage)	0.03428	592
Formula 2 (concentration)	0.03151	592
Formula 2 (raw data)	0.03151	592
Formula 1 (percentage)	0.02583	620
Formula 1 (concentration)	0.005133	620
Formula 1 (raw data)	0.02554	620
Formula 4	0.005901	483

Table 5.8 - Adjusted r squared values and average of phytoliths counted for the evaluation of each index, presented in descending order for values of adjusted r-squared. (raw data): when raw counts of morphotypes were used as terms in the formula; (percentage): when percentage of morphotypes were used as terms in the formula; (concentration): when concentration of morphotypes were used as terms in the formula.

5.2.3.2 Phytolith morphometry

Ball and Brotherson (1992) defined the "morphometric" approach for the study of phytoliths as “ [...] *the measuring of a feature's morphological parameters (morphometries) such as size, shape, texture, orientation, etc. Rather than relying strictly on phytolith morphotypes as a basis of classification, the morphometric approach to phytolith systematics tries to discriminate between taxa on the basis of the actual* *measurement of phytolith morphological parameters*". An innovative study in phytolith morphometry, which takes the concept of studying morphometry and re-proposes it in current terms, by providing the measurement of the short cells of grasses, has been proposed by Hoskova et al. (2021). Less frequent are studies directed at sensitive morphotypes (bulliforms, elongates, stomata, trichomes). Not only is their production ubiquitous, and less prone to species recognition (although bulliforms or elongates may be species specific in some cases), but also the relationship between their morphological characters and the environmental factors that might influence their formation has not yet been verified. Among the few examples available are the study by Ball et al. (2009) who uncovered differences within the lobes of dendritic elongates that can be traced back to the formation tissue (e.g., inflorescence or leaf blade), or the one by Madella et al. (2016) who clarified the potential of elongate phytoliths produced in glumes to discriminate between species. Environmental conditions have also been reported in literature as a possible source of variation of phytolith size (Katz et al., 2013; Madella et al., 2009; Tsartsidou et al., 2007) and the implications for archaeology were also discussed (see Shillito, 2013). While keeping in mind that genetics plays a key role in both determining cell anatomy and silicon accumulation (Out and Madella, 2016; Twiss et al., 1969), the size of sensitive phytoliths have been explored to observe whether or not changings may depend on variations in the transpiration process. Some attempts have been made in this direction by Ball and Brotherson (1992), who suggested that plants grown under different environmental conditions change significantly with respect to the amounts of silica accumulation and that the highest variation is linked to soil water content. Wang et al. (2019) showed that larger rice bulliforms likely occurred at locations with higher temperature, precipitation, and water levels, indicating hydrothermal climatic parameters as an influencing factor. The authors also claimed that changes in phytolith size may be triggered by domestication and influenced by environmental factors, such as CO_2 concentrations (Ge et al., 2010) and evapotranspiration rates (Issaharou-Matchi et al., 2016). Wang and colleagues speculated that if shifting in morphometry is merely a phenotypic response to environmental conditions, it would not be a useful indicator for domestication, but it could explain an adaptation to arid environments or water scarcity. On the basis of these considerations, what still remains to be done in the field of phytolith morphometry, is the definition of the phytolith size ranges within the same species and whether within these ranges the variations are environment-dependent, as was also brought to light by Out and Madella (2016).

The measurements

Measurements have been taken for 20 elongates, 20 stomata and for all the bulliforms encountered during counting. These three morphotypes were chosen from those traditionally considered sensitive, and thus subject to environmentally dependent variations including water availability (Jenknins et al., 2016; Madella et al., 2009). For elongates and stomata 20 morphotypes were measured as an appropriate size for a pilot study. The bulliforms being much fewer, it was decided to count those available (less than 20 per sample analysed). Every 5 phytoliths of that type (elongates and stomata) one was measured, so as to avoid bias in terms of variants analysed (e.g., elongates sinuate or dentate or clavate). In total, 1177 stomata, 1248 elongates including both forms with decorated and entire margins, and 77 bulliforms were measured. All samples whose measurements were outliers were subsequently excluded from the final count, probably because the data were incorrectly entered or measured. The row data is available in the appendices (file A5.2).

All the measurements were taken directly during counting using the measuring bar function of the Euromex Image Focus Alpha software® (version x64, 1.3.7.13814.20190120). For each bulliform, length, width, beak length and width (for flabellate), number of spikes and distance between two subsequent spikes were measured (see figure 5.6b). For each elongate, the length of three extroflexions was measured in addition to the length and width (see figure 5.6a). For the stomata, the length, width, and width of the two subsidiary cells were measured (see figure 5.6c). All measurements were taken with the phytoliths observed at a magnification of 60x and image resolution of 300 dpi. When possible (elongates and stomata, while bulliforms very rarely occur embedded in silica skeletons) phytoliths within silica skeletons were measured, to avoid all those incomplete or broken structures.

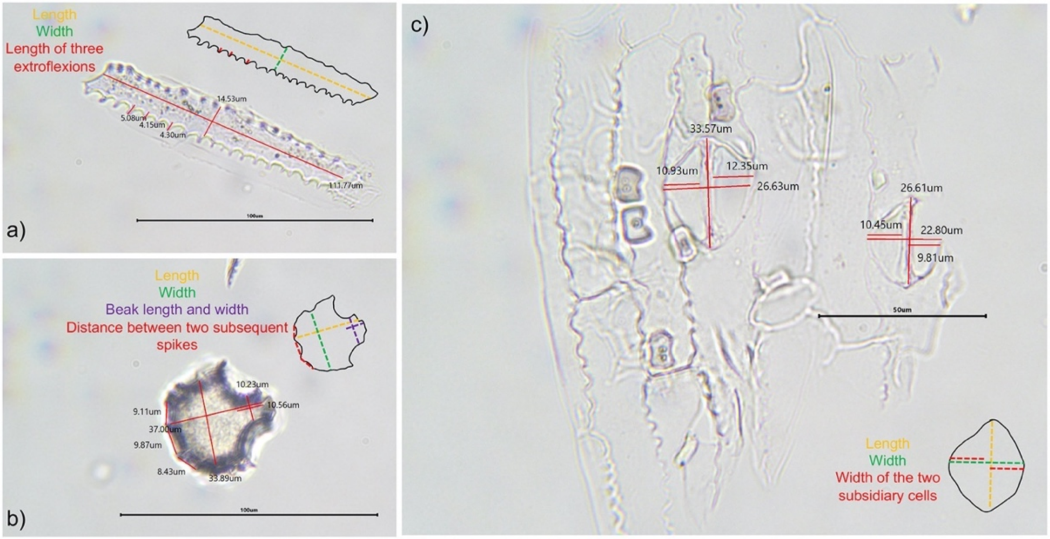


Figure 5.6 - Pictures taken during measurements. Red line bars represent the magnitudes that have been measured for the morphotypes concerned: a) elongates; b) bulliforms; c) stomata.

5.2.4 δ^{30}Si and δ^{29}Si isotopic analysis in phytoliths

The study of discrimination of stable Si isotopes in plant tissue is based on the assumption that the preferential passage of light Si isotopes contributes to a progressive isotopic fractionation of the solution moving from the uptake sites in roots to the transpiration termini in lamina. On this basis, it was hypothesised that the difference in isotopic content may depend on the amount of water absorbed by the plant and its initial concentration, through which the Si isotope poll is transported. In this study, silicon isotope analyses of phytoliths were conducted to provide a second independent proxy to evaluate water availability. As described in the previous chapters, in bulliforms, elongates and stomata mechanisms of biosilica deposition are not entirely comparable, consequently, their elemental content could vary. Furthermore, bulliforms, elongates and stomata occupy different position in the leaf tissue, implying that there could be a different level of discrimination (if any), depending on how many different Si transporters are involved in the relocation and deposition of biosilica. Given these premises, the technique was investigated to measure the isotopic content on the individual phytolith and not on bulk samples where the morphotypes are mixed. Indeed, δ^{30}Si and δ^{29}Si on phytoliths were conducted using femtosecond laser ablation multi-collector inductively coupled plasma-mass spectrometry (fsLA-MC-ICP-MS, fsLA hereafter), through a collaboration with Dr Daniel Frick of the laboratory Earth Surface Geochemistry of the German Research Centre for Geosciences in Potsdam (Germany). Traditionally, Si isotope ratios were determined after fluorination of the silicon, either by treatment with a fluorine source (Allenby, 1954) or by laser fluorination (De La Rocha et al., 1996) in a gas isotope ratio mass spectrometer (IRMS), or alternatively by using a high-resolution MC-ICP-MS (De La Rocha, 2002) on a silicon solution. These techniques, although extremely informative, are designed for bulk analysis and not for the analysis of a single phytolith cell. fsLA technique allows to collect information on the isotopic composition of a single phytolith (provided it is sufficiently thick, see Frick et al., 2016, 2019). Indeed, the machine is connected to an optical microscope so that the pathways of scanning with the laser beam of approximately 25 Im in diameter can be programmed looking at the sample directly. The laser can be continuously scanned over the sample (the phytolith) with pathways adapted to the irregular shapes of the phytolith surface, and avoiding all siliceous and non-siliceous particles in the background. To account for the fragile nature and sometimes also porous structure of phytoliths, the laser frequency (pulse repetition rate) during phytolith analysis can be dynamically adjusted to maintain a signal intensity as constant as possible within the ranges defined by the ablation of a calibrator (47Von28Si) (Frick et al., 2019). One feature that makes this machine particularly efficient is that fsLA leads to less mass bias drift between bracketing calibrators and samples, improving the precision of the isotope ratio determination (Frick et al., 2016). These properties result in significantly reduced chemical fractionation, a process through which evolving compositions are detected relative to that of the target sample (Poitrasson and d'Abzac, 2017). In addition, fsLA, by being nearly athermal, enables the accuracy of LA-ICP-MS performance (avoiding matrix effect and isobaric interferences in thick samples) allowing the analysis of low mass materials that are rarely found homogeneous, such as phytoliths (Poitrasson and d'Abzac 2017). For all the reasons listed above, fsLA was chosen to conduct the analysis of the isotopic content of individual morphotypes.

Sample preparation for laser ablation

As the technique is still entirely experimental when applied to phytolith samples, several preliminary analyses were conducted to see which was the best slide assembly technique for the analysis. In order for the laser to penetrate the inside of the target phytolith, the slides cannot be mounted with the cover slip. Two different strategies for preparing phytoliths slides for the ablation have been tested. The first was based on the protocol implemented in the fsLA laboratory, whereby phytoliths were embedded in nail polish and fixed on a microscope slide. The second, used Entellan New® as a fixing medium. While nail lacquer ensures rapid drying and prevents contamination, Entellan New® ensures proper viewing of phytoliths, enabling their classification when choosing the pathway to be lasered. Entellan New® was tested for blank contamination (absence of silicon/contaminants in the composition of Entellan), to ensure that it did not create any interference, impact and scrub resistance. Since it proved to be a good medium, it was preferred for the current analysis. To minimise the possible contamination from the support, a microscope slide made of poly-methyl methacrylate (PMMA) was udes. Each slide was divided into 6 sections, each one containing a sample to be analysed. To assemble the slide, first a drop of Entellan New® was placed in the pre-prepared section and then the residual biosilica was poured over it with the tip of a clean spatula. This prevented the phytoliths from being deposited too deep, and allowed the laser to reach them without having to pass through an overly thick Entellan blanket, which would reduce the signal. Before mounting a second sample on the same slide, 24 hours were waited until the entellan had dried, to avoid cross-contamination between the samples. Once the slide was prepared, it was fixed to an aluminium support and sample characterisation was carried out by observing the slide with a optical Euromex light microscope (Euromex iScope + Euromex scientific camera sCMEX-6).

Phytolith laser ablation

Leaf samples from five sorghum landraces (S2, S4, S5, S9 and S10) of both treatments (WW and WS) have been examined. For each leaf sample 20 bulliforms, 20 elongates and 20 stomata were analysed for a total of 650 phytolith tested for δ^{30}Si and δ^{29}Si composition. Each laser raster corresponded to either a bulliform, an elongate or a stomata or to several cells of the same morphotype. The δ^{30}Si and δ^{29}Si in situ were measured using a custom-built deep-UV (196 nm) femtosecond laser ablation system (GFZ fem2, containing a frequency-quadrupled Spectra Physics Solstice femtosecond laser, see Schuessler and von Blanckenburg (2014) for technical details) in combination with an inductively coupled plasma multicollector mass spectrometer (Thermo Fisher Scientific Neptune MC-ICP-MS, equipped with the Neptune Plus Jet Interface). The ion optics were operated at medium mass resolution with a typical mass resolving power m/Dm >6000, to resolve isobaric interferences (mainly $^{14}N^{16}O^+$ on $^{30}Si^+$). Faraday detectors (equipped with 1011 O amplifiers) were positioned to measure on interference-free, flat top peak shoulders. The gas flows, torch position and ion optics were tuned daily for highest intensity and mass bias stability, while maintaining the $^{14}N^{16}O^+$

interference on $^{30}Si^{+}$ at resolvable levels (<10 V) (Frick et al., 2019). In order to balance the small spot size required for the analysis of a single phytolith with the need to keep the laser intensity constant, thus preventing crater depth enlargement, it was decided to keep a small-size raster (50–100 lm) at a faster (than nsLA) scan speed (40 lms^{-1}) with a higher laser pulse repetition rate (frequency 20–40 Hz) (Frick et al., 2019).

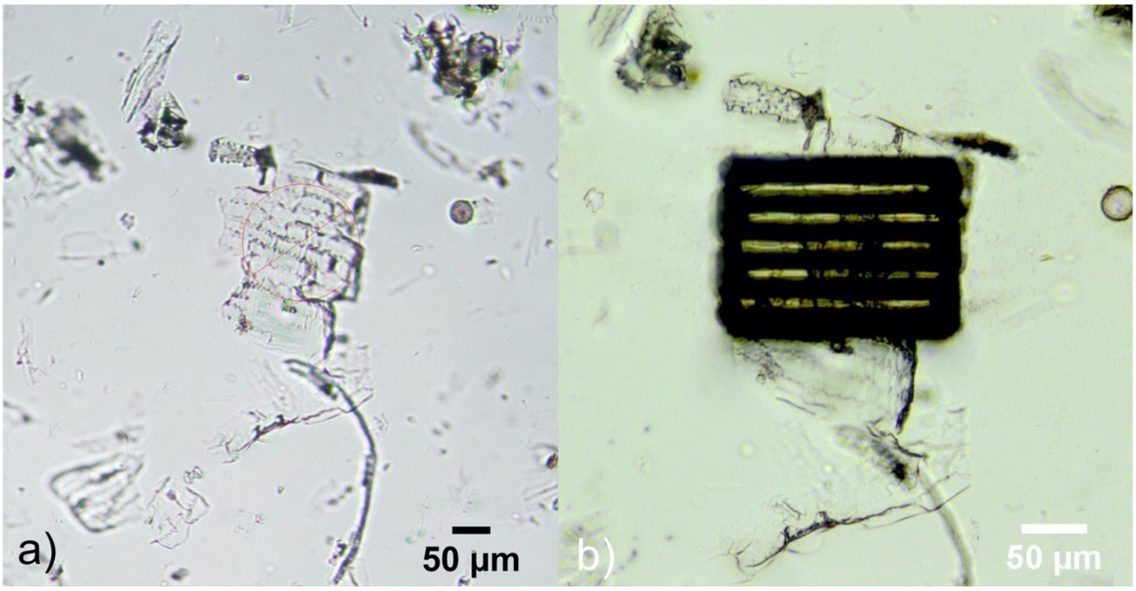


Figure 5.7 - a) Pre and b) post laser ablation of a silica skeleton composed by only elongates. The raster corresponds to one sample/spot.

The Si isotope results are reported in the δ-notation as permil deviation relative to an international measurement standard (in this study NBS28) (Coplen et al., 2002). All $\delta^{30}Si$ and $\delta^{29}Si$ from in situ fsLA-MCICP-MS analysis are reported relative to NBS28 and are available in the appendices (file A5.2).

$$\delta^{30}Si = \left[\frac{\left(\frac{\delta^{30}Si}{\delta^{28}Si}\right) \text{ sample}}{\left(\frac{\delta^{30}Si}{\delta^{28}Si}\right) \text{ standard NBS28}} - 1 \right] [\text{in } ‰]$$

$$\delta^{29}Si = \left[\frac{\left(\frac{\delta^{29}Si}{\delta^{28}Si}\right) \text{ sample}}{\left(\frac{\delta^{29}Si}{\delta^{28}Si}\right) \text{ standard NBS28}} - 1 \right] [\text{in } ‰]$$

Method validation strategy

Since there is no reference material specific for phytoliths, the choice of comparative material was made by Dr Frick, who had previously evaluated the possible matrix effects due to the presence of additional elements (e.g., carbon) within the siliceous structure of phytoliths and possible contamination due to the partial ablation of Entellan New® (Frick and Günther, 2012). Therefore, several reference materials made of silicon were chosen, such as BHVO-2 (basalt) and NBS28 (quartz δ^{29}Si = 0 and δ^{30}Si = 0, directly used as bracketing measurement calibrator, following the 'Standard-Sample bracketing' approach) in addition to materials whose isotope data had already been widely studied and published: ATHO-G (glass) and GOR132-G (glass) (Jochum et al., 2005; Schuessler and von Blanckenburg, 2014). Each of these samples were analysed in each fsLA session for quality control and to verify that the target measurement uncertainty was met. Thereby, uncertainties in the measurement results of these homogeneous materials were evaluated and then used as a benchmark to assess microscale silicon isotope heterogeneity in the unknown phytolith samples detected by in situ fsLA-MC-ICP-MS analysis, alternating the measurement of samples with that of standards.

All δ^{30}Si and δ^{29}Si results are accompanied with uncertainties at the 95% confidence level. The variability of the results analysed depends on a number of factors such as the analysis of the samples themselves, the analysis of the calibrators, but also effects such as mass bias stability and blank correction. The variance, which depends on all these factors, is obtained by calculating the standard deviation of silicon isotope ratio integration cycles acquired during < 3s of analysis (Frick et al., 2019) and expressed as the standard deviation of the mean derived from Student's t distribution at 95% confidence. Acceptable results, apart from producing a mass bias drift between the two bracketing calibrators of < 0.30‰ in 30/28Si, must follow the mass-depended terrestrial fractionation line in a three isotope plot of δ^{30}Si and δ^{29}Si within analytical uncertainties. All the measurements reported in this study met these criterias while all data that did fall outside of these standards were excluded (Frick et al., 2019).

5.3 Statistical analysis

5.3.1 Dataset description

The dataset resulting from the many experiments, varied both in composition and structure of the data (table 5.9). Species, genotypes and treatments represent nominal data. Data collected in physiology are represented by ratios whose measure unit is distinct depending on the variable (grams for biomass data, litres for transpirational data and watering). While phytolith absolute values represent variable counts, percentages and derived concentrations are compositional data. The counts also include a large number of zeros (samples that did not contain specific morphotypes in the assemblage). Furthermore, the scale of distribution of phytolith counts represent another issue: while some morphotypes (e.g., elongates) are ubiquitous and abundant, others may count in less than 10 phytoliths per sample (e.g., bulliforms). Morphometric measurements are ratios as well as isotopic data. While the scale of the isotopic data obtained is very similar, the morphometric data present the same problems as the phytolith counting data and occur on very different scales even though they all count in the same unit of measurement (micrometres). The distribution of most of these variables (especially phytolith counts) is not normal.

Table 5.9	**Variable**	**Type of variable**	**Scale**	**N° of entries**	**0s?**
Sample descriptors	*Sample*	Nominal	1-1200	1200	No
	Replication	Nominal	1-5	1200	No
	Species	Nominal	FM: 400 PM: 400 S:400	1200	No
	Genotype	Nominal	1-10	1200	No
	Plant Part	Nominal	1-4	1200	No
	Treatment	Nominal	WS: 600 WW: 600	1200	No
Physiological data	*TWT (L)*	Ratio (Bimodal)	0.28-86.06	1198	No
	TWA (L)	Ratio (Bimodal)	0-69.06	1200	Half
	TE (g/L)	Ratio (Bimodal)	0.65-26.20	1176	No
	Biomass (g)	Ratio (Bimodal)	22.241216.5	1176	No

Phytolith general analysis	*Phytolith concentration*	Ratio (Skewd on the right)	0.49-282.95	362	No
	Disarticulated phytolith	Count (Skewd on the right)	1-174	362	No
	Skeleton Phytoliths	Count (Skewd on the right)	161-2651	362	No
	Total Phytoliths	Count (Skewd on the right)	200-2657	362	No
	*Morphotypes**	Count/Compositional (Skewd on the right)	Count: 0-2336 Compositional: 0-126.25	362	Many
Morphometrics	*Length (μm)*	Ratio (Bimodal)	3.32-1264.46	2503	No
	Width (μm)	Ratio (Skewd on the right)	2.53-124.93	2503	No
	Area (μm^2)	Ratio (Skewd on the right)	45.01-11571.96	2503	No
Isotopical analysis	δ^{30}Si	Ratio (Skewd on the right)	-0.91-6.04	397	No
	δ^{29}Si	Ratio (Skewd on the right)	-0.61-3.29	397	No

Table 5.9. - Summary of the main variables composing the analysed dataset. *For the morphotypes, the count/concentration and percentages of each variant were analysed separately, here in the table is a summary is provided.

5.3.2 Tools and software

Statistical analyses for both data exploration and modelling were performed with R (version 3.5.1) using mainly standard functions of base, ggplot2 (version 3.3.5) (Wickham, 2016); tidyverse (version 1.3.0) (Wickham et al., 2019); vegan (version 2.5.6) (Oksanen et al., 2020) and MASS (version7.3-51.5) (Ripley, 2022) packages. Scripts are available in appendices (file A5.3 the R script and files A.5.3.1, A.5.3.2, A.5.3.3, A.5.3.4, A.5.3.5, A.5.3.6, A.5.3.7 are the csv UTF8 (comma-delimited) files to run the code in R).

5.3.3 Data exploration

The tests conducted can be summarised in four steps: (1) descriptive statistics for physiological data (2) analysis of variance and linear regressions for biosilica accumulation, phytolith concentration and phytolith ratios (indices developed on the basis of phytolith analysis); (3) generalised linear models on phytolith assemblages (morphotype composition); (4) ordination analysis to obtain information on the relationship between biosilica/morphotypes accumulation and environmental/physiological variables involved.

Physiological data, phytolith concentration, ratio sensitive/fixed morphotypes, silica skeleton size and phytolith morphometrics were normalised using natural logarithm to reduce the skewness (Legendre and Legendre 2012) and then linear regressions were applied to model the relationship among them. ANOVA, two-way ANOVA and Tukey’s Honest Significant Difference (HSD) tests were applied to test for significant differences between species, genotypes and treatments while boxplots were used to display the results of the analysis of variance. The analysis of phytolith counts/percentage, which are non-normal, continuous and always positive variables, were performed using generalised linear models (GLM) with Gaussian distribution. The p-value and the Akaike information criterion (AIC) model selection were used to assess the validity of the models and to identify the best-fit model (Burnham et al., 2011). AIC is considered a good index to compare models as it estimates the information loss when a model is generated by the given variable (Wagenmakers and Farrell, 2004). These models allowed to evaluate which explanatory variables (environmental variables from physiology and general variables as species and genotypes or plant part) better predict and explain differences in morphotypes concentration. Ordination analysis was used to obtain a better understanding of which variables best explained the distribution of the phytolith dataset. Canonical correspondence analysis (CCA) and Redundancy analysis (RDA) were applied to explore how phytolith morphotypes are related to the water treatment and to species (Borcard et al., 2011; Greenacre and Primicerio, 2013) and to understand whether the complete dataset (and not the single morphotype as with the GLMs) is predicted by the water treatment. Correspondence analysis was used when the dataset was composed by frequency-like (counted phytoliths), non-negative, and dimensionally homogeneous data; while redundancy analysis was applied to explore normal, positive matrices of variables, where silica accumulation values (grams of silica per biomass produced) correspond to the response variables and physiological data to the explanatory variables. Canonical ordination helped to extract the structure of the dataset by testing the hypothesis of samples being related to species and treatment (Bocard et al., 2011), as GLM models did for the single morphotypes. The CCA-RDA coordinates of the vectors representing the response variables were scaled to unit variance to avoid variables with high values to have a stronger effect on the analysis (Legendre and Legendre, 2012).

5.3.4 Archaeological modelling and predictions

The objective was to try to reconstruct on the basis of the modern phytolith assemblage results, the watering level (so to formulate hypotheses on the type of growing conditions) of the plants that produced the archaeological assemblages under study by considering the relative abundance (assemblage) of phytoliths coming from soil/sediments (see chapter 7). The model constructed for archaeology is based solely on phytolith relative abundance(s) (i.e., the percentage of each morphotype). Morphotype relative abundance was used instead of concentration because in the archaeological context phytolith concentration is relative to grams of sediment analysed and not to the biomass produced by the plants. The dataset of modern crop phytoliths was trained by applying a stepwise selection to choose the best explanatory variables (phytolith morphotypes percentage and ratio of sensitive/fixed morphotypes) to discriminate WW and WS treatment (dependent variable) in a GLM binomial model (Peduzzi et al., 1980). Logistic regressions were then used to evaluate the probability that archaeological assemblages were produced by plants that grew in WW or WS conditions (dichotomic dependent variable) (Bruce et al., 2020).

5.4 Conclusions

The experimental cultivation was designed and developed to overcome some of the issues, such as quantifying water absorbed by plants or the limitations of cultivation in pots, encountered in previous studies (see Katz et al., 2013). Experimental cultivations in pots offer the possibility to collect reliable data only up to an early vegetative growth stage beyond which the soil volume of the pots, e.g., 1 L or 2 L, becomes a limiting factor for root growth (Poorter et al., 2012) and pots are no longer sufficient to ensure a normal development of the plant at later stages. Open-field cultivations, on the other hand, do not allow transpiration to be measured nor to obtain secure data on water uptake by plants. Laboratory experiments tend to demonstrate that plant Si content is higher with high water availability but in external conditions many co-variables, including herbivory pressure, higher light intensity than in glasshouse, different atmospheric conditions (usually higher evaporative demand), nutrient availability, can influence silica content

(Katz, 2019). Growing plants under external conditions, inside lysimeters, offers the possibility of overcoming all of the above listed issues. Lysimeters have proven to overcome both the issues related to pot cultivation in the laboratory and to open field planting, offering experimental conditions that can be useful even for experimental archaeology.

C_4 plants, were capable of morphological and anatomical adaptation in response to drought (Ilyas et al., 2020). The leaves have developed some xeromorphic characters, and have changed their structure to increase drought tolerance, such as smaller and fewer stomata, thicker palisade tissues, large number of trichomes and robust vascular tissues (Ilyas et al., 2020). Grasses more generally have also developed adaptations that make them efficient plants in xeric environments. Specialised epidermal cells, called bulliform cells, facilitate leaf rolling or folding by decreased turgor pressure as plant water potential decreases also as a result of arid conditions (Kadioglu et al., 2012; Maricle et al., 2009). Leaf trichomes are often an adaptation for water conservation (Liu and Liu, 2012) as they can reduce incoming radiation (Galmés et al., 2007) or increase boundary layer resistance to water loss (Perez-Estrada et al., 2000). All these adaptations make the observation of water availability limitation in C_4 grasses particularly complicated. Indeed, C_4 grasses have developed resistance systems that avoid plants from suffering from excessive dry spells, preventing the normal phenomena of desiccation, death and reduced growth. Phytoliths provide a bridge between physiology and anatomy of C_4 species and archeology by allowing water related physiological mechanisms to be analysed. Phytoliths characteristics permit for the investigation of the anatomical adaptations of C_4 and, at the same time, offer information on plant water availability. Moreover, since phytoliths are produced as casts of cells, they allow the observation of certain phenomena that are a specific response to drought. To give an example, the size as well as the density of the stomata decrease as a physiological response to drought stress (Ilyas et al., 2020). Therefore, observing how the concentration of specific morphotypes, such as stomata, varies with the changing level of irrigation should be able to provide answers that, once modelled, can help to reconstruct the water availability, even in the driest areas.

The isotopic composition of individual phytoliths and not of a joint bulk assemblages also grants for innovative results that should provide information not yet available in literature regarding phytolith deposition processes.

To conclude, the methodology applied for this research permit to resolve certain problems relating to both the type of samples in use and the type of technique used to study them, providing useful results to answer the scientific question that led to the development of this thesis.

Appendices available at Zenodo.org

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File A5.1: the complete dataset of the experimental cultivation, including the experimental design for both seasons.

File A5.2: the complete dataset of the phytolith analysis including morphometry and isotopes data.

File A5.3: R scripts.

File A5.3.1, A5.3.2, A5.3.3, A5.3.4, A5.3.5, A5.3.6, A5.3.7: the csv UTF8 (comma-delimited) files to run the code in R.

File A5.4: metadata.

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Chapter 6 - Understanding the Relationship between Water Availability and Biosilica Accumulation in Selected C_4 Crop Leaves: An Experimental Approach

6.1 Background and Objectives

This article marks the first step of my PhD research dedicated to the study of silicon accumulation and phytolith formation in the three C_4 species: finger millet, pearl millet and sorghum. The analysis is of interest because very little is known about the process that drives the deposition of phytoliths in these three species. For sorghum, a limited amount of genetic research has demonstrated the presence of silicon transporter channels that help the flow of this element through the transpiration stream. For the remaining two millets information is scarce and genetic analyses absent. Therefore, the first objective of this PhD research, as well as the purpose of this article, was to understand whether or not the three species were comparable in their silicon accumulation process and whether the production of biosilica by these three species was actually related to transpiration or not. Answering this question seemed essential to then be able to proceed with the study of phytolith assemblage.

I proceeded by analyzing the weight of biosilica extracted from the leaves of the three species cultivated during the experimental cultivation in 2019. The leaves were chosen because most of the silicon accumulates at the end of the transpiration flow exactly in the leaves, where the greatest variety of phytoliths are produced, including the morphotypes considered to be water sensitive (see Chapter 7). Once the biosilica powerd was extracted from the leaves and weighed, it was correlated not only with transpiration rate but by means of an ordination analysis (RDA) all physiological parameters measured during the experimental sultivation, that might have influenced the production of phytoliths, were compared to identify which one was more important in explaining the variability in biosilica accumulation among samples.

6.2 Results and Conclusions

The conclusions I reached can be summarized in two main points. First, the three species are different in the way they accumulate and deposit silicon. Pearl millet and finger millet leaf phytolith accumulation is positively related to transpiration, accumulating more in WW when transpiration is high. Sorghum, on the other hand, generally accumulates much more biosilica than the other two millet species and silicon accumulation does not seem to be related to water conditions. Secondly, although sorghum generally has a stable silicon accumulation, it shows great intra-species (between the landraces considered) variability. This suggests that genetics is involved and plays a key role in the biosilica accumulation.

Since the article has already been published open access the related supplementary information (10.5281/zenodo.6320474), the list of tables and the list of figures follow the numbering of the publication and not that one in use for this thesis.

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Understanding the Relationship between Water Availability and Biosilica Accumulation in Selected C_4 Crop Leaves: An Experimental Approach

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Abstract: Biosilica accumulation in plant tissues is related to the transpiration stream, which in turn depends on water availability. Nevertheless, the debate on whether genetically and environmentally controlled mechanisms of biosilica deposition are directly connected to water availability is still open. We aim at clarifying the system which leads to the deposition of biosilica in *Sorghum bicolor*, *Pennisetum glaucum*, and *Eleusine coracana*, expanding our understanding of the physiological role of silicon in crops well-adapted to arid environments, and simultaneously advancing the research in archaeological and paleoenvironmental studies. We cultivated ten traditional landraces for each crop in lysimeters, simulating irrigated and rain-fed scenarios in arid contexts. The percentage of biosilica accumulated in leaves indicates that both well-watered millet species deposited more biosilica than the water-stressed ones. By contrast, sorghum accumulated more biosilica with respect to the other two species, and biosilica accumulation was independent of the water regime. The water treatment alone did not explain either the variability of the assemblage or the differences in the biosilica accumulation. Hence, we hypothesize that genetics influence the variability substantially. These results demonstrate that biosilica accumulation differs among and within C_4 species and that water availability is not the only driver in this process.

Keywords: biosilica; phyoliths; water availability; C_4 crops

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1. Introduction

Silicon uptake, in the form of monosilicic acid $Si(OH)_4$, depends to a great extent on water availability in the soil [1]. Silicon is radially taken up by root cortical cells either by diffusion (passive) or in an energy-dependent (active) manner [2]. A channel-type Si transporter gene, Lsi1, translocates Si across the plasma membrane from apoplast to cells [3], and Si is transported to proximal side apoplastic connections by the efflux transporter Lsi2 [4]. The xylem loading is mediated by another silicon transporter gene, Lsi6, found in the xylem parenchyma cells of the leaf sheath and blades [5]. After roots uptake $Si(OH)_4$, it is transported up to the shoots via the xylem, with the transpiration stream acting as the main motive force [6]. This model has been tested in rice (*Oryza* *sativa* L.), but the same mechanism has been discovered to be dependent on the three transporter genes located in different cell layers in maize (*Zea Mays* L.) [7], and in several vegetables such as pumpkin [8] and cucumber [9]. Far less is known of this mechanism in C_4 species. Once in the plant tissues, monosilicic acid precipitates, forming phytoliths in the cell wall or in the cell lumen [10]. Phytoliths are solid deposits of amorphous biosilica ($SiO_2{\cdot}nH_2O$) produced by living plants in and among cells [11]. In grasses, three principal mechanisms of silicification have been described: (a) a passive cell wall silicification driven by dehydration; (b) a spontaneous silica deposition driven by transpiration in the cell lumen, after the cell protoplast death, and (c) a controlled mechanism that can occur both in the cell wall or in the external side of the functional plasma membrane, templated by cell wall polymers or materials which enhance silica deposition. This mechanism characterizes the silicification of the so-called silica cells, epidermal cells filled up with silica independently of transpiration [12]. In tissues with photosynthetic activity, like leaves and to a minor degree culms, silicon polymerization due to supersaturation by transpiration-driven water loss (options a and b) seems to play a fundamental role in silica accumulation [13]. However, although transpiration has been investigated as one of the main factors, producing high concentrations of silicic acid in plant shoots [14], it is clear that it is not the only parameter that accounts for biosilica precipitation, and that localized silica deposition forming "silica cells" involves other factors (mainly compounds which enhance silica deposition) [12,15,16]. The role and function of Si in plants is not yet fully understood, and this impairs our comprehension of the mechanisms that drive its deposition. However, since the trait persists, it should confer some advantage in improved fitness [17].

Si accumulation in plant tissues varies from 0.1% up to 10% of the dry weight [18]. Even though the precise mechanical properties of Si remain elusive, it has recently been argued that Si accumulation has little if any intracellular role [19], while the biosilicification of phytoliths can act as a protection against numerous environmental stresses [6]. It has been suggested that Si could play a fundamental role in response to water stress conditions: changing the hydraulic conductivity and the osmotic adjustment [20]; forming a thin layer in the epidermal cells which gives rigidity to the tissues [21]; protecting the veins to keep water supply running in limited water conditions [22]; providing better light inception leading towards a better assimilation rate and chlorophyll biosynthesis [23,24]; reducing epidermal water losses through the formation of a Si-cuticule double layer [25]; and increasing water use efficiency in response to the impact on the stomatal movement [26]. Silicon also has a positive effect on mineral nutrient balance, improving resistance to heavy metal stresses and water storage by diluting salts in the cells [27,28]. Furthermore, silicon has been proven to have a strong impact in preventing pathogen infection [29,30].

Recently, phytoliths have been suggested as proxies to determine plant water availability during plant growth since the ratio between environmental morphotypes (transpiration-driven options a and b) and genetically controlled ones (produced in every water context and enhanced by activator substances-option c) is supposed to change under different water regimes [31,32]. We argue that before investigating phytolith morphotype assemblage compositions, which are connected to an intrinsic cell-type silicification mechanism, it is essential to clarify what the dynamics are that determine the accumulation of biosilica in grasses and if transpiration is a major driver in Si uptake and distribution. Our objective was to investigate whether or not there was a strong species-specific or genotypic variation in silica accumulation and if transpiration could account for most of the biosilica produced by the crops.

C_4 species are relatively few compared with the C_3 plants, but they account for approximately 25% of the primary production on the planet since they dominate savannah and grassland biomes of warm-temperate to tropical and arid regions [33]. Their ability to withstand high temperatures and scarce and erratic rainfall patterns is strictly connected to their photosynthetic pathway. C_4 photosynthesis increases the assimilation rate by concentrating CO_2 at the site of rubisco using a structural mechanism that is distributed between two compartments: the mesophyll and the bundle sheath cells (Kranz anatomy) [34]. This characteristic morphology reduces photorespiration and allows rubisco to operate close to its optimum even with a reduced stomatal opening, which prevents water loss but reduces CO_2 concentration. This mechanism has an energetic cost (higher request of ATP to fix CO_2 into organic acids) that C_4 crops can afford since they often develop in high-radiation environments [35], which allows them to compensate with a higher ATP supply. Indeed, C_4 plants are specifically adapted to xeric environments [36]. Due to the complexity of C_4 physiological reactions to water saving, there is still no consensus on whether water availability during plant growth can be inferred through the study of their organs, such as the grains (e.g., for the discrimination of ^{13}C) or through phytoliths (e.g., δ^{29}Si discrimination or morphotype ratios) [37,38]. Conversely, these same methodologies have already been validated for C_3 species, which are characterized by less complex water and CO_2 cycles [31,39]. Indeed, the beneficial effects of Si in different plants is well-documented on C_3 species such as *Triticum durum* (Desf.) [22] or *Triticum aestivum* (L.) [27]. More work remains to be done for C_4 crops. The few studies conducted on sorghum [40] and maize [41] demonstrate that silicon could enhance drought tolerance even in crops well-adapted to survive stress conditions. Thus, understanding the role of Si on C_4 crops' growth and productivity is important.

For this study, we chose *Sorghum bicolor* (L.) Moench (sorghum), *Eleusine coracana* (L.) Gaertn (finger millet) and *Pennisetum glaucum* (L.) R.Br. (pearl millet). Sorghum and millet varieties have been ranked by the FAO among the 150 top crops produced globally [42]. Sorghum and millets are known to be well-adapted to water-scarce situations, having a relatively short crop cycle and requiring modest water amounts [43], but they differ in their C_4 sub-pathway: sorghum is a NADP-ME species, whereas finger and pearl millets are NAD-ME subtypes [44]. NAD-ME and NADP-ME species differ in their typical Kranz anatomy, in their metabolite flow through mesophyll and bundle sheath cells, and in their plastid transporters [45]. Since the biochemical differences between the two pathways involve both the water use adaptation and photosynthetic abilities, testing both pathways allowed us to assess all the possible intra- and inter-pathway differences in biosilica accumulation.

In addition, while we can rely on data that associate sorghum with a transporter-driven Si accumulator, we have no information on the role of genetics for Si uptake in *Pennisetum glaucum* and *Eleusine coracana*. The role of silica in millets remains, therefore, to be clarified, and it is interesting to test whether it relies on genetics or on a transpiration-dependent mechanism. Indeed, *Sorghum bicolor* has been tested for the Lsi1 protein sequence by Vatansever and colleagues [46], who discovered two homologous genes codifying for the transporter proteins. A recent phylogenetic analysis of silicon transporters across the biological kingdoms shows a high level of conservation of Lsi2 in embryophytes [19], indicating an early evolution, which lets us suppose its presence in C_4 species. The study identified the presence of 5 Lsi2 homologous in *Sorghum bicolor* but highlighted the complexity and the diversity of Lsi2 transporters, leaving open different hypotheses for millets. In sorghum, Slp1 has also been localized; it is a unique amino acid compositional protein involved in the precipitation of silica in the silica cells [47]. These data suggest that Si plays a fundamental role in sorghum physiology since its deposition seems to be strictly regulated.

Reports on controlled experimental cultivations to test silica content in relation with water availability are more abundant for C_3 species than for C_4; indeed, rice and vegetables are the most cited in literature [48]. Models and existing data suggest that biosilica formation could be more sensitive to water availability in C_3 species since C_4 grasses are characterized by a specific physiological mechanism that prevents water loss and reduces the transpiration rate, which has been shown to force silica uptake and distribution along the plant [49]. Previous studies have found positive correlations between water availability and silica content in the form of phytoliths, both in C_3 and in C_4 species [14,50,51]. These studies were performed on crops grown under controlled environmental conditions indoors [50], in a greenhouse [14] or in outdoor fields, where it is difficult to keep water availability and transpiration rate under control [51]. Only Jenkins and colleagues [31,51] included more than one genotype of the same species in the experimental replication. Laboratory experiments tend to demonstrate that plant Si content is higher with high water availability but in natural ecosystems many co-variables, including herbivore pressure, higher light intensity than in glasshouse conditions, different atmospheric conditions (usually higher evaporative demand), and nutrient availability, can influence silica content [17]. To mitigate the loss of the co-variable effect in pot/greenhouse experiments but still be able to evaluate weekly plant transpiration, we set our experimental cultivation outdoors in lysimeters with ten different genotypes tested for each species.

In this paper, we aim to clarify the factors involved in silica accumulation and their variations in selected C_4 species and, in addition, provide an important reference for archaeological and paleoenvironmental phytolith studies and agronomic research. To achieve our goal we decided to test different landraces of pearl millet, finger millet and sorghum grown in different water conditions for biosilica accumulation. Our objective was to highlight any difference among the three C_4 species and between two different water regimes which correspond to an extreme water stress and an optimum irrigation condition.

2. Results

2.1. Plant Growth

All replications grew and produced leaves. Finger millet plants did not produce any panicles either in well-watered (WW hereafter) or in water stressed (WS hereafter) conditions (data available in Supplementary Information, File S1). However, WW replications of finger millet produced an abundant canopy (see Table 1), possibly indicating that biomass allocation to reproductive structures was replaced by vigorous vegetative growth. Indeed, finger millet plants transpired more and produced more biomass in WW conditions with respect to the other two species (Figure 1). Biomass has been calculated as the sum of the weights of all the plant components: stem, leaves, panicles and tillers (data and boxplots available in Supplementary Information, Figure S1). Sorghum produced more leaves and more biomass on average in WS conditions (Table 1) than the other two species, and it had higher values of transpiration efficiency (biomass produced (g)/water transpired (L)) (Figures 1 and 2 and Table 1). Sorghum plants grew for an average of 82 days, pearl millet for 73 and finger millet for 105 days. Sorghum plants flowered on average in 31 ± 20 days in WS and 39 ± 18 days in WW (5–6 weeks). Pearl millet flowered on average in 33 ± 21 days in WS and 46 ± 14 days in WW (6–7 weeks). Finger millet did not flower at all.

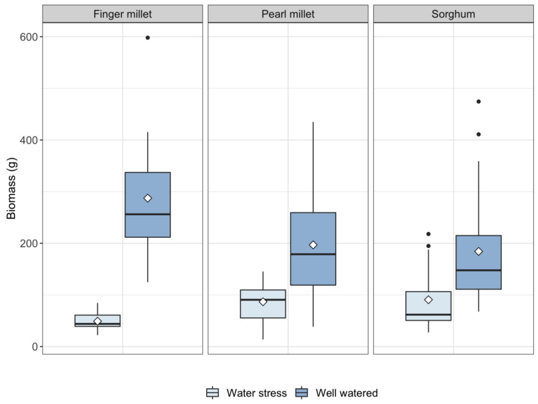

Figure 1. Boxplot of biomass production of finger millet, pearl millet and sorghum species. Biomass represents the sum of the weights of all the plant components: stem, leaves, panicles and tillers. Horizontal bar = median, white diamond = mean, black dots = outliers.

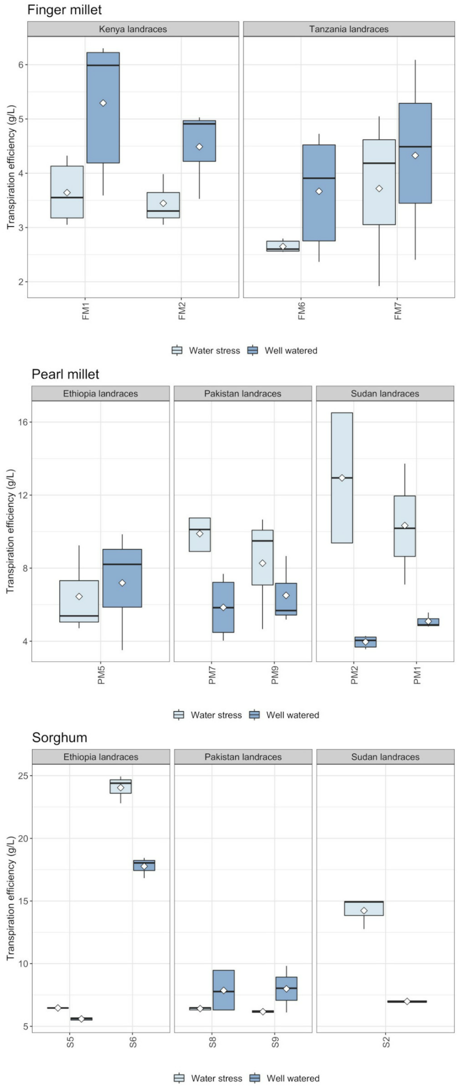

Figure 2. Boxplot of transpiration efficiency of finger millet, pearl millet and sorghum landraces by region of origin. Transpiration efficiency has been evaluated as the ratio between biomass produced/water transpired by the plant. Horizontal bar = median, white diamond = mean, black dots = outliers.

Table 1. Mean and standard deviation values for well-watered (WW) and water-stressed (WS) replications of the three crops.

	Total Water Transpired	Total Biomass	Leaf Biomass
Sorghum	WW: 20.38 ± 5.77 L WS: 8.19 ± 1.19 L	WW: 184.22 ± 112.63 g WS: 90.66 ± 60.70 g	WW: 24.02 ± 19.97 g WS: 14.75 ± 11.84 g
Pearl millet	WW: 26.78 ± 6.51 L WS: 6.72 ± 2.68 L	WW: 196.84 ± 100.57 g WS: 86.71 ± 38.81 g	WW: 10.76 ± 5.42 g WS: 8.03 ± 6.04 g
Finger millet	WW: 32.52 ± 5.88 L WS: 9.42 ± 1.87 L	WW: 287.42 ± 111.90 g WS: 49.10 ± 16.56 g	WW: 31.31 ± 11.38 g WS: 5.56 ± 1.71 g

2.2. Silica in Leaves

Sorghum was by far the species that accumulated the most biosilica, especially under WS conditions (between 5.07% and 11.88% of the dry weight), 2 times more with respect to pearl millet (2.67–6.87%) and 4 times more than finger millet (1.29–3.01%). Moreover, sorghum accumulated almost the same amount of biosilica in WS (mean and SD = 7.94 ± 1.96%) and WW (mean and SD = 8.42 ± 2.93%) conditions, while the other two species of millet presented almost double the percentage in WW conditions (pearl millet WW mean and SD = 7.51 ± 2.35%, WS mean and SD = 4.6 ± 1.47%; finger millet WW mean and SD = 3.74 ± 1.27%, WS mean and SD = 2.37 ± 0.55%) (Figure 3). Considering all the landraces, finger millet and pearl millet accumulated more biosilica in WW conditions with respect to the WS treatment, and the difference is statistically significant (adjusted *p*-value pearl millet WW-pearl millet WS = 0.0006 and finger millet WW-finger millet WS = 1.78×10^{-5}). By contrast, the difference in biosilica accumulation between the two treatments in sorghum is not significant, with an adjusted *p*-value of 0.6021.

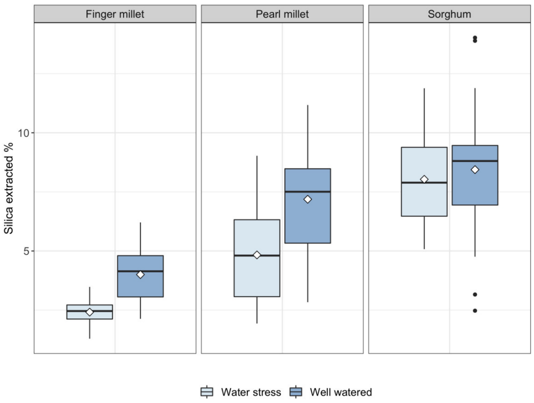

Figure 3. Boxplot of silica accumulation % values of finger millet, pearl millet and sorghum by water treatments. Horizontal bar = median, white diamond = mean, black dots = outliers.

The effect of water treatment on biosilica accumulation in each genotype is shown in Figure 4. Most of the finger millet and pearl millet genotypes (i.e., FM2, FM6, FM7, PM5, PM7, PM9) had statistically significantly higher biosilica content in the WW treatment with respect to WS. For the remaining landraces of millets, the treatment did not have a significant effect on the biosilica accumulation, although they followed the same trend, accumulating more in well-watered conditions. By contrast, sorghum landraces had almost the same value of biosilica accumulation in WW and WS conditions apart from S6, which accumulated less in WW conditions, and S8, which accumulated less in WS conditions. Finger millet and pearl millet displayed low variance of biosilica accumulation across genotypes both in WW and in WS condition (σ^2 of finger millet WW = 1.33, finger millet WS = 0.30; pearl millet WW = 3.90, pearl millet WS = 3.90). By contrast, in sorghum, there is a lot of variation among genotypes, especially in WW conditions (σ^2 of sorghum WW = 8.92, sorghum WS = 3.03).

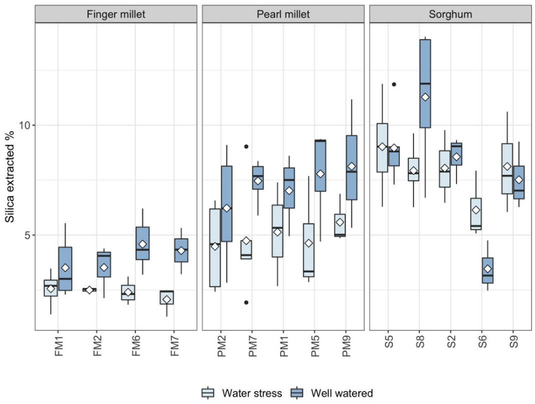

Figure 4. Boxplot of silica accumulation % values for the landraces of finger millet, pearl millet and sorghum by water treatments. Horizontal bar = median, white diamond = mean, black dots = outliers.

We found a significant relationship between silica accumulated by finger millet and pearl millet accessions and the total water transpired by the plants (Figure 5), while the relationship was not significant for sorghum. In finger millet and pearl millet, biosilica accumulation followed the same trend even if we analyze the different landraces separately (regressions available in the Supplementary Information, Figure S2), showing a comparable response to water treatments for all of them. On the contrary, sorghum landraces showed a more variable response to total water transpired: positive for S8 (from Pakistan) and negative for S6 (from Sudan). The three remaining landraces did not show significant interaction with the total water transpired.

Since the relationship between total water transpired and total silica extracted (%) was significant but not high, we decided to compare different predictors to observe which had the strongest effect on biosilica accumulation. The model that explained the highest variability in the dataset included the interaction effect of water treatment and genotype (Table 2). When the dataset with all the accessions was tested for the effect of water treatment alone, the explained variability was too low (5%) to accept the model, showing that biosilica accumulation was not exclusively associated with the water regime. The explained variability increased substantially when the species was used as an explanatory variable. The value of the adjusted R-squared increased by 10% when the genotypes were used as predictors and by 26% when the interactive effect of genotypes and treatment was used, showing that there was a high variability among genotypes and in how genotypes responded to water treatment.

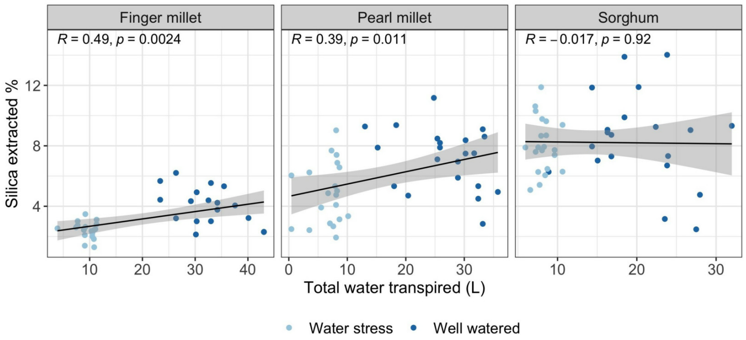

Figure 5. Linear regression tested on the 4 landraces of finger millet, 5 landraces of pearl millet and 5 landraces of sorghum. Total water transpired (L) is used as the independent variable, and % silica extracted from leaves is used as the dependent variable. Gray bands represent 95% confidence intervals.

Table 2. Comparison of linear and multiple linear regression models to account for silica accumulation variability (% of silica extracted) in the dataset, including the three species of finger millet, pearl millet and sorghum.

Model Predictors	Adjusted R-Squared
Water treatment	0.050
Species	0.475
Genotype	0.540
Water treatment + Genotype	0.608
Water treatment × Genotype	0.635

2.3. Silica Extracted and Physiological Parameters

We used a redundancy analysis to test which physiological parameters, including biosilica accumulation, characterize the response of the plants to the water treatments (Figure 6). The triplot captures ca. 50% of the variance and shows a gradient where WW samples (on the left side of the triplot) are characterized by higher values of biomass, leaf biomass (“Leaves”), stem biomass (“Stem”), biosilica accumulation (“SilicaPercentage”), panicle biomass (“Panicle”) and flowering time (“Flowering”), while WS samples (on the right side of the triplot) produced more leaves in comparison to the total biomass (“LeavesPercentage”) and have higher values of transpiration efficiency.

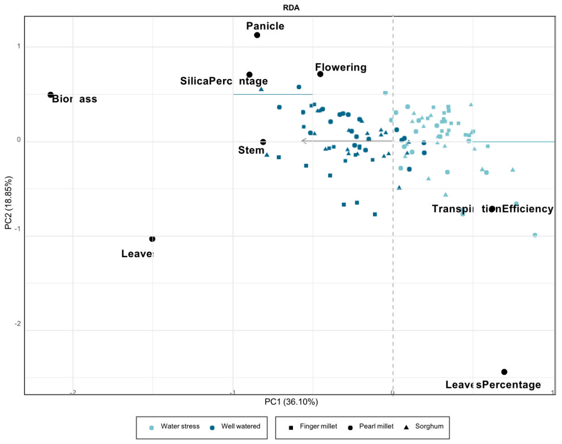

Figure 6. Triplot showing the results of the redundancy analysis. Gray arrow represents the explanatory variable (water treatment), black dots represent the variables included in the analysis (physiological parameters and biosilica accumulation), and blue spots indicate the samples which correspond to the single plant. Light blue = WS and blue = WW. Triangle = sorghum, circles = pearl millet, squares = finger millet.

3. Discussion

The results from the phytolith extraction suggest that sorghum accumulates more biosilica with respect to the two species of millet. Pearl millet and finger millet show a positive relationship between total water transpired and biosilica accumulated. On the contrary, overall sorghum production of biosilica is not influenced by the water treatment. The water treatment alone does not explain either the physiological response of the plants or the differences in biosilica accumulation. The genetics related to the different genotypes influence the variability substantially.

3.1. Biosilica Accumulation, Transpiration and Water Availability

Biosilica accumulation was positively related to total water transpired in pearl millet and finger millet for all the accessions under study (Figures 3–5). This difference could be explained by a passive (transpiration-driven) silica accumulation mechanism, controlled by the transpiration stream in the shoots of millets. We hypothesize that millets probably lack an energy-dependent transport process which loads the silica in the xylem, facilitating the influx and efflux out of the cortical cells [4] even when the transpiration rate is low. Thus, the accumulation of Si in the leaves may be mostly related to environmental parameters such as the water transpired, which involves the passive diffusions of the silicic acid with water and other uncharged solutes [4]. This finding implies that the silica accumulated by millets could be a good proxy for plant water availability. By contrast, sorghum landraces showed a null correlation with total water transpired, and no differences in silica accumulation between well-watered and water-stressed replications can be observed (Figures 3–5). This result suggests the presence, in sorghum, of an active mechanism that transports the silicic acid actively up to the leaves independently of the water conditions (transporter-governed Si uptake). We may speculate that this amount of silica is physiologically useful for the plant and could play a role in giving structural support to the tissues [21,22,25], contributing to the osmotic adjustment and the biosynthetic mechanisms [20,23,24,26], and/or influencing the mineral balance by protecting tissues from toxic elements and insect/fungi attack [27–30]. As a consequence, the transfer, mediated by the presence of the transporter Lsi1 and Lsi2 (and Lsi6) is maintained efficiently and constantly [15,46]. These findings support previous results by Verma and colleagues [52] that showed how higher levels of Si accumulation are associated with better performance in water deficit conditions, as it increases photosynthetic leaf gas exchange and improves plant biomass [52]. In conclusion, while silica accumulation, and consequently phytolith production (i.e., number of silicified cells), in sorghum is not a good proxy for water availability, it may have a physiological role that explains its relatively limited biomass reduction under WS conditions.

3.2. *Biosilica Accumulation and Genotypic Variation*

The multiple linear regression models showed that water availability was not the only factor that accounted for the variability in biosilica accumulation (Table 2). Species and genotypes predicted the amount of silica accumulated better than the treatment alone, as it was previously suggested that phytolith production was influenced more by the phylogenetic position rather than by environmental effects [53]. However, it is interesting to note that our results show that biosilica accumulation is best explained by the interactive effect of genotypes and watering, suggesting that both variables had a substantial effect in determining the variability in biosilica accumulation. Si uptake and distribution in grasses is a dynamic process that depends on Si availability [54], the plant's internal Si demand (physiology) [54], the expression gene involved in Si uptake and accumulation along the plant canopy [55], and external factors which affect the silica distribution, such as the transpiration stream that drives the passive uptake [56]. In the previous section, we argued that the absence of an active mechanism of Si uptake, controlled by genetics, could make the environmental effect prevail in finger and pearl millet. The fact that all landraces of millets analyzed in this work display comparable responses (Figures 3 and 5) supports the hypothesis that transpiration is the variable that played the most important role in biosilica accumulation. The presence of Si transporters in sorghum has been previously suggested [57], and our results show that silica accumulation exceeded the transpiration stream rather than being dependent on it for most of the landraces (Figures 3 and 5). Indeed, we prove the existence of a substantial difference in biosilica accumulation between millets and sorghum that clarifies why the effect of species and genotypes determined the silica accumulation better than the treatment alone. The strong effect that genotypes have in explaining the variability in the dataset (Table 2) could also be related to the specific genetic mechanism that sorghum landraces display for biosilica accumulation. The wide expression profile of Lsi1 and Lsi2 transporter proteins and their regulation, which can be related to different absorption rates of monosilicic acid, is known in the literature [58]. Furthermore, the presence of mutants unable to absorb high quantities of silica in sorghum has been suggested by Markovitch and colleagues [59], which possibly justifies the low biosilica accumulation of the landrace S6 (Figure 4). While silica accumulation in millets responds to the water availability, in sorghum, there is an extra variable that could play a role in biosilica formation and that could account for the high variability rate: the genetics of the silicon transporter.

3.3. *Biosilica Accumulation and Plant Physiology*

The redundancy analysis triplot (Figure 6) showed two important trends: WW samples accumulate more biomass, and WS replications are more efficient in terms of transpiration ("TranspirationEfficiency"). Biosilica accumulation is positively related to biomass accumulation, and it increased when the plant was heavier, affecting the relation of biosilica with transpiration efficiency. Therefore we hypothesize that the effect of the biomass on the biosilica accumulation is consistent, and we argue that it should be related to the effect that the treatment has on the biomass accumulation. Indeed, the RDA triplot revealed that the overall water transpired by the plants has a direct effect on the physiological response (combination of the physiological parameters, including biosilica accumulation) of the landraces under study and, as a consequence, that biosilica accumulation is a parameter that could be taken into consideration to distinguish the two water treatments. As we argued above, Si accumulation in the leaves could possibly play a role (a) to retain leaf water potential by influencing the stomata functioning that silicify more in WW conditions [49], or (b) to defend the leaf surface from biotic and abiotic stress agents [60–64], contributing actively to the better performance of the WW replications. These results seem to indicate that the biosilica accumulation process is stimulated by the physiological conditions and the developmental stage of the plants, as suggested by Mitani and Ma [65]. We need to highlight that this trend has exceptions represented by most of the landraces of sorghum, which accumulate more biomass in WW conditions, but their biosilica accumulation pattern did not change between the two treatments (explanation above). Nevertheless, the fact that most sorghum landraces showed a constant rate of Si absorption independent from the water treatment does not exclude the hypothesis that Si plays a role on the physiological performance of the plant; on the contrary, it validates this hypothesis once more,.

4. Materials and Methods

4.1. Selection of Landraces

To exclude the possibility of changes in the genetics of Si absorption introduced in the breeding process, we decided to work with traditional landraces to observe the variability in biosilica production in un-improved genotypes. Landraces were chosen according to two criteria: (a) area of origin, under the assumption that we need to investigate dryland crops which might have suffered different local adaptations and evolution processes, and (b) climate of origin, to cover all the possible variability within an arid environment. Thus, samples were chosen by selecting their coordinates of origin using the Climatic Research Unit TS3.10 Dataset [66] and https://en.climate-data.org/ (accessed on 1 October 2018), which provide high resolution data on world temperature, precipitation and relative humidity. The databases provide five independent climate variables (mean temperature, average sun hours, precipitation, rainy days and relative humidity) covering the global land surface. They also estimate maximum and minimum temperatures as well as secondary variables, such as potential evapotranspiration. CRU TS3.10 has been used to intersect the climate variables (provided in the form of grids) with the origin coordinates of the varieties on Google Earth. We obtained landraces of pearl millet (*Pennisetum glaucum* L.R.Br), finger millet (*Eleusine coracana* L.Gaertn) and sorghum (*Sorghum bicolor* L.Moench) seeds from the collection of the ICRISAT (Hyderabad, India) gene bank (Table 3).

Table 3. Selected landraces from the ICRISAT genebank with a. the acronym used to identify them and b. their accession number. Climatic data are expressed as the annual mean. Precipitation and rainy days represent the total annual condition. Climatic indices are specific to the region of interest from which the samples come.

	Sudan	**Ethiopia**	**Pakistan**	**Kenya**	**Tanzania**
Climatic data					
Mean temperature	32.79 ºC	27.63 ºC	27.34 ºC	30.03 ºC	24.89 ºC
Average sun hours	10.9 h	10.5 h	10.7 h	10.5 h	10.3 h
Precipitation	70 mm	519 mm	152 mm	213 mm	602 mm
Rainy days	13 days	60 days	15 days	25 days	63 days
Humidity	25.16%	37.16%	44.66%	47.91%	49.33%
Sorghum	S2: IS23075	S5: IS11061 S6: IS38025	S8: IS35215 S9: IS35216		
Pearl millet	PM1:IP13327 PM2: IP9859	PM5: IP2367	PM7: IP18019 PM9: IP18021		
Finger millet				FM1: IE2511 FM2: IE3476	FM6: IE4450 FM7: IE4456

4.2. Experimental Cultivation in Lysimeters

Plants were cultivated in lysimeters (PVC tubes of 2 m in height and 30 cm in diameter) inside concrete pits in ICRISAT, Patancheru, India (17°31′ N 78°16′ E). The lysimeters were filled with a mixture of 1:1 Alfisol-Vertisol, and several crops had been cultivated previously in the same soil mixture inside the lysimeters. At the time of the experiment, the lysimeters filled with the same soil had been fallowed for about 8 months. As the lysimeters were placed outdoors (but covered by a rain-out shelter in case of rain), the experimental conditions allowed for simulating real field conditions regarding plant spacing (11 plants m^{-2}), soil availability for water exploration (2 m depth of soil available for each plant) and general growing conditions. In addition, lysimeters provide control over water availability and transpiration rates thanks to their regular weighing, which allows transpiration assessment and possible re-watering to the desired soil water content levels [67]. The facility was chosen to produce results that can be compared with plants grown in a real field, which make sense both from an archaeological and an agriculture point of view, while the water availability and the transpiration rate have been kept under control to answer the experimental question. While the plants were in the early stages of development, cylinders were watered regularly to keep them close to 100% field capacity. When the plants had grown to c. 20 cm, about three weeks after planting, they were watered to field capacity, and then the soil surface was covered with a plastic sheet and 2 cm of low-density polyethylene granules, in order to prevent about 90% of the soil evaporation [67]. After this, the lysimeters were weighed weekly to evaluate plant water loss from transpiration [68]. Experimental cultivation in pots offer the possibility to collect reliable data only up to an early vegetative growth stage, beyond which the soil volume of the pots, e.g., 1 L or 2 L, becomes limiting to root growth [69] and the pots are no longer sufficient to ensure a normal development of the plant. Several reports and research articles proposed results based on the use of pots for experimental cultivation [70,71], and although we recognize the value of these studies, we propose an alternative methodology that can be exploited to achieve trustworthy results in crop physiology investigation as in experimental archaeology.

The experimental design included ten different landraces for each of the three species to obtain a sufficient sample size to observe the physiological parameters of the growing crops. Five genotypes of sorghum, five of pearl millet and four of finger millet were selected for the present study (Table 3). Selection was based on the physiological response to watering. The genotypes with the highest diversity in physiological parameters were selected within each species in order to assess inter-genotype variations in biosilica accumulation. The remaining 16 landraces have been cropped and stored for future analysis. To represent the range of possible water scenarios, two different water managements were tested to simulate water status in (a) rain-fed conditions in arid environments (water-stressed) and in (b) an irrigated system that acted as control (well-watered). We set five replications for each landrace and treatment, where one replication consisted of one lysimeter in which two plants were grown. The experiment followed a complete randomized block design with species-water treatment as the main block and genotypes as the sub-factor randomized within each block. Genotype replicates were randomized in the pits in order to prevent unintended environmental effects (e.g., heat gradient from the pit walls). Cropping took place in the period between February and May 2019. The complete experimental design is available in the Supplementary Information (File S1). During growth, lysimeters were weighed weekly, allowing the measurement of the transpiration rate and the application of water according to treatment. Flowering dates were also recorded, and biomass was measured after harvest. The physiological parameters measured and available in the Supplementary Information (File S1) are: total water used (TWU), which corresponds to the total water transpired by the plant in liters; total water added (TWA), corresponding to the liters given to the plant during irrigation; “Biomass”, which is the sum of stem, leaves, panicles and seeds weights for each plant in grams; “Leaves”, which is their weight (in grams); “LeavesPercentage”, which consists of the percentage of leaves with respect to the total weight of the plant; "Stem" weight (in grams); "Panicle" weight, corresponding to the chaff and seed weight in grams; "Flowering" time, representing the number of days that each plant took to bloom; and "Transpiration Efficiency", which is the rate between biomass in grams/water transpired in liters. Temperature and relative humidity were collected every 30 min by 2 recorders (Gemini Tinytag Ultra 2 TGU-4500 Datalogger) placed in the two different pits in the crop canopy. Temperature was maintained at 32.28 ± 0.10 °C, and relative humidity was maintained at 42.57 ± 0.23% Rh (measurements are available in Supplementary Information, File S1).

WW plants were irrigated weekly till maturity to maintain the crop at 80% of the soil field capacity, which is optimal for plant growth. WS replications received water until the flowering time to simulate the rain-fed scenario, when water is available only at the beginning of the life cycle but is scarce during the reproductive stage [72]. WW plants have been watered once per week: sorghum plants received an average of 34.99 ± 0.93 L in total, pearl millet received 35.80 ± 1.16 L, and finger millet received 48.93 ± 0.82 L. WS plants received 11 L each across species, distributed in the first 2 months after sowing and before stress imposition. Taking into consideration the diameter of the cylinders (30 cm), it roughly matches to 153 mm of water. Precise data about total water added are available in the Supplementary Information (File S1). An ethnographic investigation by Lancelotti and colleagues [38] recorded instances where pearl millet was grown in a terminal water stress condition (when water scarcity affects the reproduction stage and the grain filling), which is an extremely common growth condition in semi-arid tropics. To simulate this field condition, we set a late-water-stress imposition experiment [73]. It is known that the reproductive stage is particularly sensitive to water deficits and that water availability during and after anthesis is critical [74], so through the imposition of a late water stress, we simulated a realistic rain-fed scenario. WW plants were harvested when the panicles were mature, and the WS replications were harvested when their transpiration rate dropped below 10% of the initial value, indicating full stomatal closure (and further water losses only due to cuticular conductance) [75,76]. Harvesting was done according to genotype, i.e., we harvested all the plants of the same genotype on the same day, when at least ${}^{3}{}_{5}$ replications reached maturity.

4.3. Extraction Method from Leaf Samples

Practically all silica in plants is found as opal silica. As a consequence, we consider phytoliths good proxies for biosilica accumulation, and we proceeded with their extraction. For each landrace, we selected and analyzed two plants of each of the three replications in both treatments. We selected three replications out of five to reduce the sample size, while counting on more than 30 samples per species, as previously suggested to meet statistical representativeness by Jenkins et al. [31,51]. In total, we analyzed 36 samples of finger millet, 18 WW and 18 WS, and 40 samples of sorghum and pearl millet, with 20 WW and 20 WS each. Leaves are the organs through which the transpiration stream goes and where it is assumed to produce the strongest variability for silica accumulation. Samples were prepared for extraction by washing leaves in an ultrasound bath at room temperature to remove all the external sources of silica, and by letting them dry for two days in a ventilated room. Once completely dried, 0.1 g of leaf material was ashed at 500 °C for 12 h in a ceramic crucible covered with a lid to avoid carbonization and prevent cross-contamination. Carbonates were removed using HCl (10% *v*/*v*). Organic matter was oxidized with H_2O_2 (10% *v*/*v*) at 40 °C until the reaction subsided. The residue of silica was rinsed with distilled water and dried at 60 °C. When the powder was completely dry and cool, the extract was weighed to measure the biosilica accumulation of each plant. To facilitate direct comparison among samples, biosilica accumulation has been transformed into percentage. The full protocol used for extraction can be found at https://www.protocols.io/view/phytolith-extraction-and-counting-procedure-for-mo-b6streen (uploaded on 1 April 2022).

4.4. Statistical Analysis

We evaluated the distribution of silica extracts by performing Shapiro's test for normality and Lavene's test for equality of variance [77]. ANOVA, two-way ANOVA and Tukey's honestly significant difference (HSD) tests have been performed to evaluate the significant difference of the variance of treatments, species and genotypes, to evaluate specific pairwise comparisons between treatments and to compare the models. Linear regressions have been performed to model the relationship between biosilica accumulation and total water transpired. Even though response variables (physiological parameters and silica accumulation) were represented by a bimodal curve (juxtaposition of WW and WS normal distributions), linear regression models were used as the distribution of the model residuals was normal [78]. Multiple linear regression models were used to evaluate the variable/s with the strongest effect on bioslica accumulation. Redundancy analysis (RDA) was applied to summarize the variation of the dataset (including the physiological parameters measured and biosilica accumulation) using water treatment as an explanatory variable and conditioned by species and genotypes (covariables). Since the physiological parameters have different units, and as a consequence they are distributed along specific ranges, they have been scaled to unit variance to avoid variables with high values to have a stronger effect on the analysis [79,80]. Statistical analyses and data visualization were conducted in R (version 3.5.1) using standard functions of base, ggplot2 (version 3.3.5) [81] and vegan (version 2.5.6) [82] packages; all the scripts are available in Supplementary Information (File S2).

5. Conclusions

The results presented in this paper allows us to conclude that:

- Water availability plays a fundamental role in determining biosilica accumulation in finger millet and pearl millet, which seem to be passive accumulators where transpiration-driven biosilica production prevails over genetic-mediated silica deposition. Therefore, we maintain that biosilica accumulation in finger millet and pearl millets is a good proxy for water availability;
- Based on the results obtained, different sorghum genotypes absorbed and accumulated silica differently. The relatively high magnitude of variability in response to water treatment suggests that biosilica accumulation in sorghum is not a good proxy for plant water availability. Indeed, sorghum is seemingly characterized by a transporter-governed mechanism, which possibly determines a high variability among genotypes. In the literature, the topic is rather controversial. The results of this paper lead to new perspectives, highlighting that not all the sorghum genotypes respond equally to biosilica accumulation;
- Both environmental conditions and genetic variability play distinct roles in biosilica accumulation, even within the same species.

Nevertheless, we want to highlight that different results have been published, especially in relation to archaeological studies, and this might derive from the experimental settings. Pot-based experiments, possibly conducted under light limitations within glasshouses, may be flawed because of the strong influence of light on transpiration stream and plant development. Therefore, we suggest that experimental cultivation using a standardized methodology is now needed. It is also needed to respond to archaeological and palaeoenvironmental questions.

Supplementary Materials: The following are available online on https://doi.org/10.5281/zenodo.6320474, File S1: Experiment dataset, File S2: R script, Figure S1: Biomass boxplots, Figure S2: Linear regressions.

Author Contributions: Conceptualization, F.D., C.L., M.M., V.V.; methodology, V.V., J.K.; formal analysis, F.D., J.R.-P.; investigation, F.D, J.K.; resources, V.V., J.K.; writing—original draft preparation, F.D.; writing—review and editing, C.L., M.M., V.V., J.R.-P.; visualization, F.D., J.R.-P.; supervision,

C.L., V.V., M.M.; project administration, C.L.; funding acquisition, C.L., M.M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study received ethical approval for the transport of plant material from India to Spain and its laboratory treatment, by the Institutional Committee for Ethical Review of Projects (CIREP-UPF) Approval code 2017/7662/I on 31st October 2017.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in the article or its supplementary material.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Sample Availability: Samples of plant tissues used for the analysis are available from the authors.

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Chapter 7 - Phytoliths as indicators of plant water availability: the case of millets cultivation in the Indus Valley Civilization

7.1 Background and Objectives

The work aims to reconstruct water availability of archaeological C_4 cereals cultivated through the observation of phytolith assemblages. A methodological framework has been built to provide the tools to answer the archaeological question first by studying the production of phytoliths in modern plants and then applying the model to the archaeological assemblage. Indeed, this research aims first at clarifying whether leaf phytolith assemblages and concentration, silica skeleton size and ratio of sensitive to fixed morphotypes can be related to different water regimes in *Eleusine coracana* Gaertn., *Pennisetum glaucum* (L.) R. Br., and *Sorghum bicolor* (L.) Moench. Secondly, it was decided to apply the model to archaeological samples to test its validity and applicability. To this purpose it was decided to exploit a C_4 phytolith dataset from layers dated to the Mature Harappan period (2500-1900 BCE) coming from four different archaeological sites: Harappa, Kanmer, Shikarpur and Alamgirpur. Using millet phytolith samples from Mature Harappan period layers seemed interesting because, to date, there is an open debate on the consumption and spread of such crops throughout the Indus Civilization. During the Mature Harappan period, small millets represented anywhere from 3 to over 50% of the recovered cereals. However, their use is difficult to justify since their grain processing is very labour intensive, apart from the fact that their productivity is lower when compared to that of C_3 species.

This research can be summarised in two strands: first, an analysis of the phytolith production of the leaves of cultivated plants in 2019 was made, focusing on morphotype assemblages, phytolith concentration, silica skeletons size, ratio of sensitive to fixed, which are different indices from the weight of the extracted biosilica in the previous article (chapter 6). The samples analysed for modern crops are the same investigated in Chapter 6. Secondly, having come to the conclusion that phytoliths are efficient in discriminating water treatments, I decided to proceed with the application of the model elaborated, to the archaeological assemblages of phytoliths retrived from a total of 16 contexts (ashes, floors, fireplaces, pits, streets and plasters) for each archaeological site under study.

7.2 Results and Conclusions

I came to the conclusion that it is possible to construct a statistically significant model to observe the level of watering from the composition of phytolith assemblages. The model elaborated takes into account: (1) that the concentration of some morphotypes (bulliforms and elongates) is substantially more predictive than the concentration of others; (2) that the predictive morphotypes in the model are not necessarily those usually considered sensitive in literature; (3) the indices proposed in literature only worked for finger millet and pearl millet, showing that there can be some variability in biosilica accumulation mechanisms even between C_4 species. The prediction made on the archaeological phytolith assemblage suggested that C_4 species may have grown (wild or cultivated) in water scarcity contexts, suggesting some possible hypotheses on their management.

Since the article is in the process of being published the related supplementary information (10.5281/zenodo.7120448), the list of tables and the list of figures follow the numbering of the publication and not that in use for this thesis.

7.3 Phytoliths as indicators of plant water availability: the case of millets cultivation in the Indus Valley civilization

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Abstract

The interpretation of crop water management practices has been central to the archaeological debate on agricultural strategies and is crucial where the type of water strategy can provide fundamental explanations for the adoption and use of specific crops. Traces of water administration are difficult to detect and are mostly indirect, in the form of water harvesting or distribution structures. Attempts have been made to infer plant water availability directly from archaeobotanical remains. Current evidence suggests that the ratio of sensitive to fixed phytolith morphotypes can be used as a proxy for water availability in C_3 crops, as well as in sorghum and maize. Nevertheless, the controversy on whether genetically and environmentally controlled mechanisms of biosilica deposition are directly connected to water availability in C_4 crops is open, and several species remain to be tested for their phytolith production in relation to water levels. This research aims at clarifying whether leaf phytolith assemblages and concentration, silica skeleton size and ratio of sensitive to fixed morphotypes can be related to different water regimes in *Eleusine coracana* Gaertn., *Pennisetum glaucum* (L.) R. Br., and *Sorghum bicolor* (L.) Moench. We cultivated 5 traditional landraces for each species in lysimeters, under different watering conditions and analysed their phytolith content/production in leaves. Results show higher proportions of long cells, bulliforms and stomata produced in well watered conditions. The model built on the basis of phytolith composition has been then applied to interpret archaeological phytolith assemblages recovered from a single phase at four different sites of the Indus Civilisation: Harappa, Kanmer, Shikarpur and Alamgirpur. The results show that most probably C_4 crops grew under water stress conditions, providing new data on the interpretation of ancient agricultural management in the Indus Valley.

Keywords

phytolith; water availability; C_4; millets; Indus valley civilization

1. Introduction

Archaeologists have long appreciated the importance of identifying past water management systems, which marked a key change in the socio-ecological trajectory of human societies and their land use. Indeed, most ancient civilizations were dependent upon sophisticated techniques of water management for agricultural intensification and technological development (Mithen, 2010). Research on water management techniques for agriculture has been crucially contributing to our understanding of the evolution of land use (Beckers et al., 2013). In arid regions the subject of crop water management in the past is rather controversial and difficult to untangle. In pre-industrial drylands pastoralism is often considered to be the primary livelihood strategy, with irrigated and floodplain agriculture as main cultivation strategies,while rain-fed agriculture tends to be considered a secondary activity due to water scarcity (Giosan et al., 2012). Lancelotti et al. (2019), on the other hand, argued that rain-fed cultivation might have played a so-far under recognized role in the development of food production in arid areas, even where rainfall is normally considered too scarce for crop cultivation. Despite many modern examples testify to the existence of successful rain-fed systems (Salmon et al., 2015), such practices have received little attention in archaeology, possibly for the difficulties in identifying them from the archaeological record. Water management is usually inferred from the presence of related technology, such as canals and tanks (see Madella and Lancelotti 2022). However, in the last few decades, water management practices have been also inferred from archaeobotanical remains such as phytoliths (e.g. Jenkins et al., 2020), and charred seeds isotopic signatures (e.g. Lightfoot et al., 2019).

1.1 Plant micro-remains: phytoliths

Translocation of Silicon in the plants structures is mediated by the water flux, with the transpiration stream acting as the main motive force (Ma and Yamaji, 2015). This opal silica deposition in the plant is related to water availability. Two types of Silicon deposition have been described so far (Hodson, 2019): one at the level of the cell wall, and one that occurs within the lumen. In both cases, the precipitation of Silicon to form bio-opals may depend on spontaneous dehydration phenomena or may be stimulated by molecules designed to activate the deposition process (Kumar et al., 2017). A recent phylogenetic analysis of Silicon transporters across the plant kingdom showed the extensive presence of the channel Lsi1 and a high level of conservation of the Lsi2 in embryophytes (Coskun et al., 2019), indicating an early evolution for such transporters and their possible presence in all Poaceae. The presence of these Silicon carrier channels has been tested in rice (*Oryza sativa* L.) and homologous transporters (Vatansever, 2017) have been found in maize (*Zea Mays* L. Sp. Pl.) (Mitani et al., 2009), and in several vegetables such as pumpkin (Mitani et al., 2011) and cucumber (Sun et al., 2017). This discovery suggests that the deposition of phytoliths may not depend entirely on environmental factors such as water abundance, and that genetics certainly plays a key role in the accumulation of Silicon in shoots. Since Silicon polymerization is also due to supersaturation by transpiration-driven water loss (Schaller et al., 2013), we hypothesise that in the tissues with photosynthetic activity, such as leaves, phytolith production, and indices based on their relative abundance, can be tested as proxies for water availability.

Several indicators based on phytoliths production have been developed to assess water availability. Rose and Weiner (1994) suggested using the dimension of silica skeletons, under the assumption that a greater water absorption by the plant led to a greater silica uptake and deposition, which allows for the formation of larger silica conjoined structures (Miller Rosen and Weiner, 1994). The methodology has been applied in archaeological contexts by Katz et al., (2007) producing positive results (Katz et al., 2007). Madella et al., (2009) used the ratio of fixed versus sensitive morphotypes (elongates + stomata/ bilobates, crosses, polylobates, rondels, saddles) and tested it in bread wheat (*Triticum aestivum* L.), emmer wheat (*Triticum dicoccum* L.) and barley (*Hordeum vulgare* L.) (Madella et al., 2009). This method rests on the assumption that leaf phytolith assemblage is composed by genetically determined (short cells) and environmentally controlled morphotypes (stomata and elongates). The methodology was applied by Weisskopf et al. (2015) to detect water availability from cultivated rice (*Oryza sativa* L.) fields (Weisskopf et al., 2015), and by Jenkins et al., (2016) by cultivating barley (*Hordeum vulgare* L.) and durum wheat (*Triticum durum* Desf.) to prove the effectiveness of the ratio in these additional cereals (Jenkins et al., 2016). Both studies demonstrated distinctive phytolith patterns between plants cultivated in wet and dry conditions.

Jenkins et al. (2020) proposed for the first time to apply the same ratio to sorghum (*Sorghum bicolor* L. Moench*)*, with positive results. The same ratio has been exploited by Ermish and Boomgarden (2022), who tested how sensitive to fixed phytoliths ratio and long-cells proportion of maize (*Zea Mays* L.) respond to wet-dry conditions (Ermish and Boomgarden, 2022). The results highlighted strong differences between well irrigated and less-irrigated C_4 crops, proving that the methodology is effective even in crops with reduced water availability. Additionally, the palaeoenvironmental study of Bremond et al., (2005) hypothesised that the more plants transpire and/or suffer water stress, the more silicified bulliform cells they would produce (Bremond et al., 2005). This research is noteworthy because it places for the first time the bulliforms flabellate of the Chloridoideae in the shortlist of possible morphotypes whose production is influenced by transpiration.

1.2 Finger millet, pearl millet and sorghum

Given these favourable examples, it was decided to expand the C_4 species tested by analysing how finger millet, pearl millet and sorghum phytolith production and composition respond to different levels of irrigation. These three cereals belong to the C_4 species that today account for approximately 25% of the primary production of the entire planet (Sage and Zhu, 2011). C_4 are characterised by their physiological ability to withstand high temperatures and scarce and erratic rainfall patterns thanks to their specific photosynthetic pathway, which increases assimilation rate and reduces photorespiration by concentrating CO_2 at the site of the Rubisco (Bräutigam et al., 2014).

Finger millet, pearl millet and sorghum are native to Africa, but they probably followed different trajectories of domestication. Sorghum has been domesticated relatively late (c. around 50 BCE) even if it was probably cultivated in a wild form a few millennia earlier (Fuller and Stevens, 2018). The human population of east Sahel probably domesticated sorghum as a fodder grass once they adopted sedentarism and pastoral practices, abandoning hunting and gathering as a main economy (Winchell et al., 2018). Pearl millet is the oldest domesticated crop of Africa (c. 3000 BCE) (Manning et al., 2011), with a single domestication centre either in western Sahel or in the region included between eastern Mali and western Niger (Dussert et al., 2015). Presumably wild pearl millet attracted the attention of the local non sedentary pastoralists because of its prolific production of small grains, and its resilience to gazing (Mercuri et al., 2018). Finger millet, pearl millet and sorghum are traditionally considered to be among the first African millets to enter the Indian sub-continent along with italian millet (*Setaria italica* (L.) P. Beauv.) (Pokharia et al., 2014).

In South Asia, domesticated pearl millet was present in the Saurashtra peninsula (Gujarat) by at least 1700 BCE and possibly arrived even earlier around 1900 BCE (Manning et al., 2011). While the evolution of big-grain pearl millet happened independently in India, it has been suggested that the small-grain varieties are a derivation of Sahelian forms, rapidly disseminated eastward (Winchell et al., 2018). The earliest evidence of sorghum in the Indian peninsula was found in Kunal, nearby Banawali and Rojdi, and is assumed to be as early as Late Harappan period (2000-1700 BCE) (Fuller and Boivin, 2009). Finger millet arrival inland is disputed but the most accepted hypothesis places its introduction in the Mature Harappan Rodji (Fuller, 2003). It has been hypothesised that in northwest South Asia, African millets eventually prevail on the local species because of their higher productivity under intensive cultivation as well as taste (Weber and Fuller, 2006).

1.3 The archaeological application

Once it was verified that the methodological frame developed from phytolith assemblages of modern millets could work to indicate watering levels, it was decided to apply the model to archaeological samples to test its validity and applicability and finally providing an answer to the primary archaeological question on water management in past agricultural societies. To this purpose it was decided to exploit a C_4 phytolith dataset from layers dated to the Mature Harappan period (2500-1900 BCE) coming from four different archaeological sites: Harappa, Kanmer, Shikarpur and Alamgirpur. Using millet phytolith samples from Mature Harappan period layers seemed interesting because, to date, there is an open debate on the consumption and spread of such crops throughout the Indus civilization. During the Mature Harappan period, small millets represented anywhere from 3 to over 50% of the recovered cereals (Pokharia et al., 2014). However, their use is difficult to justify since their grain processing is very labour intensive (Arunachalam et al., 2005), apart from the fact that their productivity is lower when compared to that of C_3 cereals (such as barley and wheat) (Pearcy and Ehleringer, 1984). We assume that a possible explanation could be directly related to water availability and management. On the one hand the adoption and increasing use of these cereals can be related to the implementation of an agricultural system based on double cropping (*rabi* or winter *& kharif* or monsoon season) (Weber et al., 2010). While legumes and C_3 cereals such as barley or rice, could be cropped during the wet seasons or near water reservoirs, small millets could have been grown in the submarginal dry areas with limited rainfall (Weber and Fuller, 2006), or sown during summer, the warmest and driest season. An alternative theory sees millets involved in the process of adaptation to increasingly arid climates. Multi-proxy records indicate the onset of drier climate during the Late Harappan (Prasad et al., 2007). The progressive use of millets may have been in response to aridity (Pokharia et al., 2014). It is therefore of great interest to try to understand the water conditions under which this type of cereal survived during the late Harappan period, for adding crucial information in the reconstruction of land use.

1.4 Aims

The work aims to reconstruct water availability of archaeological C_4 cereals. To achieve this goal, a methodological framework that provides the tools to answer the archaeological question needed to be built. In this work, we focused on trying to understand whether phytoliths could serve this purpose. First, it was necessary to check if phytolith production was related to plant water availability. Based on available literature, it was tested whether:

1) phytolith assemblage composition is related to water levels;
2) phytolith concentration is related to water levels;
3) ratio fixed to sensitive morphotypes is related to water levels;
4) silica skeletons size ratio is related to water levels.

Only in a second instance, once the methodology was confirmed, the model built on the modern assemblage was applied to see how likely it was that C_4 plants at Harappa, Kanmer, Shikarpur and Alamgirpur had grown under irrigated conditions.

2. Materials and methods

2.1 Modern samples

We selected five landraces of pearl millet (*Pennisetum glaucum* L.R.Br), four of finger millet (*Eleusine coracana* L.Gaertn) and five of sorghum (*Sorghum bicolor* L.Moench) from the gene bank ICRISAT collection (Hyderabad, India) (Table 1).

	Sudan	**Ethiopia**	**Pakistan**	**Kenya**	**Tanzania**
Sorghum	S2: IS23075	S5: IS11061 S6: IS38025	S8: IS35215 S9: IS35216		
Pearl millet	PM1: IP13327 PM2: IP9859	PM5: IP2367	PM7: IP18019 PM9: IP18021		
Finger millet				FM1: IE2511 FM2: IE3476	FM6: IE4450 FM7: IE4456

Table 1 (7.1) - Selected landraces from the ICRISAT genebank with a) the acronym used to identify them and b) their accession number.

Traditional landraces were preferred to observe the variability of phytolith production in un-improved crops, which should exclude any possible recent modern change in the genetics of biosilica accumulation. Landraces were selected according to the area and climate of origin. The specific landraces were chosen using the Climatic Research Unit TS3.10 Dataset, a Google Earth Pro application that divides the entire world into high-resolution climate grids (Harris et al., 2013) combined with climate data from Climate-Data.org (https://en.climate-data.org). Thus, the landraces selected come either from the area of interest (Pakistan), or from African areas from which domestication might have started (Kenya and Tanzania) (Fuller and Boivin, 2009), or from areas in East Africa

(Sudan and Ethiopia) where the climatic parameters are comparable to those in the Indus valley, covering a good range of variability.

The experimental cultivation took place at ICRISAT, Hyderabad, India (17°31' N 78°16' E) between February and May 2019. To reproduce field conditions while keeping a tight control on water-related parameters, the plants were cultivated in lysimeters (PVC tubes of 200 cm in length and 25 cm in diameter). Lysimeters, positioned in two parallel pits about half a metre apart, simulate real field conditions regarding plant spacing (11 plants/m^2), soil availability for ground water exploration (2 m of soil available for each plant), and general growing conditions as the tubes are placed outdoors (but covered by a rain-out shelter in case of rain). The tubes were filled with a mixture of 1:1 Alifisol-Vertisol. Two different water managements were tested to simulate water availability in (a) rain-fed conditions in arid environments (water stressed, WS hereafter) and (b) irrigated conditions, which also acted as control (well watered, WW hereafter). WW plants have been watered weekly to maintain 80% of soil field capacity, which is the optimum for crops well adapted to dry climates (Zaman-Allah et al., 2011). WW sorghum plants received an average of 34.99 ± 0.93 L in total, WW pearl millet 35.80 ± 1.16 L and WW finger millet 48.93 ± 0.82 L. With WS replicas, the intention was to imitate a real rain-fed scenario where water is available at the beginning of the plant life cycle but then scarce/absent during the reproductive stage (Portmann et al., 2010). For this reason, considering the diameter of the cylinders (25 cm), and that the minimum rainfall average corresponds to approximately 150-155 mm of water (Climate-Data.org, https://en.climate-data.org), we calculated that the WS replicas should receive 11 litres of water. So, WS cylinders received 11 L each which were administered gradually, every other day, during the first 2 months of plant growth, before the critical flowering time occurred (late stress imposition) and the reproductive stage began.

We cultivated five replications for each treatment per landrace (n = 70, total = 140 replicas). Genotype replicates were randomised in the two pits in order to prevent unintended environmental effects (e.g. heat gradient from the pit walls). To simulate real plant spacing in the field, two plants of the same genotype (same replication) were planted in each cylinder. When the plants had grown to c. 20 cm, about three weeks after planting, they were watered to field capacity and then the soil surface was covered with a plastic sheet and 2 cm of low-density polyethylene granules, which prevented about 90% of evaporation from the soil (Vadez et al., 2011b). After this, the lysimeters were weighed weekly to calculate plant water loss from only transpiration (Vadez et al., 2011a). Data on temperature and relative humidity were collected every 30 minutes by 2 recorders (Gemini Tinytag Ultra 2 TGU-4500 Datalogger) placed in the crop canopy. Temperature maintained at 32.28 ± 0.10 ºC and relative humidity at 42.57 ± 0.23 % RH. We harvested WW plants when the panicles were mature (when at least 3/5 replications reached maturity) and the WS plants when their transpiration rate dropped below 10% of the initial value, indicating stomatal closure (cuticular transpiration) (Schuster et al., 2017). Panicles, leaves and stems were first dried in ICRISAT at 60-70 ºC for one week and then brought to the Laboratory for Environmental Archaeology of the University Pompeu Fabra (Barcelona, Spain) where samples were processed and phytoliths extracted. Further and more detailed information about the experimental cultivation can be found in D'Agostini et al. (2022).

2.2 The archaeological sites

Sediment samples for phytolith analysis were recovered from layers dated to the Mature Harappan period (2500-1900 BCE) at four sites of the Indus Valley Civilization: Harappa, Kanmer, Shikarpur and Alamgirpur. The samples should be representative of the same level of agricultural technologies/practices even with significant differences on the type of occupation. Samples have been collected and processed by Carla Lancelotti during her PhD project. The results of her thesis along with more detailed information about the micro remains recovered from the sites are all available in Lancelotti (2010) and Lancelotti (2018).

The archaeological site of Harappa (30°37' N 72°53' E) is situated in the Pakistan province of Punjab, on an elevated terrace (*doab*) on the left bank of a channel of the Ravi River. Today the main river flows 10 km north of Harappa, but it appears to have meandered in the vicinity of the site before and during the settlement occupation during the 3rd millennium BCE (Kenoyer, 1998). The samples have been recovered from deposits dated to the central part of the Urban period (phase 3B of mound E; Lancelotti 2018). The archaeological mound of Kanmer (23°23' N 70°52' E), locally known as Bakar Kot, stands to the north of the modern village of Kanmer in the Kachchh District (Gujarat). About 2 km away from the ancient settlement there is an ephemeral stream (*nullah*), known as Aludawaro Vokro. Rajaguro and Shuhsama (2008) suggested that the *nullah* was permanently active during the Harappan times. Presently the only source of water is a large natural tank located in the southeast of the mound, but there is no information on whether such a basin was in use during the Indus period. Shikarpur site (23°14' N 70°40' E), locally known as Valamiyo Timbo, is an archaeological mound located on the southern part of the modern village of Shikarpur (Gujarat), along the margin of a narrow creek that runs eastwards towards the Rann of Kutch. Two water gullies cut the mound into three ridges. The archaeological site of Alamgirpur (29°00.206' N 77°29.057' E), locally known as Parasuram-kakhera, is located in the east area of the modern town in Meerut district (Uttar Pradesh), 45 km northeast of New Delhi. It is situated in the plain between the river Ganga and Yamuna and sits on a consolidated sand dune.

2.3 Phytolith extraction, classification and counting

2.3.1 Modern phytolith samples

Leaves are the organs where transpiration rate is highest and therefore where the maximum production of phytoliths unfolds, including most of those related to hydration mechanisms, such as stomata and bulliforms. For each genotype we selected two replicas and one plant for each replica (n = 56), in order to optimise sample size and physiological parameters variability observed during the fieldwork. Aiming at taking into account possible inter-replicate variability in one of the selected genotypes grown under WW conditions, as two plants were grown within each cylinder, we decided to analyse both plants corresponding to one replicate (+n = 6, total = 62). Therefore, we analysed 18 samples of finger millet, 10 WW and 8 WS; and 22 samples each of sorghum and pearl millet with 12 WW and 10 WS each.

The protocol used for the extraction of phytoliths couples a dry ashing technique with a subsequent wet oxidation. From each sample 0.0001 g of silica residue was mounted on a microscope glass slide with Entellan New® mounting media. Phytoliths were observed using a Euromex light microscope (Euromex iScope + Euromex scientific camera sCMEX-6) at x400 magnification. Since the extractions preserved the silica skeletons and most of the phytoliths were therefore embedded in silica sheets, we based the total count on a minimum number of silica skeletons (50), where all phytoliths in the resulting field of views were counted, whether articulated or disarticulated. The procedure for the extraction and counting of phytoliths, has been summarised in

D'Agostini et al. (2022) and it can be fully consulted on protocols.io (dx.doi.org/10.17504/protocols.io.q26g74mb8gwz/v2).

We counted an average of 619 cells for each slide (raw data is available in the Supplementary Information-File S1). The applied protocol ensures reaching the minimum statistically meaningful number of silica cells per sample by counting more than 300 phytoliths per slide, as suggested by Strömberg (2009). Additionally, it takes into consideration both disarticulated and conjoined cells, respecting richness and evenness distribution. Classification of morphotypes follows the available literature (especially Barboni and Bremond, 2009; Gu et al., 2016; Mercader et al., 2010). Nomenclature follows the International Code for Phytolith Nomenclature (ICPN) 2.0 (International Committee for Phytolith Taxonomy (ICPT) et al., 2019). Figure 1 shows some examples, while descriptions and additional pictures of morphotypes are available in the Supplementary Information (File S2).

For each slide we evaluated and tested: the concentration of phytoliths in millions per gram of dry leaf, the concentration of each morphotype in millions per gram of dry leaf, the percentage of each morphotype, the sensitive/fixed ratio (following the indication of Jenkins et al. (2020)), and the silica skeleton size ratio. The formulas used in this work are:

$$\text{Phytoliths extracted} = \frac{(\text{total silica extracted} \times \text{total phytoliths per slide})}{\text{total silica mounted}}$$

$$\text{Concentration} = \frac{\left(\frac{\text{phytoliths extracted}}{\text{dry leaf weight}}\right)}{1000000}$$

$$\text{Sensitive/fixed ratio} = \frac{(\text{elongates} + \text{stomata})}{(\text{crosses} + \text{bilobates} + \text{polylobates} + \text{rondels} + \text{saddles})}$$

$$\text{Silica skeleton size ratio} = \frac{\text{number of cells in a silica skeleton}}{\text{total number of phytoliths}}$$

2.3.2 Archaeological phytolith samples

A total of 16 contexts for each archaeological site were sampled, giving a total of 64 phytolith assemblages. Phytoliths were extracted from sediment samples by following the procedure described in Lancelotti et al. (2018) which is based on Madella et al. (1998). Phytoliths were identified through comparison with published material and a reference collection of phytoliths recovered from the leaves of local species (Lancelotti, 2010). A minimum of 350 single cell phytoliths were identified for each sample and silica skeletons were counted separately. The effect of taphonomy was tested using the methodology proposed by Madella and Lancelotti (2012). The phytolith assemblages were found to be representative and comparable with the modern ones (see Lancelotti 2018). In order to obtain a dataset comparable with the experimental one, all morphotypes uniquely belonging to C_3 species and all morphotypes produced in inflorescences were excluded from the archaeobotanical dataset. The remaining data include some redundant morphotypes that are present both in C_4 in and C_3. Hence, the phytoliths selected are: elongate entire, elongate sinuate, bulliforms (both blocky and flabellate), stomata, and all the short cells (bilobates, crosses, polylobates, rondels, saddles) except for globulars. All silica skeletons formed by elongates entire and sinuate were also included for analysis. The full selection dataset used for this work is available in the Supplementary Information (File S1). Ubiquitous morphotypes were included because they are present in C_4 and should they derive from C_3 the analysis would not be compromised as this methodology has already been proved successful in C_3 species.

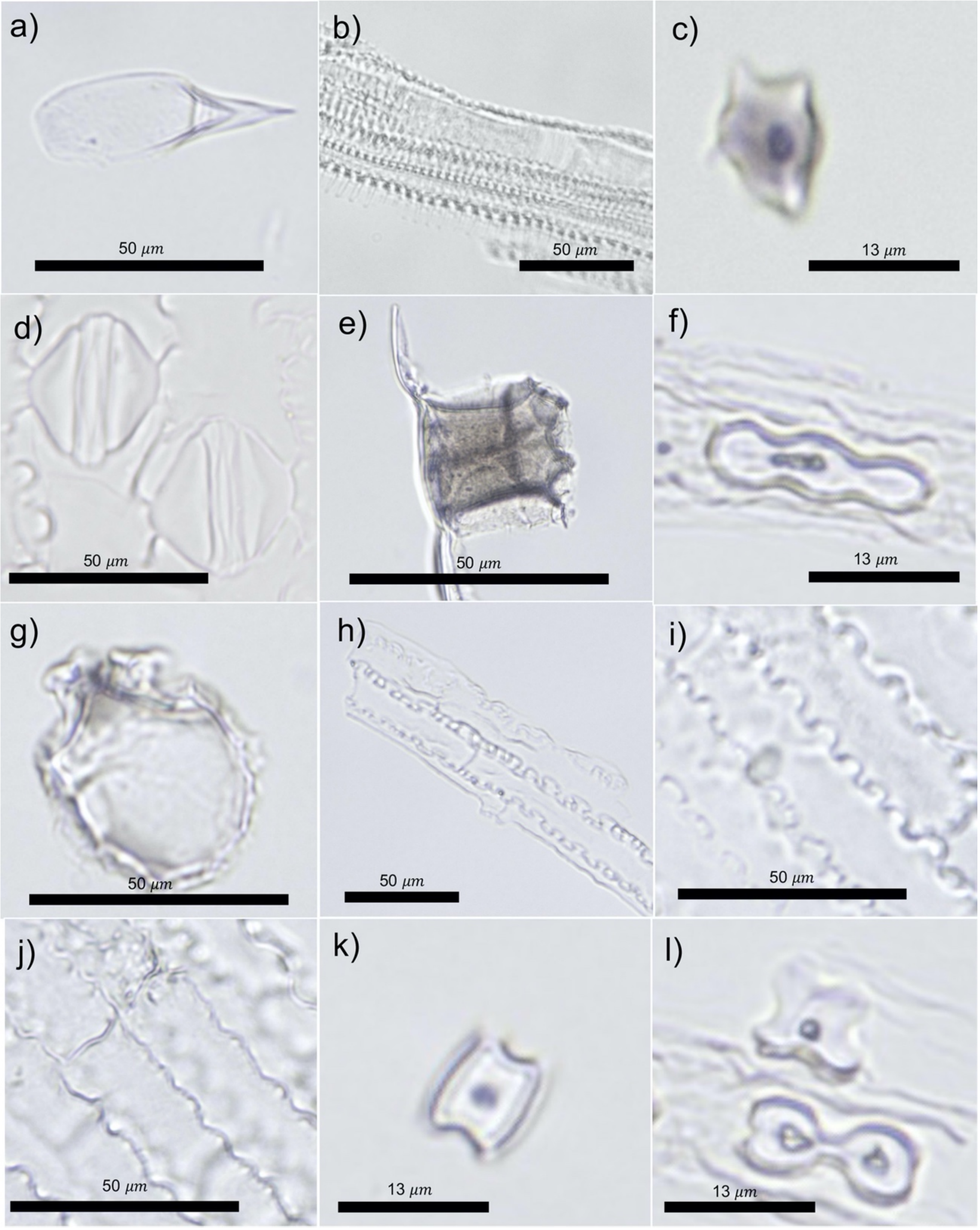
a)
50 μm
b)
50 μm
c)
13 μm
d)
50 μm
e)
50 μm
f)
13 μm
g)
50 μm
h)
50 μm
i)
50 μm
j)
50 μm
k)
13 μm
l)
13 μm

Figure 1 (7.1)

Figure 1 (7.1) - Main phytolith morphotypes recovered from the leaf tissue of the three species finger millet, pearl millet and sorghum. Magnitude x400 and x600. IPS: Inner Pericranial Surface. a) Acute bulbosus (finger millet) - IPS view; b) Tracheary anulate structures (finger millet) - IPS view; c) Rondel (pearl millet) with two spikes in the apex - side view; d) Two stomata (sorghum) in a silica skeleton - IPS view; e) Bulliform parallel (sorghum) attached to a silica skeleton - side view; f) Polylobate (pearl millet) in a silica skeleton - IPS view; g) Bulliform flabellate (finger millet) - side view; p) Silica skeleton of two elongates entire (pearl millet) - IPS view; h) Silica skeleton of elongates clavate (pearl millet) - IPS view; i) Silica skeleton of elongates crenate (sorghum) - IPS view; j) Silica skeletons of elongates sinuates (pearl millet) - IPS view; k) Saddle (finger millet) - side view; l) One cross (above) and one bilobate (below) in a silica skeleton (pearl millet) - IPS view.

2.4 Statistical analysis

Statistical analyses were performed with R (version 3.5.1) using standard functions of base, ggplot2 (version 3.3.5) (Wickham, 2016), vegan (version 2.5.6) (Oksanen et al., 2020) and MASS (version7.3-51.5) (Ripley, 2022) packages. Scripts are available in Supplementary Information (File S3 and on GitHub github.com).

The tests conducted can be summarised in three steps: (1) phytolith assemblages of modern plants were tested by analysing individual morphotypes separately to understand which morphotype is most efficient as a proxy for water availability; (2) concentration and ratios (sensitive/fix, skeleton size) were tested as proxies for water availability; (3) finally, once the model based on the phytolith assemblage of modern crops had been constructed, the archaeological application was carried out.

(1) After calculating each morphotype concentration, generalised linear models (GLM) with Gaussian distribution (the dataset is composed of non-normal variables where the output is a continuous non-negative variable) were used to evaluate which explanatory variable better predicts phytolith composition. Total phytolith and single morphotype concentration were tested as dependent variables while the treatment (WW, WS) and the species (finger millet, pearl millet and sorghum) were considered as independent variables. The p-value and the Akaike information criterion (AIC) were used to assess the validity of the models and to identify the best fitting model (Burnham et al., 2011).

(2) Total sample concentration, ratio of sensitive to fixed morphotypes and the silica skeleton size ratio were tested as possible proxies for water availability. The response variables (concentration and ratios) were normalised using natural logarithm to reduce skewness (Legendre and Legendre, 2012), then linear regressions were applied to model the relationship between phytolith ratios, concentration and transpiration, using ANOVA to test their significant difference. In addition, GLMs were used to evaluate which explanatory environmental variable measured during the experiment (see D'Agostini et al., 2022) better predicts the phytolith concentration, the ratio of sensitive to fixed morphotypes or the silica skeleton size ratio. Although concentration and ratios were normalised, the GLM was still used as the remaining environmental variables are distributed according to a bimodal curve. The independent environmental variables tested were: total water transpired (sum of all the litres transpired by the plants during growth), transpiration efficiency (biomass/total water transpired), last litres of water transpired, genotypes and biomass.

(3) For comparing modern and archaeological assemblages, we used phytolith percentage and not phytolith concentration. Although the concentration of phytoliths gives us a more precise measure of the accumulation of each morphotype in relation to the biomass analysed, the percentage allows not only to compare the results with other studies, but above all, with the archaeological assemblage, whose concentration does not correspond with the concentration of phytoliths derived from modern samples. To the percentages of the individual morphotypes, the ratio of sensitive to fixed phytoliths, which had yielded positive results in previous publications (Ermish and Boomgarden, 2022; Jenkins et al., 2020) was also added to the model. Logistic regressions were used to evaluate the chance that archaeological samples grew in WW or WS conditions (Bruce et al., 2020). The dataset of modern crop phytoliths was trained by applying a stepwise selection to choose the best explanatory variables (phytolith morphotypes percentage and ratio sensitive/fixed morphotypes) to discriminate WW and WS treatment (dependent variable) in a GLM binomial model (Peduzzi et al., 1980).

3. Results

3.1 Phytolith assemblage in modern species

The concentration of each morphotype is almost always higher in WW than in WS. Exceptions are polylobates, which have higher concentrations in WS for all the three species; and elongates clavate and bulliform flabellate, which have a higher concentration in WW only for pearl millet (Table 2). Sorghum is characterised by a high concentration of acute bulbous, bilobates, crosses, elongates clavate and stomata. Acute bulbous, crosses and elongates clavate have particularly high concentrations even in WS conditions. Finger millet is characterised by a high concentration of elongates dentate, entire and sinuate and saddles. Pearl millet has a high concentration of elongates clavate and entire and crosses, but compared to the remaining species, the concentrations of these three morphotypes are lower. Overall, morphotypes highlighted in previous studies as water sensitive (trichomes, bulliforms, stoma and elongates) are more abundant in WW conditions.

If we consider all three species together, water sensitive morphotypes (sum of bulliforms, stomata and elongates crenate, dentate, entire and sinuate) are predicted by the treatment (Table 3). Bulliforms are the morphotypes with the lower AIC and p-value. None of the fixed morphotypes (bilobates, crosses, polylobates, rondels, saddles) are predicted by the water treatments. However, species are good predictors of the morphotype, and the model demonstrates how different species produce morphotypes in different concentrations. (Table 3). Sorghum is distinguished from the remaining species by the concentration of almost all morphotypes (apart from bulliforms flabellate, elongates crenate and sinuate). Concentration of elongates dentate and entire, rondels and saddles distinguish pearl millet from finger millet.

Table 2 (7.2) Morphotype	Well-watered *(per g of dry leaf in millions)*	Water-stress *(per g of dry leaf in millions)*
Phytolith concentration	**21.6754 ± 13.8624**	**17.0006 ± 14.8692**
Acute bulbosus	**0.8243 ± 0.7500**	**0.7270 ± 0.8447**
Sorghum	1.3838 ± 0.9430	1.5061 ± 0.9292
Pearl millet	0.5579 ± 0.4276	0.3674 ± 0.3849
Finger millet	0.4726 ± 0.0847	0.2025 ± 0.4209
Bilobates	**0.5964 ± 0.8471**	**0.4687 ± 0.5322**
Sorghum	1.0723 ± 1.0455	0.8660 ± 0.6120
Pearl millet	0.6059 ± 0.7094	0.4304 ± 0.3442
Finger millet	0.0139 ± 0.0297	0.0200 ± 0.0286
Blockies	**0.1220 ± 0.1946**	**0.0481 ± 0.1110**
Sorghum	0.1826 ± 0.1645	0.1229 ± 0.2138
Pearl millet	0.1037 ± 0.0140	0.0073 ± 0.2337
Finger millet	0.0713 ± 0.0111	0.0058 ± 0.2138
Bulliforms flabellate	**0.0244 ± 0.0561**	**0.0085 ± 0.0168**
Sorghum	0.0432 ± 0.0854	0.0139 ± 0.0233
Pearl millet	0	0.0053 ± 0.0114
Finger millet	0.0310 ± 0366	0.0058 ± 0.0128
Crosses	**1.4729 ± 1.7005**	**1.3242 ± 1.5292**
Sorghum	2.6574 ± 1.2617	2.3420 ± 1.7253
Pearl millet	1.4714 ± 1.9281	1.3144 ± 1.2134
Finger millet	0.0531 ± 0.0605	0.0640 ± 0.0664
Elongates clavate	**6.7093 ± 10.2841**	**7.5099 ± 11.1163**
Sorghum	16.4743 ± 11.1091	19.0966 ± 11.4680
Pearl millet	2.4956 ± 5.4887	1.7735 ± 2.4207
Finger millet	0.0476 ± 0.1131	0.1969 ± 0.3141
Elongates crenate	**0.1825 ± 0.3015**	**0.0633 ± 0.1488**
Sorghum	0.1789 ± 0.3710	0.0213 ± 0.0453
Pearl millet	0.1757 ± 0.2515	0.0584 ± 0.1396
Finger millet	0.1949 ± 0.2960	0.1218 ± 0.2264
Elongates dentate	**0.8560 ± 0.9326**	**0.3247 ± 0.3838**
Sorghum	0.5884 ± 0.5830	0.1010 ± 0.1175
Pearl millet	0.5154 ± 0.6362	0.4189 ± 0.4732
Finger millet	1.5859 ± 1.1987	0.4864 ± 0.3853
Elongates entire	**2.8426 ± 3.4397**	**1.1869 ± 0.9328**
Sorghum	0.7267 ± 0.9134	0.4978 ± 0.3438
Pearl millet	2.5596 ± 1.2667	1.5309 ± 0.8375
Finger millet	5.7213 ± 5.0119	1.6184 ± 1.1145
Elongates sinuate	**6.6661 ± 5.1782**	**4.5050 ± 3.9180**
Sorghum	7.2338 ± 6.4446	6.8265 ± 4.6219
Pearl millet	5.7275 ± 2.9102	3.6539 ± 3.6949
Finger millet	7.1113 ± 5.9480	2.6670 ± 1.2635
Polylobates	**0.0405 ± 0.0705**	**0.0738 ± 0.1050**
Sorghum	0.0663 ± 0.0863	0.1410 ± 0.1462
Pearl millet	0.0484 ± 0.0721	0.0615 ± 0.0463
Finger millet	0	0.0053 ± 0.0151
Rondels	**0.2041 ± 0.2855**	**0.1218 ± 0.1723**
Sorghum	0.0367 ± 0.0824	0.0070 ± 0.0153

Pearl millet *Finger millet*	0.1932 ± 0.3149 0.4182 ± 0.2846	0.0916 ± 0.0980 0.3032 ± 0.2099
Saddles *Sorghum* *Pearl millet* *Finger millet*	**0.4174 ± 0.7264** 0.0093 ± 0.0323 0.0936 ± 0.2194 1.2955 ± 0.8101	**0.2399 ± 0.5277** 0 0.0146 ± 0.0340 0.8215 ± 0.7291
Stomata *Sorghum* *Pearl millet* *Finger millet*	**0.7478 ± 0.7588** 1.3474 ± 0.9246 0.3951 ± 0.3082 0.4517 ± 0.4560	**0.3870 ± 0.5224** 0.8473 ± 0.6679 0.1408 ± 0.0639 0.1194 ± 0.0537

Table 2 (7.2) - Mean and standard deviation of single morphotype concentration for WW and WS on the overall assemblage and for each species considered separately. The numbers underlined in gray correspond to the samples where the concentration increases in WS conditions, while all the remaining morphotypes have a higher concentration under well-watered conditions.

Table 3 (7.3)	**Morphotype**	**x = TREATMENT**		**x = SPECIES (FM VS PM and S)**	
SENSITIVE FORMS	***Acute bulbosus***	**p-value** **AIC**	0.633 151.32	***PM p-value*** ***S p-value*** ***AIC***	0.5520 9.47e-07 122.54
	Bulliforms (sum of blockies + flabellate)	**p-value** **AIC**	0.0555 -32.75	***PM p-value*** ***S p-value*** ***AIC***	0.9962 0.0323 -33.759
	Blockies	**p-value** **AIC**	0.0798 -45.441	***PM p-value*** ***S p-value*** ***AIC***	0.7292 0.0298 -46.304
	Bulliforms flabellate	**p-value** **AIC**	0.156 -209.72	***PM p-value*** ***S p-value*** ***AIC***	0.2034 0.4579 -210.36
	Elongates (sum of all elongates)	**p-value** **AIC**	0.219 483.58	***PM p-value*** ***S p-value*** ***AIC***	0.787 1.08e-06 452.1
	Elongates clavate	**p-value** **AIC**	0.769688 473.44	***PM p-value*** ***S p-value*** ***AIC***	0.366 1.25e-10 423.77
	Elongates crenate	**p-value** **AIC**	0.0613 5.4846	***PM p-value*** ***S p-value*** ***AIC***	0.62129 0.49623 10.621
	Elongates dentate	**p-value** **AIC**	0.00648 142.25	***PM p-value*** ***S p-value*** ***AIC***	0.00868 0.00242 141.03
	Elongates entire	**p-value** **AIC**	0.0164 299.66	***PM p-value*** ***S p-value*** ***AIC***	0.0231 8.2e-05 291.23
	Elongates sinuate	**p-value** **AIC**	0.0738 370.58	***PM p-value*** ***S p-value*** ***AIC***	0.815 0.206 372.94

	Stomata	**p-value** **AIC**	0.03707 128.96	***PM p-value*** ***S p-value*** ***AIC***	0.8913 2.52e-05 109.31
FIXED FORMS	***Bilobates***	**p-value** **AIC**	0.49124 139.63	***PM p-value*** ***S p-value*** ***AIC***	0.0117 7.54e-06 120.86
	Crosses	**p-value** **AIC**	0.721 240.17	***PM p-value*** ***S p-value*** ***AIC***	0.00187 1.51e-07 213.08
	Polylobates	**p-value** **AIC**	0.141 -121.8	***PM p-value*** ***S p-value*** ***AIC***	0.047157 0.000326 55.292
	Rondels	**p-value** **AIC**	0.18656 3.6088	***PM p-value*** ***S p-value*** ***AIC***	0.00112 1.49e-06 -17.341
	Saddles	**p-value** **AIC**	0.2851 125.48	***PM p-value*** ***S p-value*** ***AIC***	5.40e-10 1.23e-10 77.93

Table 3 (7.3) - p-value and Akaike information criterion (AIC) of the gaussian generalised linear models tested using treatment (WW-WS), species (sorghum, pearl millet and finger millet) as the independent variables (x = TREATMENT; x = SPECIES). Light grey cells show the almost significant values; dark grey cells highlight statistically significant results (p-value < 0.05 and low AIC values) i.e. those morphotypes whose concentration is predicted by the independent variable (or treatment or species). In the case of the species, the p-value < 0.05 reports when the PM or S proved to be statistically different from the FM by concentration of the morphotype.

3.2 Phytolith concentration and ratios

Total phytolith concentration between finger and pearl millet is not statistically different (p-adjusted value of 0.88), sorghum produces different (e.g. higher) concentrations of phytoliths if compared with finger millet and pearl millet (p-adjusted value of <0.05 in both cases). Sorghum shows to be a different phytolith producer in respect to the millets, as highlighted also in Table 3, hence, it was decided to keep the three species separate with the aim of highlighting any possible difference among species and landraces.

3.2.1 Concentration

Phytolith concentration is positively related to total water transpired in finger millet and pearl millet (Figure 2). In sorghum the relationship between such variables is not statistically significant (p-value 0.33). The results of the ANOVA tests confirm the hypothesis that finger and pearl millets produced a higher and statistically different concentration of phytoliths in WW conditions both together (p-adjusted WW versus WS = 0.000378) or considered separately (Figure 2), while in sorghum the two treatments are comparable (p-adjusted WW versus WS = 0.8304956). The GLM model, tested to understand which physiological parameter best explained the variability of the concentration of phytoliths identified the last transpiration value as the most significant explanatory variable (p-value = 0.00144), followed by the total water transpired (p-value 0.00359), and the transpiration efficiency (p-value 0.97779).

The variance for phytolith concentration within genotypes in sorghum is very high: σ^2 is 196.511; while it is only 64.83 for pearl millet and 104.40 for finger millet. Some of the landraces appeared to be sensitive to the water treatment (S2, S5, S6) but with a very irregular response: in S5 phytolith concentration is higher in WS conditions while in S2 and S6 concentration is higher in WW conditions (Figure 3).

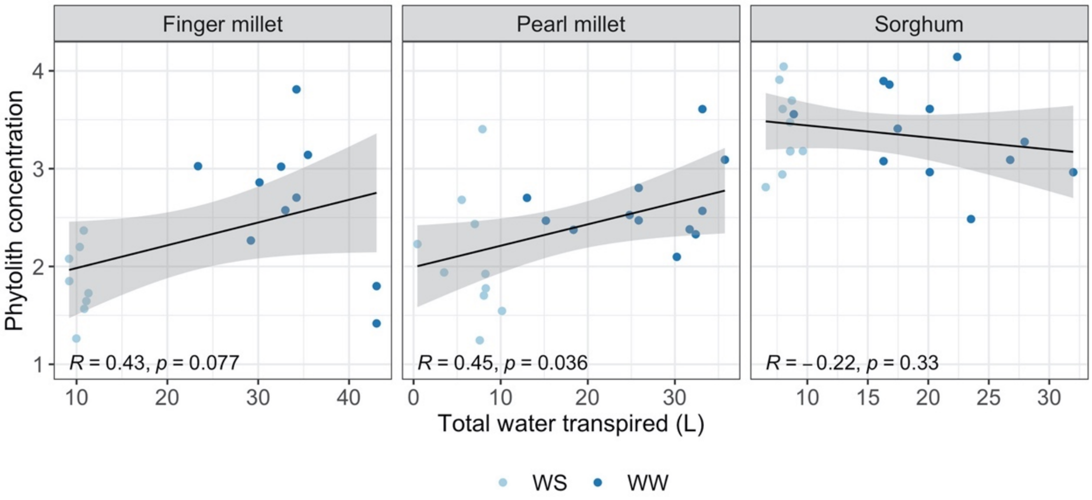

Figure 2 (7.2) - Linear regression for phytolith concentration (nº of cells per gram of dry leaf in millions) tested on finger millets, pearl millet and sorghum. Total water transpired (L) is used as an independent variable, and phytolith concentration extracted from leaves normalised with natural logarithm as dependent variable. Grey bands represent 95% of confidence intervals.

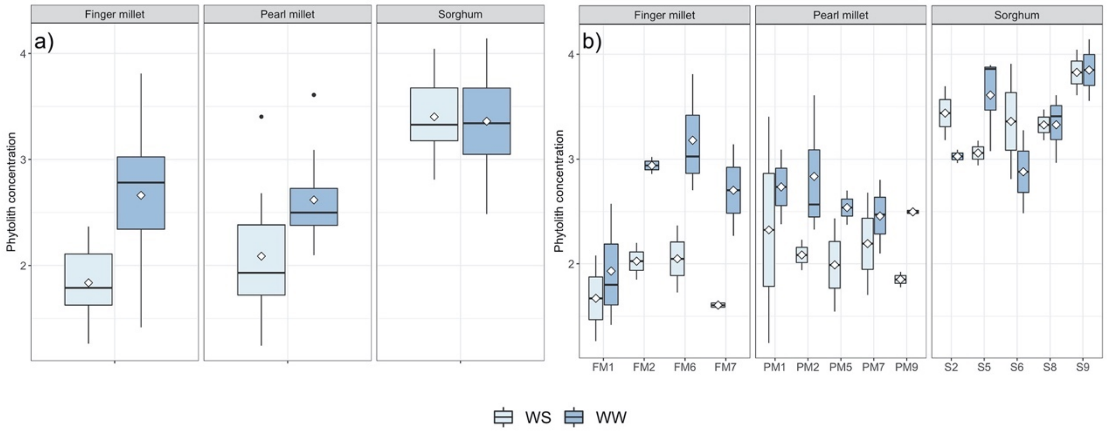

Figure 3 (7.3) - Boxplots of phytolith concentration (nº of cells per gram of dry leaf in millions) by water treatments sorted for a) species and b) landraces. Horizontal bar: median, white diamond: mean, black spot: outliers.

3.2.2 Sensitive to fixed morphotypes

The ratio of sensitive to fixed morphotypes is not related to total water transpired in any of the species (Figure 4). The GLM model tested to understand which physiological parameter best explained the variability of the ratio of sensitive to fixed morphotypes identified the last transpiration value as the most significant explanatory variable (p-value 0.0305).

Sorghum varieties are less variable than the pearl millet and finger millet: σ^2 of sorghum is 0.1439, 0.3360 for pearl millet and 0.4833 for finger millet. Most of the sorghum landraces (S2, S5, S6, S8) separate the two treatments (but not significatively) and only in S5 and in S9 the ratio has a higher value in WW conditions (Figure 5).

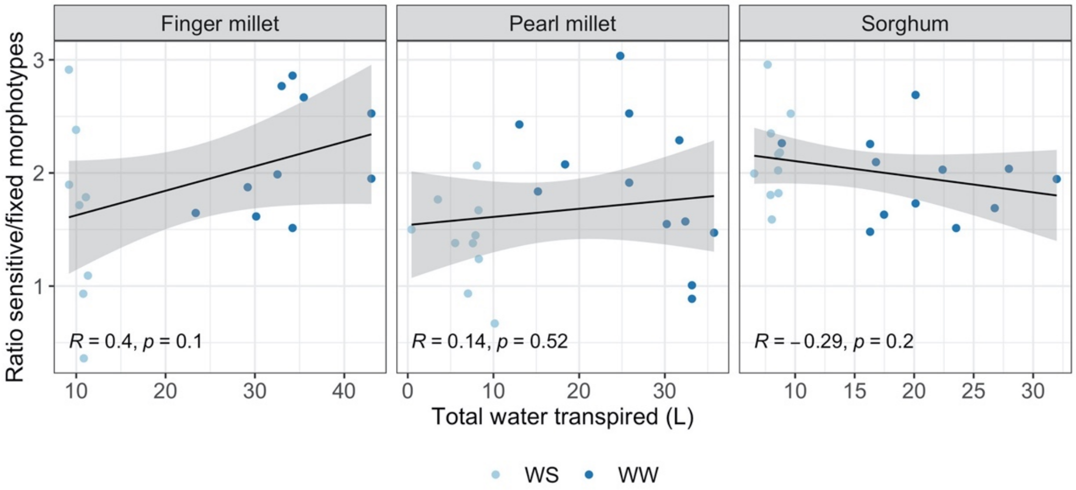

Figure 4 (7.4) - Linear regression for the sensitive to fixed phytoliths ratio tested on finger millet, pearl millet and sorghum. Total water transpired (L) is used as an explanatory variable, and the ratio, normalised using natural logarithm, as dependent variable. Grey bands represent 95% of confidence intervals.

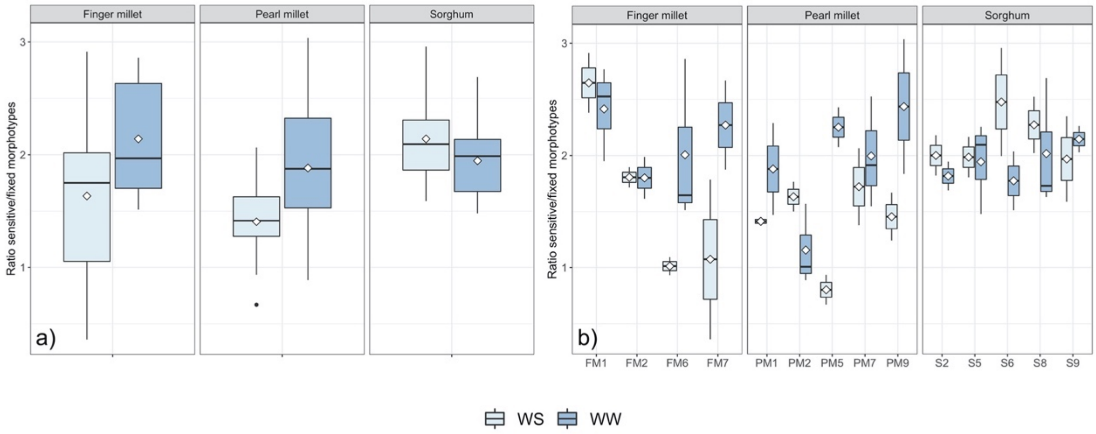

Figure 5 (7.5) - Boxplot of the ratio sensitive to fixed morphotypes by water treatments sorted for a) species, and b) landraces. Horizontal bar = median, white diamond = mean, black spot = outliers.

3.2.3 Silica skeleton dimensions

Silica skeleton size ratio (violin boxplots available in the Supplementary Information-Figure S1) do not seem to relate to water availability in any of the species. The variability of the ratio is particularly high, especially in finger millet, where is higher than the variability within the treatments: σ^2 of the three species in WW is 0.0068 and in WS is 0.0063; σ^2 of sorghum replicas in WW is 0.00038 and in WS is 0.00157; σ^2 of pearl millet replicas in WW is 0.00080 and in WS is 0.00165; σ^2 of finger millet replicas in WW is 0.0088 and in WS is 0.00783.

3.3 Comparison with the archaeological samples

Figure 6 shows the results of the stepwise model (stepwise AIC = 84.51 while full model AIC = 95.87) where the best explanatory variables (blockies, stomata and polylobates) chosen by the automatic procedure have been used as predictors to evaluate the possibility that the archaeological phytolith assemblage derives from WW crops (1 means WW, 0 means WS). The probability that most of the samples derived from WW plants is around 40%, meaning that there is a 60-70% chance that they represent plants grown in water-stress conditions.

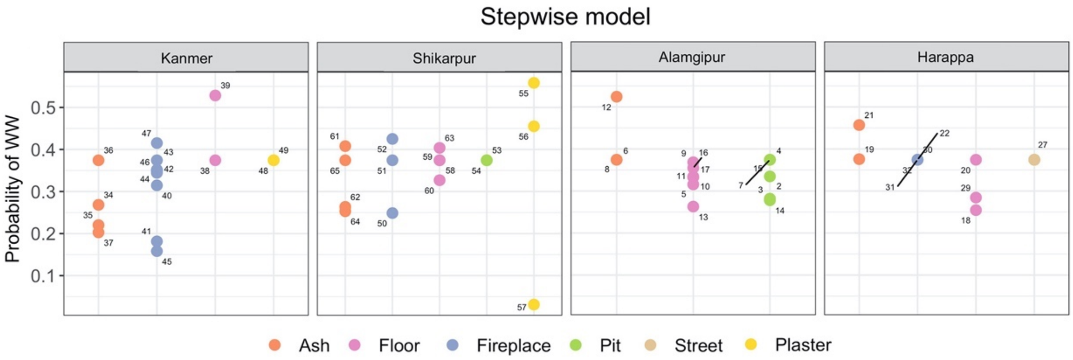

Figure 6 (7.6) - Plot of the probability of each archaeological phytolith sample to be derived from a well watered crop-phytolith assemblage. Each dot is labeled with the number corresponding to the archaeological sample.

4. Discussion

We constructed a methodological framework to test whether different leaf phytolith indices (phytolith concentration, morphotypes concentration, and sensitive/fixed ratio), produced in a group of C_4 species with a key role in prehistoric agriculture in arid areas, can be used as a proxy for plant water availability. To this end, we analysed archaeological phytolith assemblages from distinct Indus Valley sites in order to understand the water practices habits according to which available C_4 crops grew. The results of the study will therefore be discussed starting from the outcomes on the experimental plants and then analysing the extent to which they contributed to the archaeology of the Indus Valley.

4.1 Modern phytolith assemblage

The results from the experimental phytolith assemblage indicate that the concentration values of several morphotypes can be predicted by both the treatments and the species, indicating that phytoliths can be used as proxies for water availability in millet species. The concentration of most morphotypes is higher in well-watered conditions (Table 2), supporting the hypothesis by Schulz-Kornas et al. (2017) from which when there is an increase in silica supply plants produce more phytoliths instead of bigger and/or heavier ones (Schulz-Kornas et al., 2017). In addition, most of the sensitive morphotype concentrations are well predicted by the treatments (Table 3). These outcomes support previous results in different species (Ermish and Boomgarden, 2022; Jenkins et al., 2016, 2020; Madella et al., 2009). Silica skeleton size, however, did not show any variation related to plant water availability and therefore might not be a reliable proxy for water availability in the studied landraces. Only sorghum seems to produce silica skeletons of larger dimension, suggesting that high silica accumulators, as in the case of sorghum, have a bigger chance to produce conjoined silica cell structures.

Nonetheless, phytolith production exhibits high variability among and within the species which differ both in the morphotypes produced and in Silicon accumulation trends (Table 3, Figures 2-3-4-5). On the one hand, phytolith composition assemblages are discriminating the three species: sorghum is characterized by high concentrations of polylobates and crosses, especially in WS conditions, and by an almost total absence of saddles and rondels, supporting results in experiments by Novello and Barboni (2015) and Jenkins et al. (2020). Saddles and elongates are produced in high numbers in the leaves of finger millet. Bilobates, polylobates and crosses are almost absent in pearl and finger millets. These results align with previous studies that indicate that short-cell could differentiate Pooideae from Chloridoideae while bulliforms and hairs are more generic and redundant morphotypes (Barboni and Bremond, 2009; Neumann et al., 2017).

On the other hand, the accumulation of phytoliths seems to follow different trends. Sorghum is the species that not only produced the highest concentration of phytoliths (more than double if compared with the other two species) but also produced more sensitive morphotypes, especially in WS conditions, with low variability among landraces. Neither phytolith concentration nor sensitive morphotype production alone are good predictors of the water availability in sorghum. We can assume that phytoliths in sorghum play a key role in implementing the fitness of the species, being their accumulation constant and independent of treatment, as also suggested by Katz et al. (2019). The beneficial effects of Si in plants are well documented for C_3 species such as *Triticum durum* L. (Meunier et al., 2017) or *Triticum aestivum* L. (Daoud et al., 2018). Studies conducted on S*orghum bicolor* (L.) Moench (Hattori et al., 2005) and *Zea mays* L. Sp. Pl. (Liang et al., 2005) demonstrated that Silicon could enhance drought tolerance even in crops well adapted to survive stress conditions. It is known from literature that epidermal phytoliths can play a role in giving a structural support to the tissues (e.g. elongates) (Meunier et al., 2017; Rodrigues et al., 2003); they can contribute to the osmotic adjustment and the biosynthetic mechanisms (e.g. stomata) (Cooke and Leishman, 2016; Goto et al., 2003; Hosseini et al., 2017); and/or influence the mineral balance by protecting tissues from toxic elements and insect/fungi attack (Fauteux et al., 2005; Mateos-Naranjo et al., 2013; Oliva et al., 2021). Thus, as phytoliths can play a key role in implementing plant physiology response to biotic and abiotic stresses we can assume that phytolith production can be regulated and stimulated. On this consideration we can presume that in sorghum, silicification of elongates, trichomes, stomas, bulliforms and crosses can be genetically controlled. Nevertheless, we also observed a surprising variability for phytolith concentration among the sorghum genotypes, with some positively and some negatively related to water availability (Figure 3). Available literature indicates that the wide expression profile of Lsi1 and Lsi2 transporter proteins and their regulation can be related to different absorption rates of monosilicic acid (Coskun et al., 2021). Furthermore, the presence of mutants unable to absorb high quantities of silica in sorghum has been suggested by Markovich et al. (2019). We then suggest that the high variability in phytolith production in sorghum is probably in connection to such genetic features more than on the environmental factors. Notwithstanding, sorghum phytolith assemblages contributed to obtaining statistically significant results to discriminate treatments. Specifically, although taken separately sorghum seems to show no correlation between watering and phytolith production, the total database comprising the three millets (including sorghum) shows that phytolith production can be correlated with watering and that robust predictive models can be created with them. Sorghum was not removed from the model both because there was evidence that sorghum was present in the archaeological record at the sites of interest (through independent proxies e.g. seeds) and because there was an intention to produce an inclusive model, containing phytoliths of as many species as possible, in order to approximate the archaeological assemblage. Indeed, it is often impossible to reach full identification to genus/species level in archaeological assemblages and these can be formed by plants that are more responsive (such as finger millet and pearl millet in our study) and plants that are less responsive (such as sorghum in our study). Thus, including sorghum in the model makes it stronger when applied to an unknown archaeological dataset. In finger millet and pearl millet, the concentration of phytoliths is actually positively related to watering, as was also discussed in our previous work regarding biosilica accumulation, where we hypothesise a biosilica accumulation driven by mostly transpiration (D'Agostini et al., 2022). The ratio of sensitive/fixed morphotypes does not seem to show positive relationships for millets, underlining a great variability between landraces (Figure 5) in the relationship between the different morphotype classes. We can therefore conclude that fixed morphotypes are useful to distinguish the three species; phytolith concentration and total composition predicts the treatment while sensitive/fixed morphotype ratio is not a good proxy for water availability. We can deduce that since the biosilica accumulation trends are different for each species and since the production of fixed shapes is not constant between species, a common ratio for the three millet is not efficient in highlighting environmental changes in a mixed dataset. The full assemblage, on the other hand, which includes all morphotypes, allows for a model sensitive to water changes.

4.2 Archaeological assemblage and interpretation

Results from the model applied to the archaeological phytolith dataset indicate that most of the cereals grew in water scarcity conditions (Figure 6). All sites gave the same result without any particular differences, despite the fact that the water and climatic conditions are somewhat different among settlements. Harappa and Alamgirpur were possibly located near watercourses, with extensive *doabs* where it was very easy for agricultural practices to take place, perhaps aided even by irrigation systems (Kenoyer, 1998). In contrast, Kanmer and Shikarpur stood most likely on drier sites with no alluvial plains to exploit for agricultural purposes (Lancelotti 2010). In spite of these differences, and considering that the presence of domesticated C_4 cereals has been reported at each of these sites (Harappa: *Panicum* L. Sp. Pl.; Kanmer: *Pennisetum galucum* e *Setaria* Sp. P. Beauv.; Shikarpur: *Brachiaria ramosa* L. Stapf., *Coix lacryma-jobi* L., *Digitaria* Sp.

Haller, *Eleusine coracana*, *Setaria* Sp. P. Beauv.; Alamgirpur: small millets in general) (Bates 2019; Bates et al., 2021) the results obtained in this study seem to emphasise that, at these sites, cereals C_4 grew under a low water regime. Several hypotheses can explain this result: in areas with ample water availability (e.g. Harappa and Alamgirpur) there could be a system of double cropping (*rabi* and *kharif*) with C_3 cereals (e.g. wheat and barley or rice) and vegetables grown under a water regime while others species more resistant to drought, such as C_4 employed during the hot and less rainy period (Petrie et al., 2016). This is supported by ethnographic evidence from the area, where millet crops are mostly rain-fed (Giosan et al., 2012). On the other hand, it is also possible that more drought-tolerant resistant cereals were cultivated alongside C_3 throughout the year, but were used in drier areas of the settlement, growing in a controlled or uncontrolled manner. The results of the model also do not rule out the hypothesis that the area included between southern Pakistan and northwestern India has experienced some form of drying and that the cultures have therefore adapted to more consistent dry periods over time, as suggested by Wright et al. (2008) (Wright et al., 2008). The authors suggested that after a millennium of riverine agriculture based on the overbank flooding, around 2800-2500 BCE, the rivers failed to deliver the usual balance of water and forced farmers to base their agricultural system on the rainfall. The same study suggests that around 2000 BCE the reduction of rainfall could lead to a minimum mean peak of 240 mm. All this information matches with the results obtained in this study, which hypothesised the possible presence of C_4 cereals grown in water stressed conditions with less than 300 mm of available water. Whether the adoption of millets was due to a question of progressive aridification, or whether there was an opportunistic cultivation of C_4 to exploit drier areas or that C_4 grew wild in arid parcels of the sites, what emerges from these findings is that most likely C_4 grew in water scarcity conditions.

5. Conclusion

Firstly, we constructed a methodological framework using phytolith assemblage to observe changes in water availability. Albeit the miscellaneous assemblage of landraces highlighted different trends in phytolith production, the complete dataset allows us to make predictions on the water availability with a good fitting. Even if sorghum, in all likelihood, is an active biosilica accumulator which does not respond only to water availability for phytolith production, it contributed to construct a solid database for archaeological comparison if considered together with the other millets. Bulliforms and stomata, specific sensitive morphotypes are well predicted by the treatments and gave sensible results when tested as explanatory variables in the archaeological dataset. Fixed morphotypes on the contrary are better associated with the species. These findings confirm the validity of leaf phytoliths to identify past water availability for the three species under study when considered together. Nevertheless, further experimental cultivations have been set to assess whether phytolith production are influenced by different growing conditions, where environmental variables as relative humidity, soil composition and silica availability, light intensity and exposition could lead to a variation on sorghum, pearl millet and finger millet biomass production and transpiration rate which in turn could influence phytolith production. Secondly, we aim to answer an important archaeological question about water management in the Indus Valley. Through the study of the dataset of phytoliths from the four archaeological sites Harappa, Kanmer, Shikarpur and Alamgirpur, we can hypothesise the presence of millets grown under water stress regimes, leaving open the hypothesis of double cropping/opportunistic management, where millets were possibly planted in marginal areas with little access to water. The hypothesis would be best confirmed with the addition of alternative proxies such as isotopes or the study of macro remains. However, these results mark a preliminary finding that may open up new theories on millet consumptions in the Indus Valley.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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RAINDROPS has received ethical approval from the Institutional Committee for Ethical Review of Projects (CIREP) at Universitat Pompeu Fabra (ethics certificate n. 2017/7662/I). The work for this paper was carried out when F.D., J.R.P., M.M. and C.L. were members of the CaSEs (UPF), a Research Group of the Catalan Agency for Research (AGAUR SGR 212).

Supplementary Information

Supplementary data to this article can be found online at Zenodo.org with the DOI 10.5281/zenodo.7120448; and in the github.io repository DAgostini2022-PALBO.

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Chapter 8 - Is the phytolith ratio of sensitive to fixed morphotypes a valid index for assessing plant water availability in millets? An alternative approach using the complete morphotype assemblage to build a predictive model

8.1 Background and objectives

A ratio (sensitive to fixed morphotypes) was proposed to discriminate plant water availability through phytolith. The effectiveness of this ratio was tested on various species but not as yet in millets. The aim of this study was to assess the efficacy of this approach/index in millets, considering that in recent years alternative theories on the silica deposition patterns in grasses have been proposed. This study represents a continuation of the work published in previous articles (chapter 7), and it incorporates the results of the second experimental cultivation, which completes the phytolith dataset used.

For this study, phytolith samples extracted from plants cultivated under controlled settings in 2019 and 2020 in ICRISAT have been analysed. The plants were grown in the same season and subjected to the same water treatments. On these plant samples, analysis on phytolith concentration and ratios of sensitive to fixed morphotypes in leaf tissues (divided into young leaves and old leaves) and chaff were conducted. The ratio of sensitive to fixed morphotypes for the three species did not relate to watering, but the concentration of many morphotypes (both sensitive and fixed) did. Therefore, a model was developed to make predictions on watering based on the complete phytolith assemblage.

8.2 Conclusion

The results confirmed the observations previously described in chapter 6, that the three millet species under analysis have different modes of silica deposition. This conclusion needs to be taken into consideration in archaeological studies as it shows that millets cannot be considered a uniform category in terms of silica accumulation. The study also shows that the ratio of sensitive to fixed morphotypes does not closely mirror different water levels available to the plants. This index should not be used as it currently is and, in this article, it has been suggested an alternative approach using a more complex model that considers the silica deposition mechanisms involved in each morphotype formation. The model is useful in archaeological contexts because it allows the assessment of the level of watering of the grasses based on the concentration of morphotypes, proving that phytoliths are good environmental proxies. The proposed model has great applicability in archaeological contexts as was demonstrated in Chapter 7.

8.3 Is the phytolith ratio of sensitive to fixed morphotypes a valid index for assessing plant water availability in millets? An alternative approach using the complete morphotype assemblage to build a predictive model

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Abstract

This work investigates the relationship between phytoliths from C_4 millets (finger millet, pearl millet and sorghum) and the water conditions under which these plants grow. The subject is of broad interest: from an archaeological point of view, the debate on the past use and dispersal of millets remains open in different regions and finding a proxy to help recognise their growing conditions is crucial for the reconstruction of their domestication and acquisition. The study of phytoliths formation in relation to water availability in the three species used in this work has not been studied in depth. A phytolith ratio of sensitive to fixed morphotypes has been previously proposed as a general approach for assessing water availability directly from phytolith assemblages. The ratio has been tested on both C_3 and C_4 species (wheat, barley, rice, maize and sorghum), but never comparing three C_4 species grown under the same experimental conditions. Thus, we developed two experimental cultivations to explore the crops growth under controlled water conditions (irrigation and water-stress). Phytolith concentration and the ratio of sensitive to fixed morphotypes for each species was investigated first. Secondly, an alternative predictive model was elaborated which considered the morphotype concentrations that better predict water availability. The results presented in this study show that there is variability in phytolith production according to the water regimes, but these variations are not always reflected by changes in the sensitive to fixed ratio. The species under study showed to produce phytoliths following different mechanisms and, at least in sorghum, there appears to be no correlation between phytolith concentration and transpiration levels. Therefore, we suggest considering the entire assemblage of phytoliths produced (not just the proportion of sensitive forms) and we advocate for the revision of the classification of sensitive *versus* fixed phytoliths.

Keywords

Phytoliths; water availability; transpiration; C_4 plants; sorghum; pearl millet; finger millet

1. Introduction

The study of C_4 millet crops, occupy a substantial portion of the archaeological debate on the development of land use in past societies (Matuzaite-Matuzeviciute et al., 2022; Stevens et al., 2021; Winchell et al., 2018). Archaeologists are concerned not only with the type of millet species that were consumed, but rather with the type of management that they required (Briggs et al., 2006; Fuller et al., 2011; Stephens et al., 2019). C_4 crops management is closely related to the issue of water availability, as these types of plants are characterised by the ability to withstand high temperatures and water scarcity (Sage and Zhu, 2011; Yang et al., 2021). For this same reason, they are thought to be less sensitive to changes in precipitation or temperature (Ghannoum et al., 2002). Research based on direct botanical remains, both macro- and microscopic, requires acknowledging the plasticity of C_4 crops anatomy and biochemistry, to understand which type of phenomena they can be proxies of (Ferrio et al., 2020). Phytoliths have been indicated as reliable proxies for water availability, as they are preserve well in the archaeological record (Madella and Lancelotti 2012; Strömberg et al., 2018); they are produced by most of the cultivated cereals (Ball et al., 2016); they provide an alternative to the biochemical and morphological analysis on caryopses, seeds, and fruits (morphometry and stable isotopes) as these can be hampered by the C_4 specific photosynthetic and carbon-fixing process as well as the low level of preservation of most C_4 caryopses in the archaeological record (de Vareilles et al., 2021).

Phytoliths are associated with water availability to plants since the translocation of Si in the plant aerial structures is mediated by the water flux and it happens by diffusion, with the transpiration stream acting as the main motive force (Nawaz et al., 2019). In the tissues with photosynthetic activity, such as leaves, culms and to a minor degree inflorescences, the formation of specific morphotypes is most likely due to supersaturation by transpiration-driven water loss (Schaller et al., 2013). On such evidence, the abundance of morphotypes like stomata, bulliforms and elongates, or the indices based on their relative abundance has been proposed as a proxy for water availability. Madella et al. (2009) tested the ratio of fixed versus sensitive morphotypes in bread wheat (*Triticum aestivum* L.), emmer wheat (*Triticum dicoccum* L.) and barley (*Hordeum vulgare* L.). This approach rests on the assumption that leaf phytolith assemblages are composed by morphotypes originating from genetically determined (short cells) and environmentally controlled (stomata and elongates) deposition of silica. The same methodology was applied by Weisskopf et al. (2015) to detect water availability in cultivated rice (*Oryza sativa* L.), and by Jenkins et al. (2016) in barley (*Hordeum vulgare* L.) and durum wheat (*Triticum durum* Desf.). Both studies demonstrated distinctive phytolith production patterns between plants cultivated in wet and dry conditions. Jenkins et al. (2020) applied the same approach for the first time to a C_4 grass, sorghum (*Sorghum bicolor* L. Moench*)*, with positive results. Ermish and Boomgarden (2022) tested how sensitive to fixed morphotype ratio and long-cells proportion of maize (*Zea Mays* L.) responded to wet-dry conditions. The results highlighted strong differences between well irrigated and less-irrigated C_4 plants crops. Additionally, a palaeoenvironmental study of Bremond et al. (2005) hypothesised that the more plants transpire and/or suffer water stress, the more silicified bulliform cells they produce. This research was important because it brought to attention for the first time that the production of bulliforms flabellate in Chloridoideae could be influenced by transpiration.

New discoveries in both genetics and physiology have complicated phytolith morphotypes classification into sensitive and fixed categories considerably. Specifically, alternative/middle-way strategies in the accumulation of Si have been proposed that do not solely rest on transpiration and that could differ according to the species involved or the tissue of deposition. A number of transporter channels were recognised as mediators of Si translocation from the roots to the xylem, facilitating the entry of the silicic acid into the vessels (Vatansever et al., 2017). Lsi1 has been discovered to translocate Si across the plasma membrane from apoplast to cells (Deshmukh and Bélanger, 2016) and Lsi2 to carry Si into the proximal side apoplastic connections (Ma et al., 2006). The xylem loading of silica is mediated by another transporter gene Lsi6 found in the xylem parenchyma cells of the leaf sheath and blades (Gaur et al., 2020). These transporter channels were first identified in rice (Oryza sativa L.) but the same translocation mechanism has been detected in the same three (orthologues) transporter genes in maize (Zea Mays L.) (Mitani et al., 2009), and in vegetables such as pumpkin (Cucurbita pepo L. Dumort.) (Mitani et al., 2011) and cucumber (Cucumis sativus L.) (Sun et al., 2017). Ma and Yamaji (2006) suggested that the amount of silica uptake attributable to genetics depends on two principal factors which both contribute to the xylem loading: the presence/absence of channels to transport Si from cortical cells to the xylem (Lsi1-Lsi2-Lsi6 and orthologs), and the density with which they occur (Ma and Yamaji, 2006). The combination of these two factors determines the classification into active, passive or rejective (non-accumulators) silica accumulators. Although all grasses are considered high accumulators (Hodson et al., 2005), their ability to accumulate Si through specific transporters remains uncertain. Sorghum bicolor (L.) Moench has been tested for Lsi1 protein sequence by Vatansever et al. (2017) who discovered two homologous genes codifying for the transporter channels (Vatansever et al., 2017). A recent phylogenetic analysis of Si transporters across the biological kingdoms shows a high level of conservation of Lsi2 in embryophytes (Coskun et al., 2019), indicating an early evolution which suggests that it should be present in all C_4 species. To add to the uncertain picture of the genetics of Si absorption, mutations have also recently been discovered in sorghum by Markovich et al. (2019) who proved the existence of varieties unable to absorb high quantities of silica. In sorghum, a unique amino acid compositional protein involved in the precipitation of silica in the silica cells (Slp1) has also been discovered (Kumar et al., 2017a, 2020). While data exploring the genetics of Si content are available (Markovich et al. 2019; Vatansever et al., 2017), no information on the mechanisms for Si uptake in Pennisetum glaucum (L.) R. Br. and Eleusine coracana (L.) Gaertn. is available so far.

Different ways of grouping morphotypes for classification purposes have been proposed. A first attempt of grouping morphotypes based on elemental composition and not morphological characters was proposed by Hodson et al. (2016) with the aim of defining phytoliths development in the plant tissue. This approach was then expanded in Hodson (2019), who identified three main mechanisms of Si deposition: 1) silica is deposited onto a carbohydrate matrix such as the cell wall; 2) silica is deposited in the cell lumen; and 3) silica is deposited in the intercellular spaces, which does not lead to phytolith formation. Cell wall morphotypes include macro-hairs and papillae of the glumes, elongate cells and trichomes of the inflorescence bracts; lemma, papillae, hairs, and elongate cells of leaf and culm; and endodermal cells of roots. Cell lumens phytoliths, apparently the more common form in grasses epidermis, include the silica short cells produced in leaves and the inflorescence bracts as well as bulliform cells and elongate cells of the leaves. This suggested grouping brings forward two major innovations: it redefines the sensitive and fixed morphotype categories and it shows that the same morphological category (e.g., elongate cells) could form according to different mechanisms, depending on the species or tissue of formation. Kumar et al. (2017 a,b) also proposed another phytolith classification that somehow results in bringing together the works of Madella (2009) and Hodson (2016) by proposing three different processes of silicification that intrinsically suppose a genetic or an environmental control over it. The three phytoliths grouping are:

(1) Passive cell wall silicification types, environmentally controlled: distinctive of mature and/or intensely transpiring organs, where the condensation of Si is driven by dehydration. In this case a continuous supply of Si infiltrates the cell walls, and its deposition occurs without being metabolically controlled by the cells.

(2a) A controlled cell wall silicification where silica is deposited directly on the cell wall matrix, even before the organ is exposed to the transpiration. Silicification is possibly mediated by the cell wall polymers and the process is genetically controlled.

(2b) In some of these cases, during the silicification of the cell wall the protoplast dies, allowing spontaneous silica deposition driven by transpiration in the cell lumen. In this last instance there is a first genetically controlled silicification followed by dehydration driven by transpiration.

(3) Silica cell silicification genetically controlled, where the Si is deposited on the external side of a functional plasma membrane, possibly in a volume that contains materials that enhance silica deposition, independent of transpiration (option 2 of the cell wall silicification suggested by Hodson (2019).

This alternative classification of phytoliths does not deny the fundamental role in the deposition process played by transpiration (Euliss et al., 2005; Miller Rosen and Weiner, 1994; Sangster and Parry, 1971; Yoshida et al., 1962), but indicates that genetics plays a key role in the formation of specific morphotypes only (Twiss et al., 1969). This new rationale on phytolith formation mechanisms shows that it is not always immediate to classify morphotypes as deposition processes may depend on both (1) the genetics related to Si uptake (active accumulator/passive accumulator species), and (2) the cell-district of deposition, which depends, in turn, on the tissue and on the cell-type.

In respect to the physiological role played by phytoliths, recent discoveries seem to have brought a paradigm shift suggesting that phytoliths play a fundamental function in improving species fitness. Indeed, Katz (2019) suggested that Si content is a plant functional trait since it is so widespread in the plant kingdom. Even though the precise mechanical properties of phytoliths remain elusive, it has recently been argued that Si accumulation has little if any intracellular role (Coskun et al., 2019), while biosilicification can act as a protection against numerous environmental stresses (Nawaz et al., 2019). Si has a positive effect on mineral nutrient balance, improving resistance to heavy metal stresses and water storage by diluting salts in the cells (Daoud et al., 2018; Mateos-Naranjo et al., 2013). Opaline silica has also been proven to have a strong impact in preventing pathogens infection by making difficult to digest abrasive tissues covered by silicified hairs (Fauteux et al., 2005; Oliva et al., 2021). The same genes which regulate lignin deposition govern phytolith formation (Piperno et al., 2002). Since phytoliths are energetically inexpensive in comparison to the deposition of organic carbon-based polymers and, in addition, they stimulate light inceptions and so photosynthesis (Strömberg et al., 2016), it has been suggested that phytolith deposition in arboreal plants helps young tissues to stiffen before organic materials (e.g., lignin) are deposited (Schoelynck et al., 2010). Recently it has been suggested that Si could play a fundamental role in response to water stress conditions as well. Indeed, Si could change the hydraulic conductivity and the osmotic adjustment (Hosseini et al., 2017). Opaline silica could form a thin layer in the epidermal cells that gives rigidity to the tissues, prevents water losses, and confers a beneficial support in water stressed environments (Rodrigues et al., 2003; Yoshida et al., 1962). Phytoliths can harden externally the walls of the veins to keep water supply running in limited water conditions, when vessels, due to dryness of the tissue, may lose turgidity (Meunier et al., 2017). Opaline silica can provide better light inception leading to improved assimilation rate and chlorophyll biosynthesis (Cornelis and Delvaux, 2016; Goto et al., 2003); Phytoliths formation may also impact water use efficiency acting directly on the stomata movement (Gao et al., 2020). These innovative studies, which connected the formation of phytoliths with the ability to withstand water stress conditions, form the scientific rationale for researching a relationship between phytolith assemblages and plant water availability.

Aims of the study

The aim of this work is to understand whether phytolith assemblages of finger millet (*Eleusine coracana* (L.) Gaertn.), sorghum (*Sorghum bicolor* L.Moench) and pearl millet (*Pennisetum glaucum* L.R.Br) can be used as a proxy for plant water availability. This understanding and approach can then be used in archaeological contexts to unravel agricultural technology and, eventually, to reconstruct land use in the past. Beyond clarifying the strength and wider applicability of previously proposed phytolith ratios (Jenkins et al., 2016, 2020; Madella et al., 2009;) there is the need to better understand the mechanisms involved in phytolith deposition in relation to water availability, especially with a comparative approach between and within species (Hodson et al., 2005). Thus, in this research phytolith concentration, assemblages and the relationship between sensitive and fixed morphotypes have been determined in connection to water levels.

Considering that:

1) In this study, water level is evidenced from the transpiration rate, which takes into account the specific adaptations to drought of C_4 species providing the amount of water that passed through the plant. Rainfall is a difficult variable to interpret (Jenkins et al., 2020; Katz et al., 2013) because it does not necessarily express the level of water absorbed by the plant and even less the water available to the plant (e.g., loss due to the evaporative process and the drainage capacity of the soil). Transpiration, therefore, is a useful parameter for the kind of studies carried out in this research since it overcomes the limits of measuring rainfall, but at the same time it positively correlates with water availability.

2) With the aim of obtaining a broad genetic and physiological spectrum different C_4 biochemical pathways have been analysed, sorghum being a NADP-ME and finger millet and pearl millet being NAD-ME sub-types. In addition, to each species different landraces were tested from both Africa and South Asia.

2. Materials and methods

2.1. Experimental cultivations

The experimental cultivation took place at ICRISAT, Hyderabad, India (17°31' N 78°16' E) between February and May of both 2019 and 2020. The second cultivation in 2020 represents a replica of the previous experiment. To reproduce field conditions while keeping a tight control on water-related parameters, the plants were cultivated in lysimeters (PVC tubes of 150 cm in length and 30 cm in diameter). Lysimeters, positioned in two parallel pits about half a metre apart (figure 8.1), simulate real field conditions regarding plant spacing (11 plants/m^2), soil availability for ground water exploration (2 m of soil available for each plant), and general growing conditions as the tubes are placed outdoors (but covered by a rain-out shelter in case of rain). The tubes were filled with a mixture of 1:1 Alifisol-Vertisol.

Figure 8.1 - Lysimeters in ICRISAT (Hyderabad, India) during the experiment in 2019: a) 15 days after the sowing; b) 56 days after the sowing.

Two different water managements were tested to simulate water availability in (a) drought (water stressed, WS hereafter) and (b) irrigated conditions, which also acted as control (well-watered, WW hereafter). The watering of WW was based on the transpiration rate evaluated during the plant growth according to the following procedure: when the plants had grown to c. 20 cm, about three weeks after planting, they were watered to field capacity and then the soil surface was covered with a plastic sheet and 2 cm of low-density polyethylene granules, which prevented about 90% of evaporation from the soil (Vadez et al., 2011b). After this, the lysimeters were weighed weekly in 2019 and every two weeks in 2020 to calculate plant water loss from transpiration only (Vadez et al., 2011a). WW plants were watered weekly to maintain 80% of soil field capacity, which is the optimum for crops well adapted to dry climates (Zaman-Allah et al., 2011). The average of the watering values for WW plants for both years is reported in table 8.1 and the weekly oscillation of transpiration is visible in figure 8.2. The values of water added weekly for both cultivations are available in the appendices (file A8.1). With WS replicas, the intention was to imitate a rain-fed scenario of the arid areas from which these species originate, where water is available at the beginning of the plant life cycle but then scarce/absent during the reproductive stage (Portmann et al., 2010). For this reason, considering the diameter of the cylinders (25 cm), and that the minimum rainfall average corresponds to approximately 150-155 mm of water (Climate North West Knowledge, https://climate.northwestknowledge.net accessed on 22 August 2022) we calculated that the WS replicas should receive 11 L of water. So, WS cylinders received 11 L each which were administered gradually, every other day, during the first 2 months of plant growth, before the critical flowering time occurred (late stress imposition) and the reproductive stage began.

	2019	**2020**
	WW	**WW**
Sorghum	34.99 ± 0.93 L	20.98 ± 0.54 L
Pearl Millet	35.80 ± 1.16 L	15.48 ± 0.57 L
Finger Millet	48.93 ± 0.82 L	15.92 ± 0.62 L

Table 8.1 - Average total water added (L) ± the standard error for each cylinder in the two experimental replicas after cylinders weighing started.

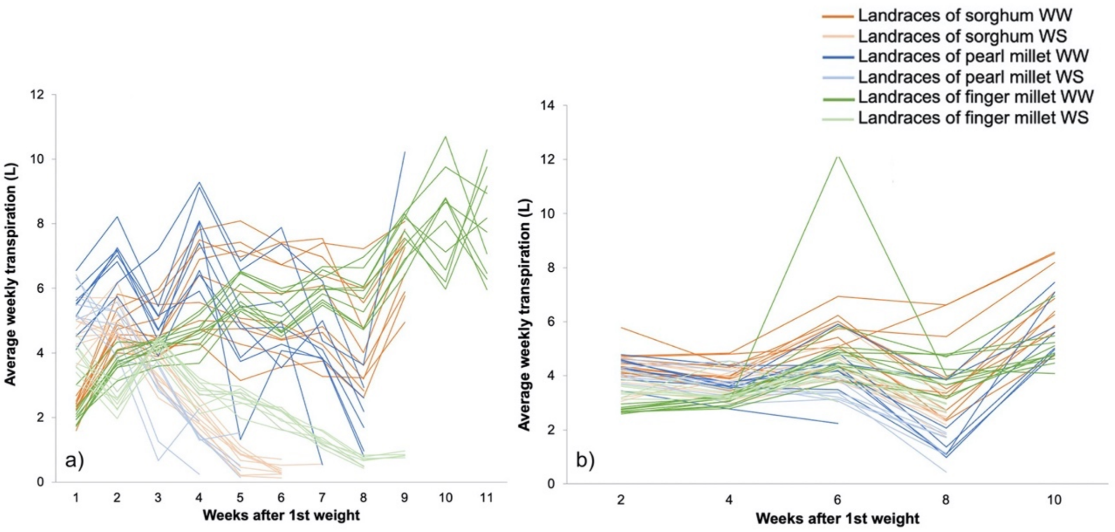

Figure 8.2 - Weekly average transpiration in a) 2019 and b) 2020. Water has been administered on the basis of the oscillation of the weekly transpiration.

To observe the variability of phytolith production in un-improved crops, traditional landraces were preferred, which should exclude any possible recent modern change in the genetics of biosilica accumulation. For both experimental cultivations, the same ten landraces for each species were selected among those available in the ICRISAT genebank, according to the area and climate of origin (for more details see D’Agostini et al., 2022a,b - chapter 6-7). For each of the ten landraces, five replications for each treatment have been setted (n = 50 replicas per each treatment, total = 300 cylinders). The experiments followed a complete randomised block design with species-water treatment as the main block and genotypes as sub-factor randomised within each block (i.e., each year). Genotype replicates were randomised in the pits to prevent unintended environmental effects (e.g., heat gradient from the pit walls). For each cultivation, genotype distribution was randomised separately, so the distribution of the replicas in the pits was different for the two years even though the landraces used are the same. The complete experimental design of both cultivations is available in the appendices (file A8.1).

During the cultivations, transpiration data were collected together with data on temperature and relative humidity that were measured every 30 minutes by 2 recorders (Gemini Tinytag Ultra 2 TGU-4500 Datalogger) placed in the crop canopy. The average temperature across the season in 2019 was 32.28 ± 0.10 ºC (max: 50.5 ºC, min: 19 ºC) and relative humidity at 42.57 ± 0.23 % RH (max: 87%, min: 13.5%). In 2020 the average temperature across the season was 28.64 ± 0.52 ºC (max: 42.8 ºC, min: 11.6 ºC) and relative humidity at 61.70 ± 1.47 % RH (max: 98%, min: 18%).The physiological parameters assessed for this study are: total water transpired (TWT) that corresponds to total litres of water transpired by the plants; total water added (TWA) that is the total amount of water, in litres, provided; Biomass that is the sum of dry stem, leaves, panicles and seeds weights for each plant in grams; and transpiration efficiency that is the rate between biomass in grams divided by water transpired in litres. All corresponding row data is available in the appendices (file A8.1), for more details see D'Agostini et al. (2022a) (chapter 6).

WW plants were harvested when the panicles were mature (when at least 3/5 replications reached maturity) and the WS plants when their transpiration rate dropped below 10% of the initial value, indicating stomatal closure (i.e., cuticular transpiration) (Schuster et al., 2017). Panicles, leaves, and stems were first dried in ICRISAT at 60-70 ºC for one week and then brought to the Laboratory for Environmental Archaeology of the University Pompeu Fabra (Barcelona, Spain) where samples were processed and phytoliths extracted.

2.2 Phytolith extraction, counting and classification

Out of the 10 cultivated landraces, two replicas for each treatment for five landraces of pearl millet (*Pennisetum glaucum* L.R.Br), four of finger millet (*Eleusine coracana* L.Gaertn) and five of sorghum (*Sorghum bicolor* L.Moench) (n = 20 per species, total = 56) were selected. One plant for each replica (cylinder) was sampled. Aiming at taking into account possible inter-replicate variability, for one of the selected genotypes grown under WW conditions, both plants grown in the same cylinder were analysed. Landraces that showed great diversity in both biomass and transpiration rate were chosen for phytolith extraction in an attempt to include all possible intra- and inter-species variability in the analysis. Selected landraces are listed in table 8.2 while table 8.3 provides a more detailed sample list.

Leaves are the organs where transpiration rate is highest and therefore where the maximum production of phytoliths unfolds, including most of those supposedly related to hydration mechanisms, such as stomata and bulliforms, that are produced neither in the chaff nor in the stem. For this reason mainly leaf samples were analysed, first bulk leaf samples (samples 2019, see D’Agostini et al., 2022b - chapter 7) and then young and old leaves separately (samples 2020) (table 8.3), to try to approximate whether there was a difference in silicon accumulation depending on the stage of leaf development as well. “New leaves” correspond to the 5 youngest leaves from the upper apex of the stem, “old leaves” are the mature leaves at the base of the plant. The chaff tissues were analysed in the knowledge that a large proportion of the phytoliths essential for genus/species recognition in cereals accumulate in these organs and are, therefore, recognisable in the archaeological record. In addition, some morphotypes are ubiquitous (e.g., trichomes or elongates entire) and morphologically identical in leaves and chaff, making it impossible to discriminate in the archaeological record which come from inflorescence and which from the leaves. As a consequence chaff samples were included in the analysis. Chaff samples have been processed for phytolith production both for sorghum and pearl millet, while finger millet did not produce any panicles neither in WS nor in WW conditions, neither in 2019 nor in 2020.

	Sudan	**Ethiopia**	**Pakistan**	**Kenya**	**Tanzania**
Sorghum	S2: IS23075	S5: IS11061 S6: IS38025	S8: IS35215 S9: IS35216		
Pearl millet	PM1: IP13327 PM2: IP9859	PM5: IP2367	PM7: IP18019 PM9: IP18021		
Finger millet				FM1: IE2511 FM2: IE3476	FM6: IE4450 FM7: IE4456

Table 8.2 - Selected landraces with the acronym used to identify them and their accession number in ICRISAT genebank.

	2019			2020		
	WW	**WS**	**Total**	**WW**	**WS**	**Total**
Sorghum	12 leaves	10 leaves	22	21 new leaves 21 old leaves 17 chaff	20 new leaves 20 old leaves 14 chaff	113
Pearl millet	12 leaves	10 leaves	22	20 new leaves 20 old leaves 20 chaff	20 new leaves 20 old leaves 15 chaff	115
Finger millet	10 leaves	8 leaves	18	18 new Leaves 18 old leaves	18 new leaves 17 old leaves	71

Table 8.3 - Samples analysed for phytolith content per each year of experimental cultivation.

The protocol used for the extraction of phytoliths combines a dry ashing technique with a wet oxidation. From each sample 0.0001 g of silica residue was mounted on a microscope glass slide with Entellan New® mounting media. Phytoliths were observed using a Euromex light microscope (Euromex iScope + Euromex scientific camera sCMEX-6) at x400 magnification (x600 to take pictures) while the mounting media was still fresh to allow rotating morphotypes. Since the extractions preserved the silica skeletons and most of the phytoliths were therefore embedded in silica sheets, the total count was based on a minimum number of silica skeletons (50). For each slide up to 50 silica skeletons were counted and phytolith included in the structure were classified. All disarticulated phytoliths encountered during the scanning were described and counted separately. At the end of the counting, the disarticulated phytoliths and those encountered inside the silica skeletons were summed together to obtain the total sum for each morphotype, and to calculate the concentration and relative percentage of each one. The procedure for the extraction and counting of phytoliths, has been summarised in D’Agostini et al. (2022a) (chapter 6) and it can be fully consulted on protocols.io (dx.doi.org/10.17504/protocols.io.q26g74mb8gwz/v2 uploaded on 30 September 2022).

An average of 604 cells for each slide was counted (row data is available in the appendices, file A8.2). The applied protocol ensures reaching the minimum statistically meaningful number of silica cells per sample by counting more than 300 phytoliths per slide, as suggested by Strömberg (2009) and Zurro et al. (2018). Additionally, it takes into consideration both disarticulated and conjoined cells, respecting richness and evenness distribution. Classification of morphotypes follows the available literature (especially Barboni and Bremond, 2009; Gu et al., 2016; Mercader et al., 2010). Nomenclature follows the International Code for Phytolith Nomenclature (ICPN) 2.0 (International Committee for Phytolith Taxonomy (ICPT) et al., 2019). The main morphotypes observed are presented in figure 8.3.

Phytolith name following the ICPN 2.0 (2019)	**Figure 3 corresponding images**
Acute bulbosus	a), b), u)
Bilobate	c)
Blocky	d), e)
Bulliform flabellate	f)
Cross	c)
Elongate clavate	g)
Elongate crenate	h)
Elongate dentate	i)
Elongate entire	j)
Elongate sinuate	k), t)
Polylobate	l)
Rondel	m)
Saddle	n), o), p)
Stoma	q)
Tracheary anulate	r)

Table 8.4 - Phytoliths types identified in sorghum, pearl millet and finger millet. Classification has been based on the International Code for phytolith nomenclature (ICPN) 2.0 (International Committee for Phytolith Taxonomy (ICPT) et al., 2019).

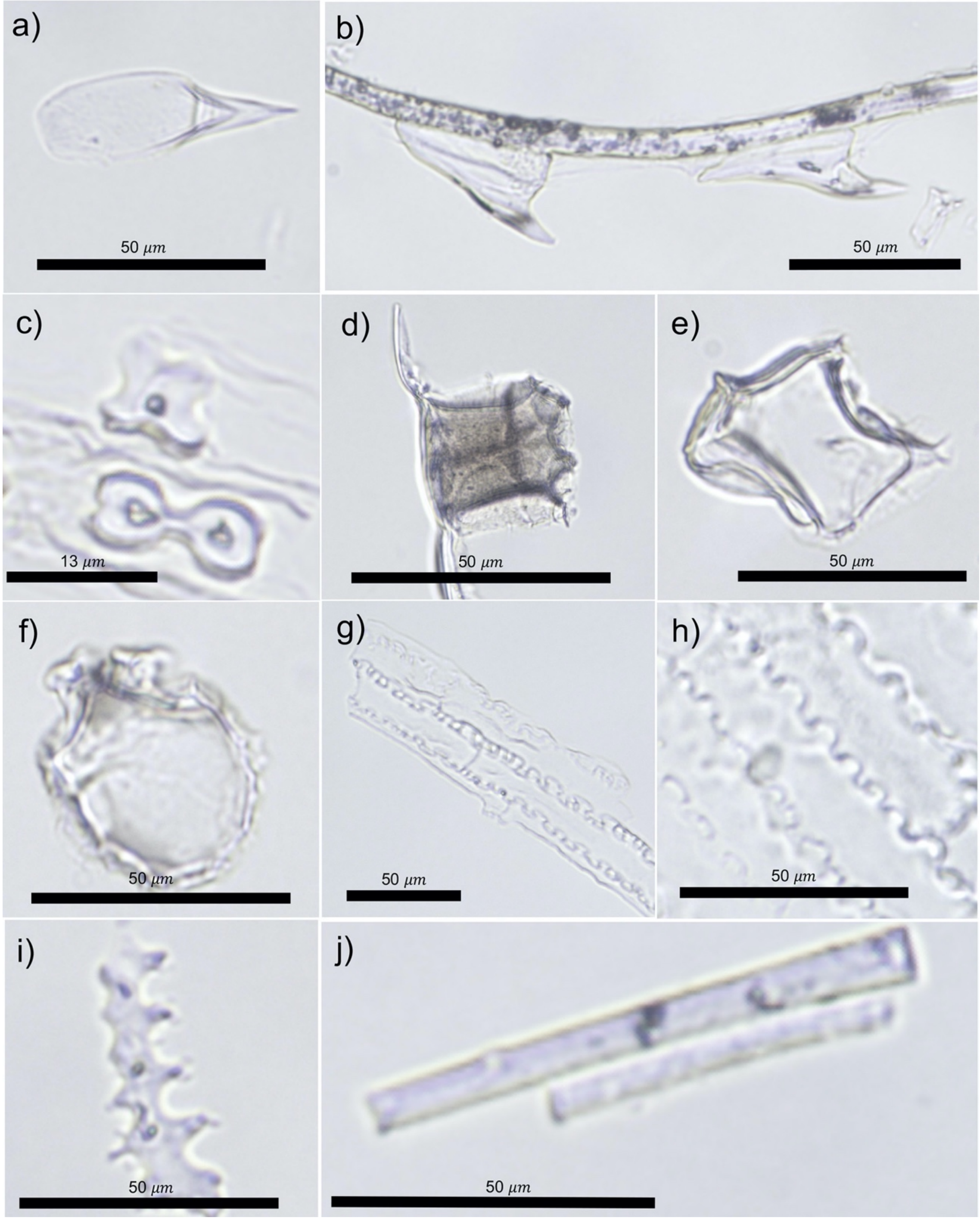
a)
50 μm
b)
50 μm
c)
13 μm
d)
50 μm
e)
50 μm
f)
50 μm
g)
50 μm
h)
50 μm
i)
50 μm
j)
50 μm

Figure 8.3

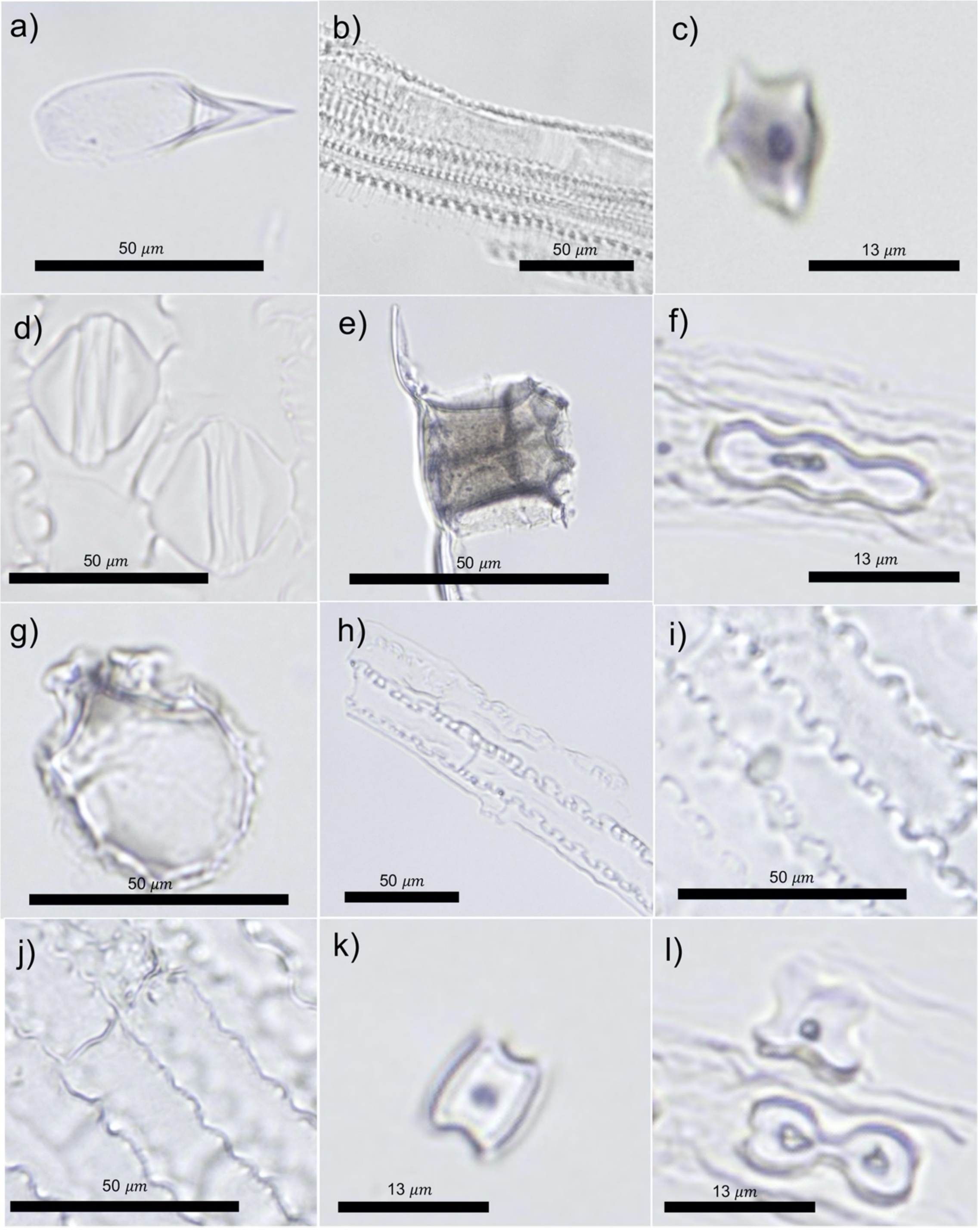
a)
50 μm
b)
50 μm
c)
13 μm
d)
50 μm
e)
50 μm
f)
13 μm
g)
50 μm
h)
50 μm
i)
50 μm
j)
50 μm
k)
13 μm
l)
13 μm

Figure 8.3

Figure 8.3 - Main phytolith morphotypes recovered from the leaf tissue of Finger millet, pearl millet and sorghum. Magnitude x400 and x600. IPS: Inner Pericranial Surface. a) Acute bulbosus of finger millet leaf (IPS view); b) silica skeleton of acute bulbosus connected by an elongate entire in pearl millet leaf (side view); c) one bilobate and one cross in a silica skeleton in pearl millet leaf (IPS view); d) silica skeleton with a blocky in sorghum leaf (side view); e) blocky in finger millet leaf (side view); f) bulliform flabellate of finger millet leaf (side view); g) silica skeleton of elongates clavate in pearl millet leaf (IPS view); h) silica skeleton of elongates crenate in sorghum leaf (IPS view); i) elongate dentate in sorghum leaf (IPS view); j) silica skeleton of two elongates entire in pearl millet leaf (IPS view); k) silica skeletons of elongates sinuates in pearl millet leaf (IPS view); l) polylobate in a silica skeleton in pearl millet leaf (IPS view); m) rondel with two spikes in the apex of pearl millet leaf (lateral view); n) saddle (short form) in finger millet leaf (side view); o) saddle (collapsed form) in finger millet leaf (side view); p) saddle (bilobed form) in finger millet leaf (IPS view); the various saddle variants have all been included in the general saddle category; q) two stoma in a silica skeleton in sorghum leaf (IPS view); r) tracheary anulate structures in finger millet leaf (IPS view); s) silicon structure normally identified as infilling (no phytoliths) very common in pearl millet to sorghum leaves (this comes from pearl millet leaves and reproduces the outward form of epidermal cells); t) silica skeletons of elongates sinuates in sorghum chaff (IPS view); u) silica skeleton of acute bulbosus connected by an elongate entire in pearl millet chaff (side view) (these are the most abundant structures encountered in pearl millet chaff).

Phytolith concentration was calculated for each morphotype identified as well as for the total silica accumulated and the ratio of sensitive to fixed morphotypes following the indication of Jenkins et al. 2020). The formulas used in this work are:

$$\text{Phytoliths extracted} = \frac{(\text{total silica extracted} \times \text{total phytoliths per slide})}{\text{total silica mounted}}$$

$$\text{Concentration} = \frac{\left(\frac{\text{phytoliths extracted}}{\text{dry leaf weight}}\right)}{1000000}$$

$$\text{Sensitive/fixed ratio} = \frac{(\text{elongates} + \text{stomata})}{(\text{crosses} + \text{bilobates} + \text{polylobates} + \text{rondels} + \text{saddles})}$$

2.4 Statistical analysis

Statistical analyses were performed with R (version 3.5.1) using standard functions of base, ggplot2 (version 3.3.5) (Wickham, 2016), tidyverse (version 1.3.0) (Wickham et al., 2019), vegan (version 2.5.6) (Oksanen et al., 2020) and MASS (version7.3-51.5) (Ripley, 2022) packages. Scripts are available in appendices (file A8.3 with the script and A.8.3.1 csv UTF8 (comma-delimited) file to run the code in R).

The tests conducted can be summarised in four steps: (1) comparison of the two cultivation years by phytolith production; (2) concentration and ratios (sensitive/fix, skeleton size) were tested as proxies for water availability for samples coming from the experimental cultivation 2020; (3) phytolith assemblages were tested by analysing individual morphotypes separately to understand which morphotype is most efficient as a proxy for water availability (species and formation tissue, samples 2020); (4) a predictive model based on the percentages of individual morphotypes produced by modern crops (from the experimental cultivations in both 2019 and 2020) had been constructed with the idea of being able to make predictions on watering levels. The results of the analyses for points 2 and 3, conducted on samples from the 2019 cultivation, are available in D'Agostini et al., 2022b (chapter 7).

(1-2) Total concentration and ratio of sensitive to fixed morphotypes were tested as possible proxies for water availability. The response variables (concentration and ratios) were normalised using natural logarithm to reduce skewness (Legendre and Legendre, 2012), then linear regressions were applied to model the relationship between phytolith ratios, concentration and transpiration, using ANOVA to test for significant differences. In addition, GLMs were used to evaluate which explanatory variable (species, treatment, plant part) better predicts the phytolith concentration, the ratio of sensitive to fixed morphotypes and the silica skeleton size ratio. Although concentration and ratios were normalised, GLMs were preferred for the analysis as the remaining variables are not normally distributed. The independent environmental variables tested were: total water transpired (sum of all the litres transpired by the plants during growth), transpiration efficiency (biomass/total water transpired), last litres of water transpired, species, genotypes, biomass production (total biomass of the plant) and plant part.

(3) After calculating each morphotype concentration, generalised linear models (GLMs) with Gaussian distribution were tested to evaluate which explanatory variable among treatment, species and plant part, better predicts phytolith composition. GLMs were used since the dataset is composed of non-normal variables where the output of the model is a continuous non-negative variable. Single morphotype concentration were tested as dependent variables while the treatment (WW, WS), the species (finger millet, pearl millet and sorghum) and the formation tissues (old leaves, new leaves, and chaff) were considered as independent variables. The p-value and the Akaike information criterion (AIC) were used to assess the validity of the models and to identify the best fitting model (Burnham et al., 2011).

(4) Since the model was developed to be applied to archaeological phytolith assemblages (see D'Agostini et al., 2022b - chapter 7), it was decided to use percentages and not concentrations of individual morphotypes. The percentage corresponding to each morphotype was evaluated to make the analysis comparable to existing publications, especially archaeological ones where calculating the concentration of phytoliths produced per biomass unit is not possible. Following previously published results (Ermish and Boomgarden, 2022; Jenkins et al., 2020), the ratio of sensitive to fixed phytoliths was added to the model. The dataset of phytoliths was trained by applying stepwise selection to choose the best explanatory variables (phytolith morphotype percentage and ratio sensitive/fixed morphotypes) to discriminate WW and WS treatment (dependent variable) in a GLM binomial model (Peduzzi et al., 1980). Logistic regression analysis was used to evaluate the chance that a single morphotype percentage indicated a WW condition of growth (Bruce et al., 2020).

3. Results

3.1 Experiment 2019 *versus* experiment 2020

While environmental conditions (temperature and relative humidity, light intensity, and average sun hours) were comparable between the two years of experimentation, transpiration showed marked differences (figure 8.2), which consequently influenced biomass production and transpiration efficiency. While WS replications remained stable between the two years, transpiring a comparable amount of water, WW crops in 2020 transpired less than in 2019 (10 L less in sorghum and pearl millet on average and 20 L less in finger millet), narrowing the gap between the two treatments. A more detailed report with weekly transpiration value is available as an appendices (file A8.1). Sorghum did not show any difference between WW and WS phytolith concentration and ratio of sensitive to fixed morphotypes both in 2019 and in 2020 (figure 8.4), despite changes in watering and transpiration rate occurred between the two years of experimentation (figure 8.2). Phytolith concentration remains stable around 38.22M of phytoliths per gram of dry plant tissue, both in WW and in WS conditions. On the contrary, pearl millet and finger millet have different phytolith concentration values between WW and WS in both 2019 and 2020, and accumulated more phytoliths in WW conditions (figure 8.4). In 2020, when the transpiration rate of both finger millet and pearl millet in WW dropped closer to that of the WS replications, the differences in phytolith concentrations between the two treatments also narrowed accordingly.

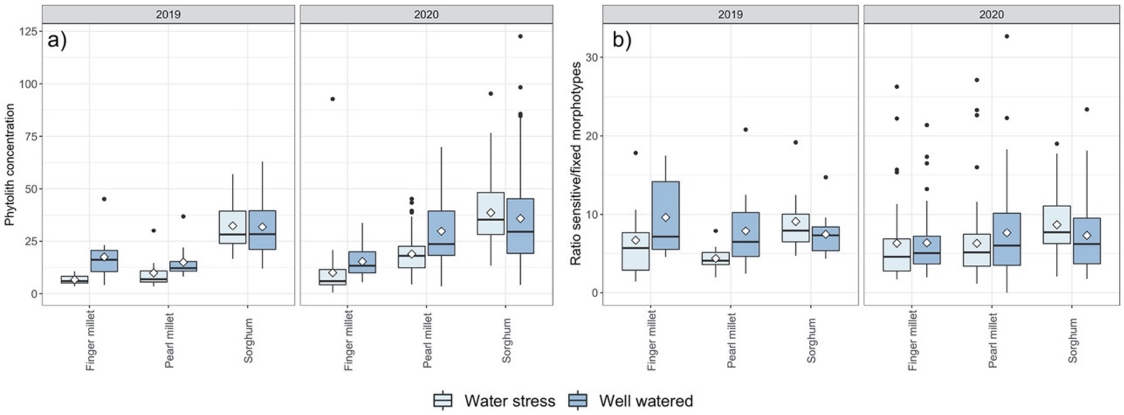

Figure 8.4 - Boxplot visualising a) phytolith concentration and b) ratio of sensitive to fixed morphotypes in crops cultivated in 2019 and 2020. Phytolith concentration and ratio are normalised with natural logarithm.

3.2 Phytolith analysis of crops cultivated in 2020

Phytoliths concentration is positively related to total water transpired for new leaves, almost positively related (p-value almost < 0.05: 0.071) for chaff samples while in old leaves, even if the trend appears slightly positive, it is not statistically significant (figure 8.5). In old leaves, sorghum data probably change the general average because sorghum accumulates more biosilica in WS conditions than in WW (figure 8.6a) and phytolith concentration is negatively related to the total water transpired significantly (p-value: 0.014, r:-0.39). Leaves and chaff of pearl and finger millet, always contain more phytoliths in WW than WS replications (figure 8.6a) and the concentration resulted almost always (exceptions are PM chaff and FM old leaves) positively related to the total water transpired (PM new leaves p-value: 0.021, r: 0.36; PM old leaves p-value: 0.009, r: 0.41; PM chaff p-value: 0.27, r: 0.19; FM new leaves p-value: 0.014, r: 0.41; FM old leaves p-value: 0.43, r: 0.14). Linear regression plots are available in the appendices (figures A8.1.1, A8.1.2, A8.1.5, A8.1.6, A8.1.9, A8.1.10). The GLM model identified species as the most significant explanatory variable for phytolith concentration (FM *versus* PM species: p-value = 0.00204, FM *versus* S species: p-value= 1.62e-10).

The ratio of sensitive to fixed morphotypes did not show any relation to the total water transpired either for species or for plant parts (see figures A8.1.3, A8.1.4, A8.1.7, A8.1.8, A8.1.11, A8.1.12 in the appendices). The GLM model tested identified both treatment, species, and plant parts as significant predictors for the increasing ratio. New leaves of pearl and finger millet showed a higher ratio in WW conditions while sorghum did not show significant differences between the two treatments (figure 8.6b). In old leaves only pearl millet displayed a higher ratio in WW conditions (figure 8.6b). Chaff of sorghum and pearl millet showed a higher ratio in well water conditions. Although small variations in the ratio of sensitive to fixed morphotypes between the two treatments for the three species have been shown, none is significant (p-value > 0.05).

The results for phytolith assemblages of 2019 crop replicas are available in D’Agostini et al., (2022b - chapter 7). However, in both experiments phytolith concentration and ratio of sensitive to fixed morphotypes trends are the same and the results obtained are comparable as also shown in figure 8.4.

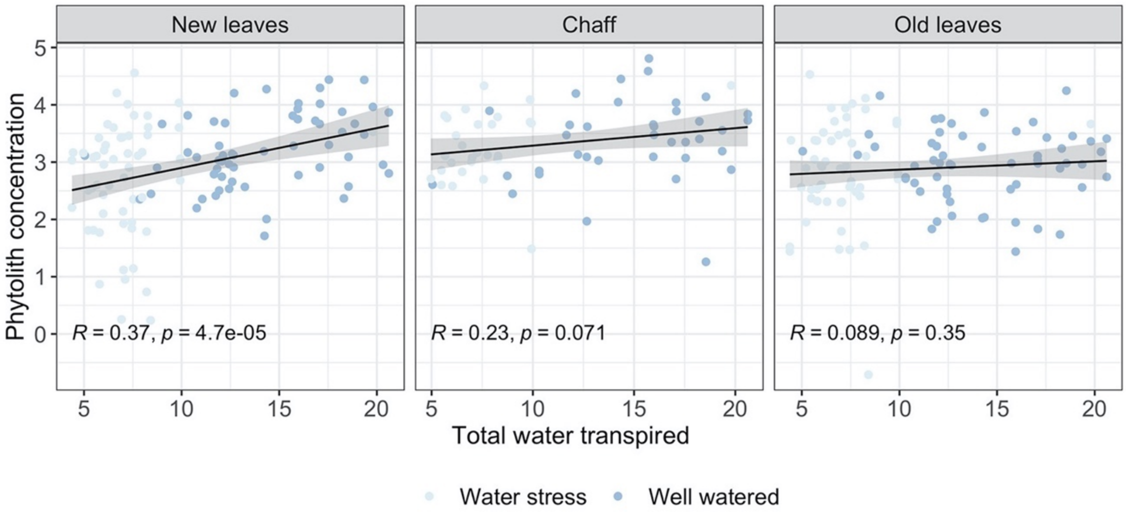

Figure 8.5- Linear regression of phytolith concentration tested for the three species together and displayed by plant part. Total water transpired (L) is used as independent variable, and phytolith concentration (per gram of dry tissues in millions normalised with natural logarithm) as dependent variable. Grey bands represent 95% of confidence intervals.

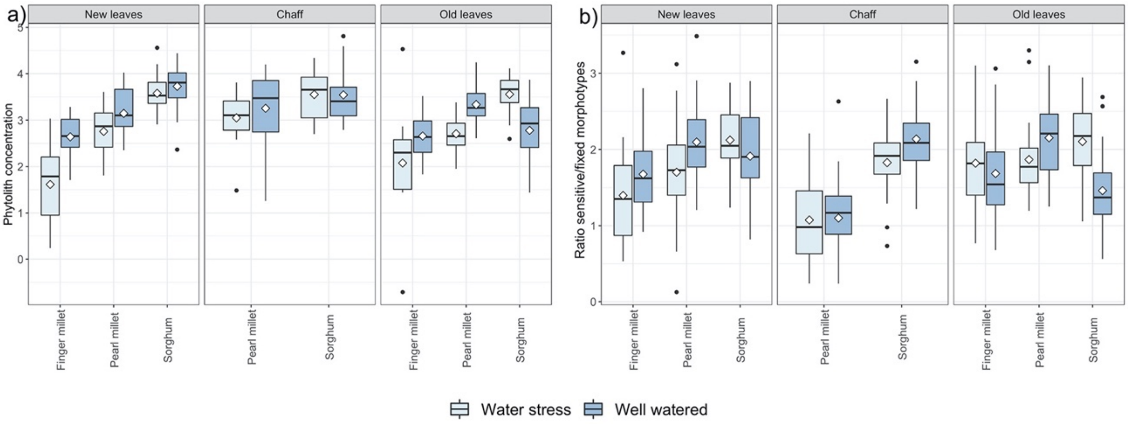

Figure 8.6 - Boxplot visualising a) phytolith concentration and b) ratio sensitive to fixed morphotypes in crops cultivated in 2020, displayed by species and plant part. Phytolith concentration and ratio are normalised with natural logarithm.

Concentration of each morphotype has been tested considering species and plant parts together and separately. In leaves (both old and new) samples, when the three species were tested together, all morphotypes were present in higher concentration in WW than in WS conditions. Considering the three species separately, old leaves of sorghum show a higher concentration of acute bulbosus, crosses, elongate clavate and entire and stomata (all, apart from crosses, considered morphotype sensitive) in WS conditions than in WW. Finger millet new leaves presented a higher concentration of elongates clavate and dentate, and rondels in WS. In chaff samples polylobates, elongates sinuate, bulliforms and stomata displayed a higher concentration in WS. However, bulliforms and stomata are almost absent in the chaff, and their concentration in these tissues is not highly indicative. Precise data of each morphotype concentration are available in the appendices (file A8.5).

GLMs tests conducted on morphotypes concentration indicate that sensitive morphotypes, (acute bulbosus, blockies, bulliforms flabellate, the general category of “bulliforms” as a sum of all types of bulliforms, elongates entire and sinuate and the general category of “elongates” as a sum of all types of elongates) are predicted by the treatment with high levels of significance (table 8.5). Bulliforms flabellate are the morphotypes with the lower AIC and p-value. Species and plant parts are good predictors of morphotype concentration too. Chaff is the tissue with the strongest prediction power, while sorghum is the species with the strongest prediction power, distinguished from the remaining two millets by the concentration of almost all morphotypes (apart from bulliform flagellates, elongates crenate) (table 8.5).

Table 8.5	**Morphotype concentration (family = gaussian)**	**x = Treatment**		**x = Species**		**x = Plant Part**	
SENSITIVE FORMS	***Acute bulbosus***	**WW p-value AIC**	0.0112 * 1871.2	**PM p-value S p-value AIC**	1.74e-07 *** 0.000712 *** 3495.2	**Chaff p-value Leaf p-value AIC**	5.71e-13 *** 0.0336 * 1793.6
	Bulliforms (sum of blockies + flabellate)	**WW p-value AIC**	0.00165 ** -346.14	**PM p-value S p-value AIC**	0.2293 0.0301 * -348.75	**Chaff p-value Leaf p-value AIC**	1.67e-05 *** 0.117 -353.16
	Blockies	**WW p-value AIC**	0.009768 ** -384.11	**PM p-value S p-value AIC**	0.386497 0.020384 * -388.83	**Chaff p-value Leaf p-value AIC**	8.16e-05 *** 0.123 -391.18
	Bulliforms flabellate	**WW p-value AIC**	0.000269 *** -1538.5	**PM p-value S p-value AIC**	0.0497 * 0.8934 -1528.8	**Chaff p-value Leaf p-value AIC**	0.00297 ** 0.59698 -1533
	Elongates (sum of all elongates)	**WW p-value AIC**	0.0301 * 3087.1	**PM p-value S p-value AIC**	0.00372 ** < 2e-16 2998.8	**Chaff p-value Leaf p-value AIC**	0.119 0.403 3088.3
	Elongates clavate	**WW p-value AIC**	0.603 2860	**PM p-value S p-value AIC**	0.00324 ** < 2e-16 *** 2744.1	**Chaff p-value Leaf p-value AIC**	0.000769 *** 0.051733 * 2850.7

	Elongates crenate	**WW p-value** **AIC**	0.1549 -517.19	**PM p-value** **S p-value** **AIC**	0.158902 0.123684 -515.91	**Chaff p-value** **Leaf p-value** **AIC**	0.908486 0.000891 *** -527.8
	Elongates dentate	**WW p-value** **AIC**	0.110 1820	**PM p-value** **S p-value** **AIC**	0.7888 0.0212 * 1814.9	**Chaff p-value** **Leaf p-value** **AIC**	2.13e-07 *** 0.685 1792.3
	Elongates entire	**WW p-value** **AIC**	2.03e-06 *** 1897.8	**PM p-value** **S p-value** **AIC**	0.77490 0.00293 ** 1910	**Chaff p-value** **Leaf p-value** **AIC**	8.01e-08 *** 0.415 1889.8
	Elongates sinuate	**WW p-value** **AIC**	0.00138 ** 2453.7	**PM p-value** **S p-value** **AIC**	0.0377 * 1.06e-06 *** 2440.7	**Chaff p-value** **Leaf p-value** **AIC**	4.8e-07 *** 0.427 2437.4
	Stomata	**WW p-value** **AIC**	0.287 808.99	**PM p-value** **S p-value** **AIC**	0.687 4.71e-11 *** 749.65	**Chaff p-value** **Leaf p-value** **AIC**	1.51e-07 *** 0.042 * 784.04
FIXED FORMS	***Bilobates***	**WW p-value** **AIC**	0.28689 1855.2	**PM p-value** **S p-value** **AIC**	0.0761 . 3.69e-05 *** 1840.3	**Chaff p-value** **Leaf p-value** **AIC**	0.75326 0.60055 1857.7
	Crosses	**WW p-value** **AIC**	0.576 1773.9	**PM p-value** **S p-value** **AIC**	1.11e-11 *** 2.44e-15 *** 1707.5	**Chaff p-value** **Leaf p-value** **AIC**	2.41e-09 *** 0.176 1720.1
	Polylobate	**WW p-value** **AIC**	0.3218 585.87	**PM p-value** **S p-value** **AIC**	0.1019 0.0477 * 584.59	**Chaff p-value** **Leaf p-value** **AIC**	0.721 0.218 586.21
	Rondels	**WW p-value** **AIC**	0.097207 . -104.77	**PM p-value** **S p-value** **AIC**	1.07e-06 *** 3.97e-07 *** -130.93	**Chaff p-value** **Leaf p-value** **AIC**	0.440579 0.095756 . -105.87
	Saddles	**WW p-value** **AIC**	0.0369 * 966.88	**PM p-value** **S p-value** **AIC**	<2e-16 *** <2e-16 *** 701.71	**Chaff p-value** **Leaf p-value** **AIC**	0.00283 ** 0.99126 961.76

Table 8.5 - p-value and Akaike information criterion (AIC) of the generalised linear models tested using treatment (WW-WS), species (sorghum, pearl millet and finger millet) and plant part (new leaves, old leaves and chaff) as independent variables. "*" highlight statistically significant results (p-value < 0.05). "." highlight almost statistically significant results (p-value > 0.05 but close to 0.05 value).

3.3 Predictive model

Table 8.6 shows the results of the stepwise model (stepwise AIC = 473.92, residual deviance: 451.92 *versus* full model AIC = 481.72, residual deviance: 445.72) where the best explanatory variables, chosen by the automatic selection (acute bulbosus, the general category of "bulliforms", elongates clavate, dentate, entire, and sinuate, crosses, polylobates, rondels and saddles) have been used as predictors of the two treatments (WW-WS). Although the model indicates that water treatments are predicted by a combination of morphotypes (those shown in table 8.6 with a significant p-value), the forms with the highest degree of significance are short cells (see p-values in table 8.6). Figure 8.7 represents the sigmoid prediction (logistic regressions) on the morphotypes which have been shown to be predictive of water availability (table 8.6). The percentages of each morphotype represented in the x-axis of each graph of figure 8.7 show that the differences between WW and WS are not marked and that in some cases only the tissues of origin is predictive. Table 8.6 (last column) also includes the results of the logistic regression when considering the three species separately. The predictive response of the morphotypes is (or can be) different for the three species.

Table 8.6 - Logistic regression result of the stepwise generalised linear model tested with binomial distribution, using phytolith morphotype percentage as predictors and treatment (WW-WS) as dependent variable. “*” highlight statistically significant results (p-value < 0.05). “.” highlight almost statistically significant results (p-value > 0.05 but close to 0.05 value). The last column defines the trend of the sigmoidal curves of the logistic regressions tested on the predictive morphotypes (graphics on the appendices, figures A8.2.1, A8.2.2, A8.2.3, A8.2.4, A8.2.5, A8.2.6, A8.2.7, A8.2.8, A8.2.9, A8.2.1).

Table 8.6	**Percentage %**	**Stepwise model p-value**	**Probability of WW when percentage of the morphotype increases**
	Ratio sensitive to fixed morphotypes	Not significantly predictive (p-value > 0.05)	
SENSITIVE FORMS	***Acute bulbosus***	0.015059 *	***Finger millet:*** decreases ***Pearl millet:*** increases ***Sorghum:*** decreases
	Bulliforms (sum of blockies + flabellate)	0.069513 .	***Finger millet:*** increases ***Pearl millet:*** increases ***Sorghum:*** increases
	Blockies	Not significantly predictive (p-value > 0.05)	
	Bulliforms flabellate	Not significantly predictive (p-value > 0.05)	
	Elongates (sum of all elongates)	Not significantly predictive (p-value > 0.05)	
	Elongates clavate	0.000237 ***	***Finger millet:*** decreases ***Pearl millet:*** decreases ***Sorghum:*** decreases
	Elongates crenate	Not significantly predictive (p-value > 0.05)	
	Elongates dentate	0.002461 **	***Finger millet:*** decreases ***Pearl millet:*** increases ***Sorghum:*** increases
	Elongates entire	0.005109 **	***Finger millet:*** increases ***Pearl millet:*** increases ***Sorghum:*** increases
	Elongates sinuate	0.001761 **	***Finger millet:*** increases ***Pearl millet:*** increases ***Sorghum:*** increases
	Stomata	Not significantly predictive (p-value > 0.05)	
FIXED FORMS	***Bilobates***	Not significantly predictive (p-value > 0.05)	
	Crosses	0.000149 ***	***Finger millet:*** increases ***Pearl millet:*** decreases ***Sorghum:*** increases
	Polylobate	0.019280 *	***Finger millet:*** increases ***Pearl millet:*** decreases ***Sorghum:*** increases
	Rondels	0.000476 ***	***Finger millet:*** decreases ***Pearl millet:*** increases ***Sorghum:*** increases
	Saddles	0.000349 ***	***Finger millet:*** decreases ***Pearl millet:*** increases ***Sorghum:*** increases

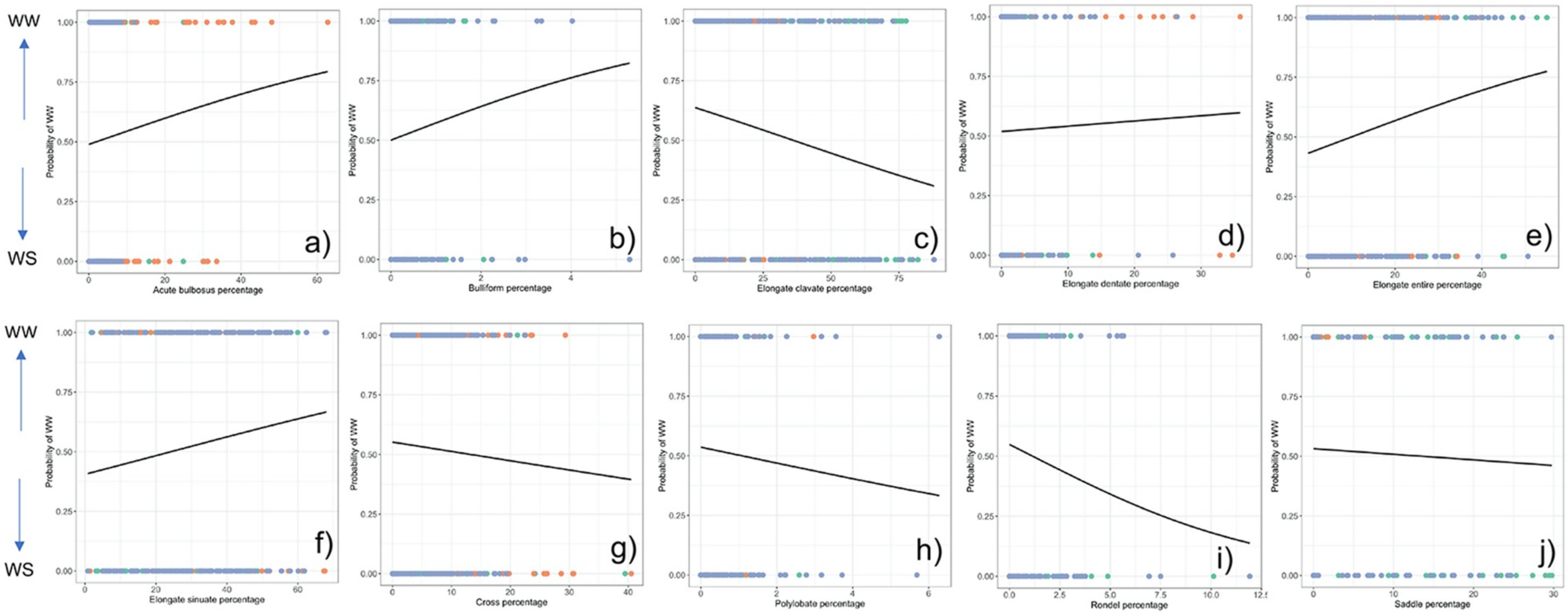

Figure 8.7 - Logistic regression plots for the morphotypes whose percentage have shown to be predictive of the water availability considering the three species together (table 6): a) Acute bulbosus; b) Bulliforms; c) Elongates clavate; d) Elongates dentate; e) Elongates entire; f) Elongates sinuate; g) Crosses; h) Polylobates; i) Rondels; and j) Saddles. X axis: percentage of each morphotype which increases along the axis. The colours of the dots correspond to the tissue of origin: blue for old leaves, green for new leaves and orange for chaff samples. Plots for morphotypes divided for species are available in appendices (figures A8.2.1, A8.2.2, A8.2.3, A8.2.4, A8.2.5, A8.2.6, A8.2.7, A8.2.8, A8.2.9, A8.2.1).

4. Discussion

The methodology used for the experimental cultivation was especially efficient in overcoming methodological problems related to rainfall measurements, allowing us to obtain reliable data on watering. Measuring and relate the production of phytoliths with the actual amount of water that has passed through the plant (total water transpired), instead of rainfall (which in this experiment corresponds to the total water added), allows to exclude all the issues concerning the complex water cycle between soil-plant and atmosphere. Previously Katz et al. (2013) and Jenkins et al. (2016), have discussed this controversy related to the measure of rainfall in connection with phytolith production, and highlighted the possible influence of additional variables, such as solar irradiation and evapotranspiration on soil water availability. Therefore, by relating phytolith production to the transpiration reduced the possible errors related to water losses by evaporation and soil retention/drainage.

The results obtained by studying phytolith assemblages show a relationship between phytolith accumulation and transpiration in the three species under study. However, the ratio of sensitive/fixed morphotypes was found not to be adequate for assessing water availability in the three species equally. Since previous studies seem to have obtained different results regarding the very same ratio (Jenkins et al., 2016, 2020), we are inclined to think that phytolith production in C_4 species is more complex than anticipated. First, we observed a taxonomic issue: different species most likely deposit Si differently as confirmed by the results obtained in the present study. Finger and pearl millets are the species that expressed a positive relationship between transpiration and the concentration of phytoliths. The rate of phytolith production remained constant in WS (when no water was added), while it varied in WW as the transpiration process fluctuated over the two years of experiment (figure 8.4-8.5-8.6).

Separate considerations must be made for sorghum. Sorghum phytolith production did not change as transpiration fluctuated and no difference in silica accumulation has been displayed either between the two years of cultivation, where significant differences have been registered in the transpiration rate, or between the two water treatments (figure 8.4). Sorghum produced a concentration of phytoliths that is always higher than the remaining species, with the one and only exception of WW old leaves (figure 8.6). This result confirms a trend noticed in our previous study (D'Agostini et al., 2022b - chapter 7). Sorghum emerged to be a different phytolith producer from the GLMs as well (table 8.5) where it distinguished by concentrations of specific morphotypes from the remaining two millets. Indeed, sorghum phytolith assemblage is characterised by a high concentration of polylobates and crosses, especially in WS conditions, and by the almost total absence of saddles and rondels, as also mentioned by Novello and Barboni (2015) and Jenkins et al. (2020). In the leaves, sorghum produced trichomes, bulliforms, stomata and elongates clavate (appendices-file A8.5) in much higher quantities than pearl millet and finger millet. In view of these results, we might speculate that sorghum has a constant Si precipitation rate, independent of the water availability (as suggested also in D'Agostini et al., 2022 a-chapter 6) and that the production of some morphotypes (possibly more efficient than others in performing structural or physiological functions) increases depending on the physiological conditions of the plant. Hence, we are inclined to think that phytolith formation plays a role in sorghum physiology, because the species seems to regulate phytolith deposition to maintain it constant and to increase the formation of specific morphotypes in certain tissues and water conditions. This result supports the statement by Katz (2019) which suggested, from an evolutionary-ecological perspective, that if silica content is persistent in the plant kingdom, it has to confer some advantages in improving fitness. According to this hypothesis, and in line with our results, it may be possible that morphotype production increases in response to drought. Indeed, silicification of stomata could help in reducing transpiration by regulating water loss (Goto et al., 2003; Cooke and Leishman, 2016; Hosseini et al., 2017; Gao et al., 2020). Crosses, according to results previously presented by Kumar et al. (2017) could deposit preferentially to the side of minor veins toughening them and allowing xylem flow to continue (Markovich et al., 2015; Kaufman et al., 1985). Elongates, arranged on the epithelial surface, would increase light inception (Cooke and Leishman, 2016) and would toughen epidermal tissue (particularly fragile in WS conditions) and simultaneously prevent both pathogen attack and further crumpling of leaf blade (Yoshida et al., 1962; Rodrigues et al., 2003; Meunier et al., 2017). Acute bulbosus, which have been proven to respond to pathogens attack (Fauteux et al., 2005; Mateos-Naranjo et al., 2013; Daoud et al., 2018; Oliva et al., 2021), have also demonstrated to be a consistent response to xeric environment (Olsen et al., 2013).

The fact that the ratio of sensitive/fixed morphotypes showed no relation to transpiration brings forward three major considerations:

(1) The ratio includes stomata among the sensitive morphotypes, but it does not include either bulliforms or trichomes (i.e., acute bulbosus). In view of the recent studies on the classification of morphotypes on the basis of their deposition mechanism (Hodson 2019) and considering that silicified stomata in a leaf that is still photosynthesising could lead to the death of the tissue itself (Kumar et al., 2017), we believe that stomata cannot fall into the sensitive category. Stomata should be included in the group of phytoliths whose deposition is most probably genetically regulated. In this case, genetic control must be exercised at least for the timing of deposition, which has to be later than leaf emergence (Motomura, 2004) in order not to cause a dysregulation of normal leaf development (Kumar et al., 2017). In contrast, bulliforms not only have a function closely related to water availability as living cells, but moreover the deposition of Si in these cells has already been related to rainfall (Wang et al., 2019). Therefore, we believe that bulliform morphotype should be considered into the sensitive category. The same can be said of trichomes whose density has been proven to be higher in a xeric environment with low soil moisture (Olsen et al., 2013).

(2) The existence of a genetic regulation of Si uptake based on Si-transporter channels has been confirmed (Vatansever et al., 2017), and that of proteins capable of triggering silica precipitation within the short cells (Kumar et al., 2020) as well. Hence, when classifying phytoliths it would be more appropriate to divide them into morphotypes whose deposition is triggered by genetics in response to water availability, and morphotypes spontaneously induced by the transpirative phenomena (Hodson 2019). Both mechanisms of morphotype deposition would depend on the hydration levels of the plant and on the taxonomy on which a plant's ability to respond to water availability relies.

(3) Several studies seem to indicate that there is a strong correlation between Si deposition and taxonomy (Ball et al., 1996, 2016; Hodson et al., 2005; International Committee for Phytolith Taxonomy (ICPT) et al., 2019). Not only have different species shown different deposition patterns, but some intra-specific variability has also been shown (e.g., sorghum) (Markovich et al., 2019), leading to the suggestion that a ratio that works for all species, in all growth environments, is potentially impossible to establish. Indices that relate phytolith concentrations to water availability levels are possible (Jenkins et al., 2016, 2020; Madella et al., 2009), but their applicability is probably restricted to certain species grown in specific environments to which the tested varieties are adapted. However, based on our results we maintain that it is possible to fit a

predictive model for water availability based on the phytolith concentrations of three different millet species, composed of landraces from different drylands areas.

The model must include all morphotypes produced by the plants, especially if several species are considered at the same time, as in this case. As it has been shown in this study, the stepwise selection applied to the dataset (table 8.5) resulted in excluding the ratio of sensitive to fixed ratio as a predictive variable but included both short cells (fixed morphotypes), elongates (sensitive morphotypes), bulliforms and acute bulbous. The results of this study show that the concentration of different morphotypes is predicted by the water availability (table 8.5), regardless of fixed and sensitive categories. Among these morphotypes, elongates clavate, crosses, rondels and saddles are good predictors of water availability when considering the complete phytolith assemblage for building the model (table 8.6). Predictive ability of phytoliths depends on morphotype concentration (figure 8.7) but not all of the morphotypes increase in percentage as watering increases (table 8.6). As mentioned above, it is likely that the production of some morphotypes could be stimulated in response to drought, and, therefore, the production of morphotypes can also increase under conditions of water stress.

5. Conclusion

According to the results presented in this article, phytolith deposition in finger millet and pearl millet is not comparable to sorghum. Taxonomy plays a more important role in the deposition of Si than water availability. Results have also demonstrated that a relationship between watering and phytoliths production exists, it is more evident in finger and pearl millet than in sorghum and it can be better predicted by the application of a logistic regression model on the complete phytolith assemblage than with the use of the ratio of sensitive to fixed morphotypes. The predictive model using all available morphotypes is recommended to overcome possible issues related to the difficulty in categorising sensitive and fixed forms, and because the ratio is not always positively related to watering for all the species analysed. Furthermore, the concentration of predictive morphotypes is not always positively related to water availability; sometimes, depending on the species, a high concentration may correspond to a higher chance of growth in water stress conditions. Phytolith deposition is a complex system that depends on many factors such as the species, the laying tissue, and the environment in which the plant grows. Therefore, we suggest that inter- and intra-species variability should be considered in future studies intended to develop predictive models based on phytolith concentration.

Acknowledgments

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Appendices available at Zenodo.org

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Figures A8.1.1, A8.1.2, A8.1.3, A8.1.4, A8.1.5, A8.1.6, A8.1.7, A8.1.8, A8.1.9, A8.1.10, A8.1.11, A8.1.12: boxplots and linear regression plots for phytolith concentration and ratio of sensitive to fixed morphotypes (dependent variables) and total water transpired (independent variable) for finger millet, pearl millet and sorghum considered separately.

Figures A8.2.1, A8.2.2, A8.2.3, A8.2.4, A8.2.5, A8.2.6, A8.2.7, A8.2.8, A8.2.9, A8.2.10: logistic regression plots for each morphotype, arranged for the three species considered separate.

File A8.1: the complete dataset of the experimental cultivation, including the experimental design for both seasons.

File A8.2: the complete dataset of the phytolith both row data, concentration and ratio of sensitive to fixed morphotypes.

File A8.3: R scripts.

File A8.3.1: csv UTF8 (comma-delimited) file to run the code in R.

File A8.4: metadata.

File A8.5: table of means and standard deviations of morphotypes concentration from plant samples grown in the experimental cultivation 2020.

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Chapter 9 - Results and discussion on phytolith morphometry and isotopical analysis- Do bulliforms tell us more than we thought?

9.1 Phytolith morphometry

9.1.1 Results

This section is dedicated to the description of the results of the morphometry taken on morphotypes commonly referred to as sensitive. As it has been described in the previous chapters (chapter 4, 5, 7, 8), the classification of sensitive morphotypes is not always straightforward and in the future these measurements would need to be repeated on other morphotypes, even non-sensitive ones. The methodology has been applied on leaf phytolith samples from the experimental cultivation set up in 2019, but future research development plans also include the measurement of phytoliths coming from the experimental cultivation 2020. The density plots (figure 9.1, 9.2 and 9.3) represent the frequency of the width or the length size with which these morphotypes occurred. As it can be seen from illustrations, the size of elongates phytoliths and stomata phytoliths does not follow a normal distribution, neither in WW nor in WS conditions. In addition, the two dimensions were not able to differentiate phytoliths from WW replications from those of WS replications since the two distribution curves (dark blue and light blue) overlap. Elongates most frequently have a maximum width of 25 μm and an average length of 50 μm, but larger sizes are not excluded even if they occur less frequently. The stomata have an average width of 20 μm, but they can measure up to 70 μm. Average length for stomata is 50 μm with some rare cases of particularly long opaline bodies up to 80 μm. Subsidiary cells are normally between 5 and 10 μm wide, with some rare exceptions of up to 20 μm. In this section, only the plots for length and width are presented, but I took more measurements for stomata (subsidiary cells), with comparable results (available in the appendices, figures A9.2.1,A9.2.2).

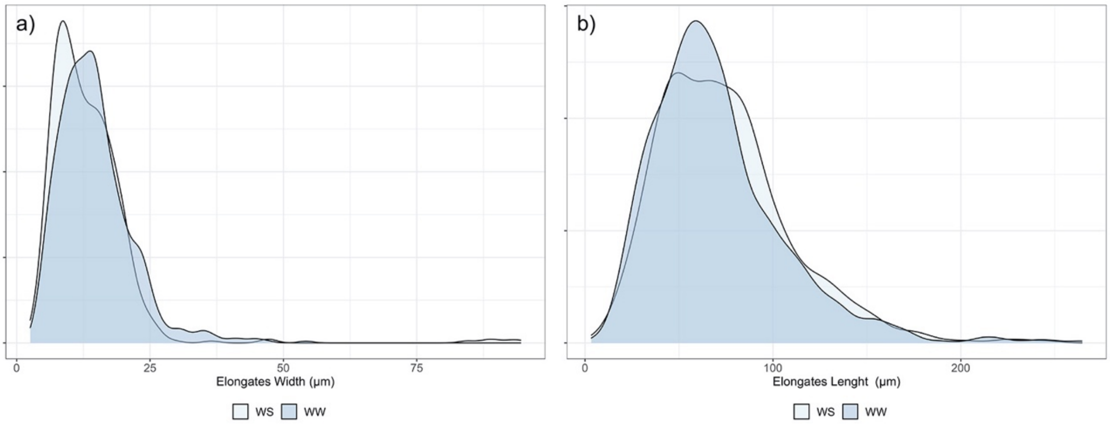

Figure 9.1 - Density plots visualising a) width and b) length of elongates coming from leaf samples of experiment 2019. Light blue curve represents water stress sample distribution while dark blue is for well watered replications. The curve is highly skewed on the right and the two distributions almost completely overlap.

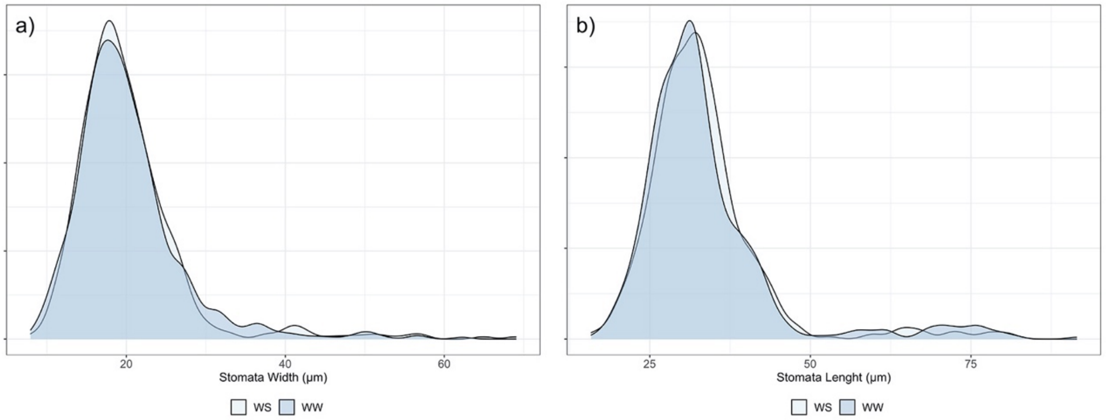

Figure 9.2 - Density plots visualising a) width and b) length of stomata coming from leaf samples of experiment 2019. Light blue curve represents water stress sample distribution while dark blue is for well watered replications. Curve is highly skewed on the right and the two distributions overlap almost fully.

Bulliforms, on the other hand, show a different trend. The size distribution curve is bimodal for the well-watered samples and slightly bimodal, with two less pronounced peaks, for the water-stressed samples. While the water-stressed bulliforms showed a width dimension dispersion always ranging between 10 and 55 µm and a height never exceeding 80 µm, bulliforms from well-watered leaves exceed these ranges and can reach widths and heights of over 100 µm (figure 9.3). Therefore, bulliforms with a height between 80 µm and 150 µm and a width between 60 µm and 125 µm are characteristic of plants grown under conditions of water abundance. The size of bulliforms is positively related to total water transpired with high levels of statistical significance (figure 9.4 and appendices figures A9.1.1, A9.1.2, A9.1.3, A9.1.4, A9.1.5, A9.1.6, A9.1.7, A9.1.8, A9.1.9, A9.1.10, A9.1.11, A9.1.12). On the other hand, the size of the beak and any peaks on the surface of the bulliform central body, seem to be unrelated to physiological values of biomass or water availability (appendices figures A9.3.1, A9.3.2, A9.3.3, A9.3.4). Figure 9.5 represents linear regressions tested on WW samples only. The analysis shows that there is a strong relationship between bulliform size and both transpired water and leaf production.

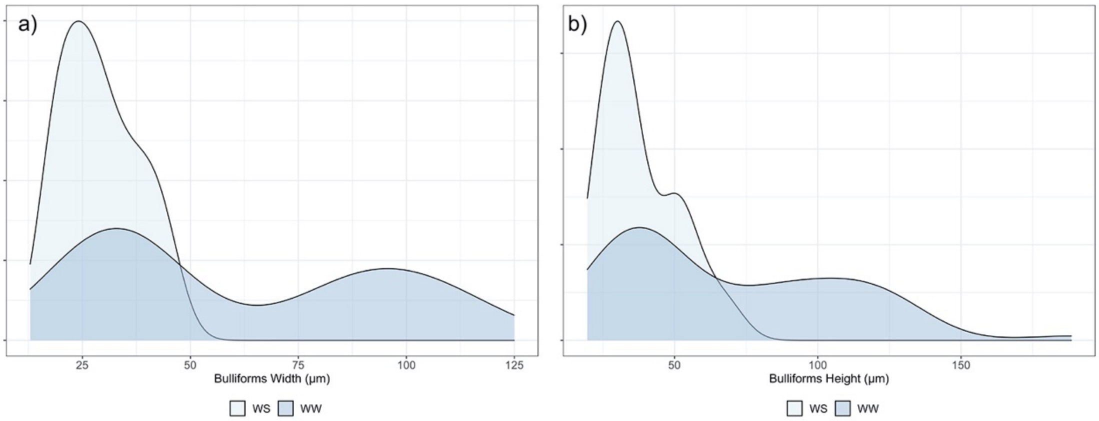

Figure 9.3 - Density plots visualising a) width and b) length of bulliforms coming from leaf samples of experiment 2019. Light blue curve represents water stress sample distribution while dark blue is for well watered replications. The bimodal distribution of well water replications is evident.

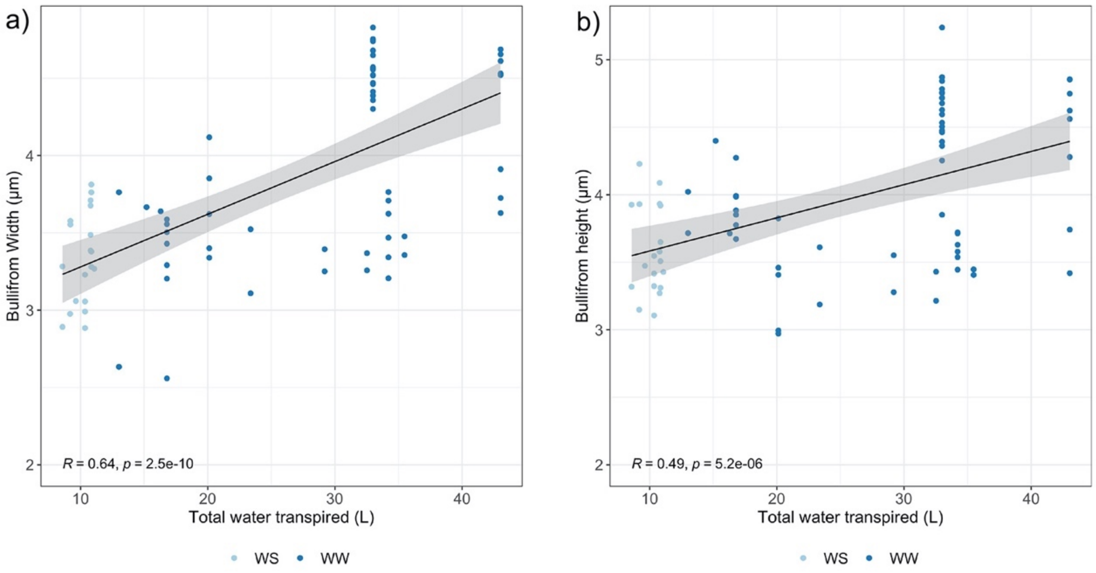

Figure 9.4 - Linear regression for bulliforms dimension sizes (a. width, b. height) tested in relation to the total water transpired (L). Bulliform measurements have been normalised with natural logarithm to apply a linear regression. Grey bands represent 95% of confidence intervals.

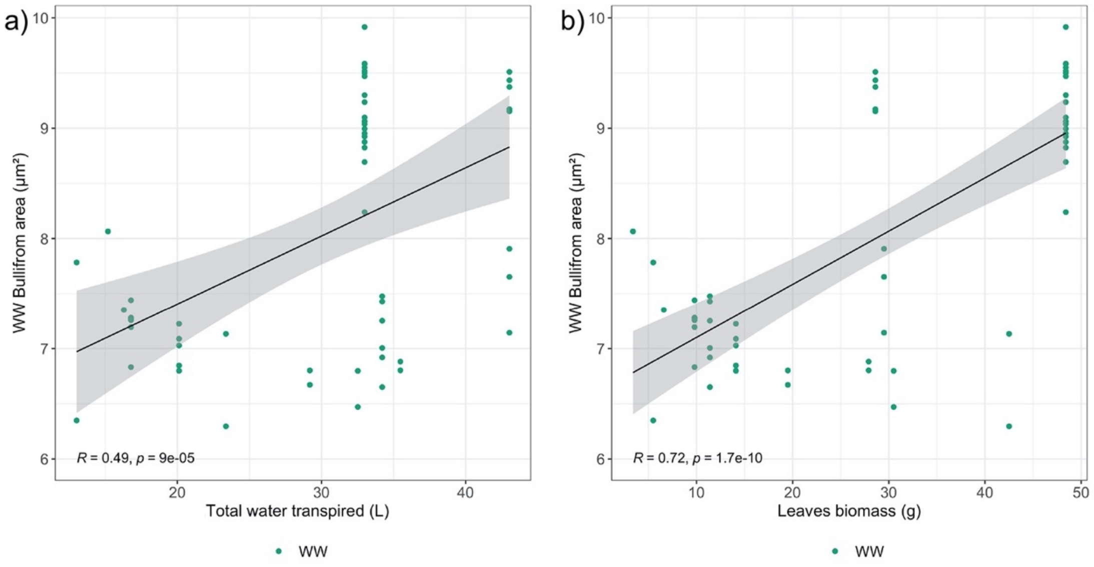

Figure 9.5 - Linear regression for bulliforms area tested in relation to a) the total water transpired (L) and b) to the total leaves biomass. Only WW replications are represented. Bulliform measurements have been normalised with natural logarithm to apply a linear regression. Grey bands represent 95% of confidence intervals.

9.1.2 Discussion

The results of this study are to be considered preliminary as they concern the analysis of leaf samples of replicates grown only in 2019. As bulliforms, elongates and stomata morphotypes are extremely ubiquitous and also produced in grass taxa other than those taken into account for this study, a wider catalogue of species, landraces and wild varieties need to be added to obtain more representative results to applied to archaeology and confirm the the link between phytoliths size and water availability exists. However, the analysis conducted so far shows that the sizes of elongates and stomata are not responsive to water treatment, while bulliforms size is able to distinguish water regimes in finger millet, pearl millet and sorghum.

Larger well-watered bulliforms occur in plants that have transpired more with higher leaf biomass, meaning that the larger the plants grew under conditions of abundant water availability, the larger the bulliforms they produced. Bulliforms robustness (width + length + length of the beak) increases as the level of transpiration rises. Differently, the beak size remains proportional to that of the elongates between which it fits, which in turn showed no water-dependent variations. Anatomically and physiologically, this phenomenon could be explained by the increased storage of water in the vacuoles of bulliforms in plants with high water availability (Beal, 1886). Therefore, as these cells are predisposed to increase leaf turgor, they increase their compressive power by increasing the size of their internal vacuoles (Chen et al., 2020; Shamah et al., 2019). The beak of the cell, which is wedged between the elongated epithelial cells, has no physiological reason to change according to water availability. The augmentation in bulliform cells size would then be reflected in the formation of larger bulliform phytoliths, confirming the theory (see Bremond et al., 2005; Chen et al., 2020; Parry and Smithson, 1958; Wang et al., 2019) that bulliform phytoliths can be the proxy that reflects the hydric conditions in which bulliform cells operate and so the plant developed. These results are in accordance with Wang et al. 2019, who found that larger rice bulliform likely occurred at geographical locations with higher temperature and precipitation.

The results for stomata and elongates, on the other hand, are more difficult to interpret for several reasons. Stomata phytoliths were chosen because in literature they are considered sensitive morphotypes due to the function performed by the stomatal cells, which are directly related to transpiration. However, the results presented earlier in this thesis (chapter 7-8), in conjunction with the recent works by Hodson (2019) and Kumar et al. (2017), showed that the deposition of stomata is much more genetically determined, and must occur at specific times in leaf development. In addition, it has already been evidenced by a large number of studies of C_4 species, that stomatal shape and size depend on tissue maturity, light intensity and atmospheric CO_2 concentration (e.g., Haworth et al., 2018; Rudall et al., 2017), while the increased efficiency in water retention is determined not only by the size and responsiveness of the guard cells but also, and above all, by the density of stomata (Bertolino et al., 2019). Moreover, even if watering had an effect on the dimensions of the guard cells, changins in stomata size would probably not be captured by phytolith deposition because Si is only deposited in mature tissue cells that have already exhausted their physiological function and are not subjected to water deficiency responses (Hodson, 2019; Rudall et al., 2017).

Elongate cells do not play any physiological role in water response, representing the epidermal surface of the tissue. Following the results obtained in this study not even the dimension of elongate phytoliths are related to water availability.

Additionally, elongates and stomata did not demonstrate dimensional variability, suggesting that there is no intra- and inter-species difference among the size of these morphotypes of the three C_4 millets under study. The low variability in the size of leaf elongates, besides hampering the determination of the relative environmental phenomena (in this case water availability), also bring to the conclusion that these morphotype dimensions may not be a good proxy for the taxonomic identification of the three species.

These results have two important archaeological implications. Firstly, bulliforms of these three grasses expand in size as watering increases (figure 9.5). Thus, the recognition of dimensional gradients in bulliforms size of finger millet, pearl millet and sorghum, in archaeological contexts, could also imply variations in watering conditions in the area. Secondly, bulliforms of finger millet, pearl millet and sorghum with heights over 70 µm and widths over 50 µm belong to plants grown in well water conditions. Although the difficulty in recognising the affiliation of these morphotypes to these three specific species (being ubiquitous in all grasses) in an archaeological context, if the presence of the these specific millets have been proven through alternative proxies, this type of analysis can be applied to propose an environmental reconstruction of the site of origin.

9.2 δ^{30}Si and δ^{29}Si isotopic analysis in phytoliths of sorghum

9.2.1 Results

The current results of Si isotopes analysis in phytoliths are highly preliminary. Here I am presenting the results on selected landraces of sorghum that had demonstrated during the experimental cultivation a great diversity of physiological responses to drought. Indeed the landraces were chosen as pilot samples to observe any compatible responses of isotopic discrimination as well. Bulliforms, elongates and stomata show δ^{30}Si between 0.5‰ and 3‰ while δ^{29}Si between 0.4‰ and 1.4‰. Generally, δ^{30}Si is higher than δ^{29}Si for all three morphotypes in both treatments. The isotope variations for each morphotype in the two different water treatments are given in table 9.1.

	δ^{29}Si			δ^{30}Si		
	WW	**WS**	**Average**	**WW**	**WS**	**Average**
Bulliforms	0.87‰	1.39‰	1.15‰	1.70‰	2.68‰	2.22‰
Elongates	0.76‰	1.18‰	0.96‰	1.40‰	2.27‰	1.87‰
Stomata	0.51‰	0.58‰	0.53‰	1.0‰	1.19‰	1.07‰

Table 9.1 - δ^{30}Si and δ^{29}Si isotopic discrimination percentage for bulliforms, elongates and stomata of selected landraces of sorghum.

The differences observed in the isotopic composition show that stomata are not responsive to water levels, elongates are weakly related, while bulliforms seem to discriminate against water availability (figure 9.6). Bulliforms discriminate ^{30}Si and ^{29}Si against the lighter counterpart ^{28}Si in well watered conditions (figure 9.6). In addition, bulliforms display a statistically significant negative relationship between the small delta (of both δ^{30}Si and δ^{29}Si) and transpiration levels (figure 9.7). In figure 9.7, also elongates show a weak, but significant, negative relationship between δ^{30}Si and δ^{29}Si and transpiration, although the figure 9.6 shows that the difference between WW and WS is not significant. However, figure 9.8 shows that there is great difference between landrace phytolith isotopical composition and that while some varieties show differences between the two treatments in isotopic composition of both elongates and bulliforms, others do not. Only S5, S9, S10 significantly separated the two treatments for discrimination of ^{30}Si and ^{29}Si in both elongates and bulliforms.

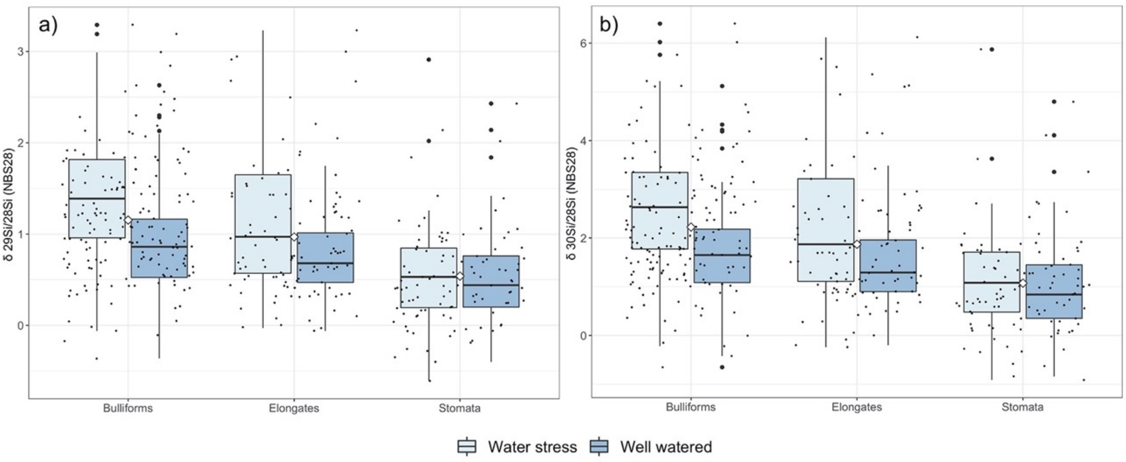

Figure 9.6 - Boxplot visualising a) δ^{29}Si b) δ^{30}Si in bulliforms, elongates and stomata extracted from the leaves of sorghum cultivated in 2019. Small black dots correspond to the single replication value, big black dots represent outliers.

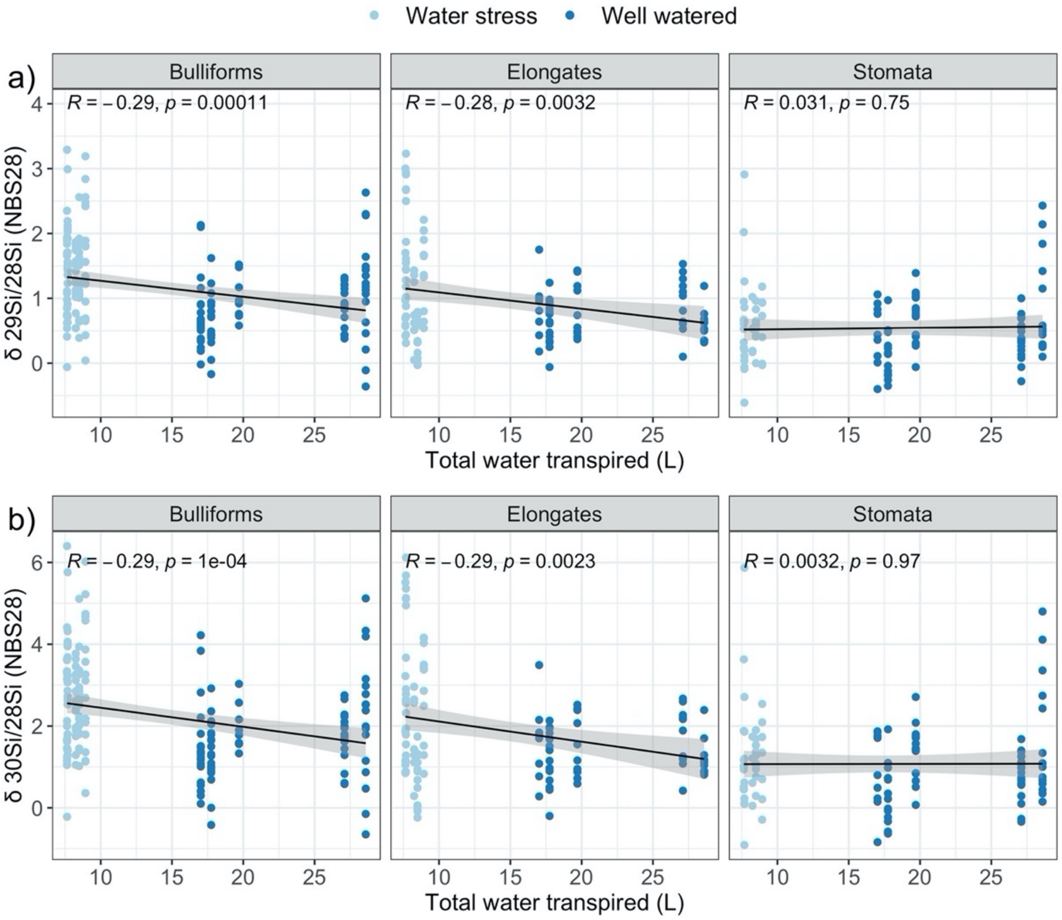

Figure 9.7

Figure 9.7 - Boxplot visualising a) δ^{29}Si b) δ^{30}Si in bulliforms, elongates and stomata extracted from the leaves of sorghum cultivated in 2019. Grey bands represent 95% of confidence intervals. The total water transpired is arranged to form three sample groups because, as also explained in chapter 5 of methodology, only a few landraces were selected for this analysis that had demonstrated different values of total water transpired. Linear regressions for all the morphotypes considered together available in appendices figures A9.4.1, A9.4.2.

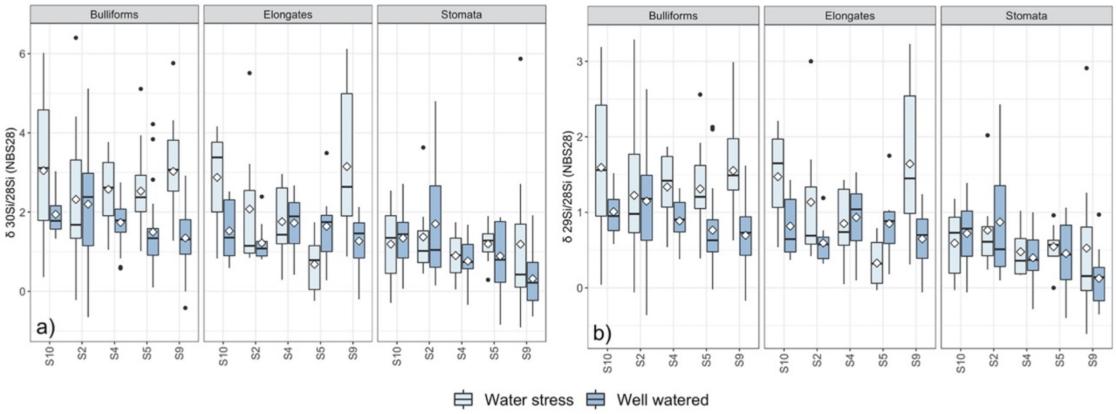

Figure 9.8. Boxplot visualising a) δ^{29}Si b) δ^{30}Si in leaf bulliforms, elongates and stomata and displayed divided by sorghum landraces.

9.2.2 Discussion

The most substantial result in terms of Si isotopic analysis achieved so far, is the development of a standard methodology to measure the composition of individual phytoliths. The data presented here is the result of more than a year of tests using different mounting materials, measurements on single and bulk phytoliths. The recovery of statistically significant results with a high degree of resolution already corresponds to an advance in the measurement technique. More importantly for understanding plant water availability, the values obtained from the isotopic analysis of Si in phytoliths are also extremely promising. The isotope ratio composition measured (0-6‰) ranges within the previously proposed values for δ^{30}Si plant phytoliths which is -2.3‰ and +6.1‰ with differences up to +3.3‰ among plants organs (Ding et al., 2008, 2005; Hodson et al., 2008; Opfergelt et al., 2006; Prentice and Webb, 2016; Sun et al., 2008). However, it must be taken into account that many of the published results were conducted on different species, not always C$_4$, under both natural and non-natural growth conditions, and measured with different technologies (such as fluorination and gas MS) and not only with fsLA-MC-ICP-MS on single phytoliths.

So far, very few results have been available on single morphotype stable Si discrimination (see Frick et al., 2019 mentioning that stomata could be a distinctive zone of fractionation). Going into more detail, these preliminary analyses on individual phytoliths of sorghum leaves have shown that, at least for this species, bulliforms can discriminate water treatment on the basis of both δ^{30}Si and δ^{29}Si content (figure 9.6). Bulliforms are the morphotype where differences between the two water regimes are most pronounced, while in elongates the intra-species variability (i.e., between landraces) seems to have a higher influence in the results, suggesting the need to widen the sample tested in terms of both replications and landraces. The level of discrimination between heavy and light isotopes, which is between 0.5‰ and 1‰ for bulliforms is negatively related to total water transpired (R:-0.29), which implies that the more the sorghum plant transpires, the fewer heavy isotopes are fixed in the leaf bulliforms (figure 9.7). The result also implies that, if the production of bulliforms by sorghum can be assumed in the archaeological record, they can be used as indicators of water availability. On the contrary, stomata demonstrate stability in the storage of heavy and light isotopes, with consistent and comparable discrimination between different water regimes. Since stomata are the last cells in which Si is deposited during the transpirative cycle (then water exits through the somatal pores), it was hypothesised that they might represent the cells with the highest level of discrimination (δ^{30}Si and δ^{29}Si), with light isotopes being preferentially deposited first while heavy isotopes continue through the transpirative flow till stomata. The results of this study show that the stage of the transpiration cycle where cells reside is not the only factor to be taken into account and that much more likely the transporters involved in silica intake for each cell have a substantial effect on the phytolith Si composition. This statement is supported by Hodson's (2019) theory that argued how Si is only deposited in these cells once the transpirative function of the leaf has ceased.

9.3 Conclusions

Morphometry as well as Si isotopes analysis have been carried out to explore the feasibility of producing a series of phytolith independent proxies to discriminate water availability.

Morphometric studies of phytoliths have progressed enormously in recent years, thanks to the development of programmes and software (e.g Hošková et al., 2021) that allow the measurement not only of length and width, but also of articulated indices (perimeter, area, concavity and convexity). In this study, only basic measurements were taken (length, width) to observe whether there was ground for a more in-depth study of morphotype-sensitive measurements or not. The measurements were taken in 2D because taphonomy in archaeological contexts can massively influence the three-dimensionality of the phytoliths, making 3D measurements unusable when applied in the context of ancient phytolith assemblages (Hošková et al., 2022). Despite the preliminary nature of the research developed here, the results obtained are informative in several respects. This research, designed to observe shifts on the size ranges related to water levels in sensitive morphotypes, also contributes with new information on the size of bulliforms, elongates and stomata for the three species under consideration. Morphometry studies on grass sensitive morphotypes are scarce (see also discussion in chapter 4) because these morphotypes are often ubiquitous and redundant, and do not allow taxonomic identification at a very high level (e.g., family) (Ball et al., 2016, 2009; Hošková et al., 2022, 2021; Out and Madella, 2017, 2016). Furthermore, the results obtained from the current work suggest to concentrate further studies on a multi-factor analysis of bulliforms. In fact, bulliforms have proven to be the morphotypes whose size changes in relation to water availability, where plants grown in water abundance produce larger bulliforms. To capture -with less/lower uncertainty- whether such observed differences are closely related to a difference in water storage by the bulliform cells or whether instead they are influenced by genetics, it should be extended the measurements to more species and landraces. The results obtained would have a great archaeological implication, allowing the use of bulliform sizes as environmental-water proxies. Although a more thorough investigation of bulliforms is necessary, this study has archaeological applicability if the presence of these three species finger millet, pearl millet and sorghum, has been established in the context. In fact, bulliform measurements exceeding 50 µm x 70 µm in these species belong to replications grown well-watered.

In respect to Si isotopes, there is a difference in fractionation when water conditions change, which is evident in the isotopic composition of sorghum bulliforms from almost all the studied landraces (apart from S2) and in elongates from a few landraces. Thus, bulliforms are confirmed as an interesting morphotype to investigate for two reasons. First, because of the positive results of the analysis, and second, from a methodological point of view. Bulliforms are large sized phytoliths, commonly well-preserved in the archaeological record and produced in all grass species. Their large size facilitates analysis while less robust (thinner) phytoliths such as elongates have to be analysed in bulk to obtain a strong signal. Bulk of archaeological elongates, preserved enough to be analysed with laser technology, and embedded in silica skeletons are not always easy to find in the archaeological records (and if they are not embedded into silica skeletons there is no certainty they come from the same plant/tissue). The isotopic fractionation of Si along the plant is still not completely understood and before this can be applied to study plant micro-remains from archaeological contexts, several issues related to the biochemistry and the genetics related to phytoliths deposition will have to be resolved. In this doctoral work I have only discussed preliminary results that were part of my direct research work, while additional analyses carried out by RAINDROPS members in collaboration with Dr Daniel Frick of the German Research Centre for Geosciences on morphotypes of different species and from different tissues (culm and chaff) are currently undergoing.

Notwithstanding the preliminary nature of the data presented here, it is evident that there is great potential for using bulliform phytoliths in archaeobotanical studies and the study of their morphometry and isotopic composition will greatly help in understanding past water regimes in archaeological and paleoenvironmental settings. These results added to those explained in the previous chapters (7-8) seem to confirm that not only that bulliform production depends largely on water availability but also that bulliforms can be used as predictors in models that use phytolith assemblages to describe water availability.

Appendices available at Zenodo.org

DOI: https://doi.org/10.5281/zenodo.7220967

Figures A9.1.1, A9.1.2, A9.1.3, A9.1.4, A9.1.5, A9.1.6, A9.1.7, A9.1.8, A9.1.9, A9.1.10, A9.1.11, A9.1.12: linear regression plots of height (“He”), length of the beak (“LB”), and width (“Wi”) (dependent variable) in relation with leaves biomass (“Leaves”), transpiration efficiency (“TE”), total water added (“TWA”) and total water transpired (“TWU”) (independent variable) for bulliforms.

Figures A9.2.1, A9.2.2: density plots for subsidiary cells size.

Figures A9.3.1, A9.3.2, A9.3.3, A9.3.4: density plots for bulliform additional parameters measured (height of the beak, length of the beak, number of peaks and length peak to peak).

Figures A9.4.1, A9.4.2: linear regression plots of the $\delta^{29}Si$ and $\delta^{30}Si$ (dependent variable) in relation to total water transpired (independent variable) for all the morphotypes considered together.

File A9.1: the complete dataset of the phytolith morphometry and isotopic data.
File A9.2: R scripts.

File A9.2.1, A9.2.2, A9.2.3, A9.2.4: csv UTF8 (comma-delimited) files to run the code in R.

File A9.3: metadata.

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Chapter 10 - General discussion: plant science and archeology to unlock water management in the past

The overarching objectives of the current work are (1) to study biosilica accumulation processes in sorghum, pearl millet and finger millet, in order to understand whether phytoliths produced by C_4 crops can be used as proxies for plant water availability; (2) to investigate intra and inter specific variability in phytolith production, and with regards to water availability during crop development; and (3) to elaborate a model that can be applied for the interpretation of archaeological phytolith assemblages in respect to ancient water management. These objectives included a series of questions which answers are discussed in this chapter.

For the sake of clarity, I specify that in the following paragraphs biosilica accumulation is defined as the amount of biosilica extracted (weight of amorphous biogenetic opaline extracts) (chapter 6) wheres phytolith production refers to the concentration of phytoliths extracted and then identified following the formula proposed in chapters 7 and 8. The two variables were compared and proved to be positively related, indicating that the extracts (biosilica accumulation) were pure phytoliths, without external sources of contamination.

10.1 Experimental archaeology

Is the developed methodology suitable to answer the three initial aims?

The methodological approach of this thesis can be divided into two major categories: 1) the experimental cultivation and 2) the analysis of phytolith from such cultivations. In both cases the approach was innovative, using techniques that have never been applied in experimental archaeology and for this very reason deserve a discussion.

Aso mentioned in chapter 8, the cultivation methodology used in this research is effective for overcoming methodological issues identified for other methods in the past, such as the quantification of the water absorbed by plants or the limitations of cultivation in pots (see Katz et al., 2013). Katz et al. (2013) and Jenkins et al. (2016), discussed the controversies related to using rainfall data (i.e., total water received by the plant) to interpret phytolith production, and highlighted the possible influence of additional variables, such as solar irradiation and evapotranspiration, on soil water availability. It should also be emphasised that different species have different water use efficiency and diversely respond to vapour pressure deficit (VPD) levels and soil water availability (Devi et al., 2010; Sinclair et al., 2017; Vadez et al., 2014). The simple measurement of rainfall would not take into account the inherent ability of species and varieties to absorb and utilise water. Therefore, assessing plant water "availability" via a measure of transpiration, as carried out in this work, is an essential methodological progress. Transpiration gives a measure of the water that passed through the plant and it takes into account the ability of species to respond differently to soil water availability, light, and atmospheric conditions. The use of lysimeters (growing cylinders) adequately solves the issues related to measuring the crop water consumption. All this while upholding a direct connection to water added or naturally received rainfall, which is the data of interest when exploring the plant water availability from archaeological realities.

Regarding the study of phytoliths, a specific methodology for extracting and counting silicified cells was developed. This specific need arose from the interdisciplinary nature of the research. The extraction of phytoliths from the experimental samples had to consider the particularity of working with dessicated tissues, with a high content of silica skeletons and organic matter, and archaeological assemblages derived from sediment samples subjected to taphonomy. Thus, it was necessary to find an extraction method that would produce comparable results. In this study, the challenge was to extract as clean as possible opaline silica (phytoliths) from samples with a high content of organic matter and without damaging the phytoliths that had then to be observed and counted to be compared with archaeological datasets. The procedure developed, which allowed the extraction of almost pure phytoliths, brought with it a further problem: the abundance of silica skeletons in modern phytolith samples that is not common in archaeological assemblages. Silica skeletons or articulated phytoliths cannot be counted as a single cell morphotype. The tissue phytoliths of the three millets analysed presented as large silica structures. On average silica skeletons were composed of 50 or more phytoliths, primarily elongates morphotypes. In the archaeological record, however, silica skeletons tend to disarticulate and elongates can be highly affected by taphonomy (Cabanes and Shahack-Gross, 2015). Short cells, on the other hand, heavily silicified and with lower surface area/volume ratio, respond better to taphonomic processes, and normally represent a good portion of the archaeological assemblage. Thus, a phytolith sample from fresh tissue is normally characterised by the abundance of elongates whereas an archaeological sample normally consists of many short cells. In this study, a protocol to compare these two types of samples (i.e., fresh plant material rich of elongates *versus* archaeological samples extracted from soils samples rich in short cells) was elaborated, which maintained the richness and the evenness of both types of samples and kept the abundance of each morphotype homogeneous.

10.2 The effect of genetics

Do finger millet, pearl millet and sorghum accumulate biosilica through the same pathway?

All the results obtained from the analyses seem to suggest that the three species have different mechanisms of biosilica accumulation and phytolith formation. Pearl millet and finger millet showed the closest relationship between total water transpired and biosilica accumulated. Indeed, the observed rate of phytolith production in finger and pearl millet is constant in WS plants (when water was never added during cultivation), while it varied in WW plants as the transpiration process fluctuated between the two years of experimentation. Also, phytolith production in the transpiration organs -new leaves, for example- of these two species showed a positive relationship with transpiration rate. Conversely, in sorghum no difference in phytolith production and biosilica accumulation has been registered either between the two lots of plants cultivated in the two different years (where significant differences have been registered in the transpiration rate) or between the two water treatments. Therefore, sorghum biosilica accumulation was not substantially influenced by the environmental conditions in which the plants grew. These results have an important impact on both physiology and archaeology.

First, the experimental results show that, despite taxonomic proximity, the three species possess substantial differences in Si accumulation mechanisms. Ma and Yamaji (2006) suggested that the amount of silica uptake, attributable to genetics, depends on two principal factors which both concur to the xylem loading: the presence/absence of channels to transport Si from cortical cells to the xylem (Lsi1-Lsi2-Lsi6 and orthologs), and the density with which they occur. The combination of these two factors determines the classification into active, passive or rejective (non-accumulators) silica accumulators.

Although all grasses are considered high accumulators (Hodson et al., 2005), their ability to accumulate Si through specific transporters remains unverified for many species. In *Sorghum bicolor* some research has already been carried out and the species has been tested for Lsi1 protein sequence by Vatansever and colleagues (2017) who discovered two homologous genes codifying for the transporter channels. However, mutations have also recently been discovered by Markovich et al. (2019) who proved the presence of varieties unable to absorb high quantities of silica. Slp1, a unique amino acid compositional protein involved in the precipitation of silica in the silica cells, has also been localised in sorghum (Kumar et al., 2020, 2017a, 2017b). In this study, sorghum not only accumulated a large amount of Si in comparison with the other two species tested, but the rate of accumulation proved to be independent of environmental factors such as transpiration. Thus, this result is in line with the presence of a mechanism in sorghum that transports the silicic acid actively up to the leaves, independently of transpiration (transporter-governed Si uptake) and placed the species among high silica accumulators. Possible mutant varietis of sorghum are also present in the landraces studied as shown by contradictory biosilica accumulation rate (see for example landrace S6, chapter 6, figure 4). For pearl millet and finger millet the information seems more complex to interpret. Although the amount of Si accumulated also places the two species within the high accumulator group (Hodson et al., 2005), it is also true that the relative production (g of Si/g of dry matter) in comparison to sorghum is lower. Based on what is proposed by Ma and Yamaji (2006), it might be speculated that the density of the transporters facilitating Si uptake is lower (albeit existing) in pearl millet and finger millet and that these millets depend largely on passive transport along the transpiration stream, although the dynamics of the mechanism remain to be clarified. Unfortunately, it cannot be substantiated with data on the genetics of Si uptake because no information on the correspondent loci in *Pennisetum glaucum* and *Eleusine coracana* are available so far. However, since finger millet and pearl millet biosilica accumulation seems to rely more on environmental factors (transpiration), it can be proposed that they are more suitable than sorghum to investigate the response to water availability. When such results are viewed in respect to their applicability to understand past water availability, the contribution of each of these species in the archaeological assemblage cannot be inferred through a simple evaluation of the relative abundance of the corrispective phytoliths. Most of the morphotypes produced by these three species are not species-specific. Therefore, the key new information arising from this work can be summarised as: first, that even in species closed related (in this case *Poaceae*) phytolith production can vary and that there are species that accumulate more biosilica and species that accumulate less biosilica; second, that phytolith deposition is influenced by water availability in some species (e.g., finger millet and pearl millet), while in others is less dependent on environmental factors (e.g., sorghum, whose relative abundance of phytoliths can be directly related to biomass). Hodson et al. (2005) have already explored silica accumulation in different families and emphasised important differences among taxas. Therefore, it is important to stress that to clearly understand biosilica -and therefore phytoliths- production of millets for archaeological and paleoenvironmental scopes there is the need of exploring more in depth biosilica accumulation at the genus, if not at the species, level. For this purpose, particularly important would be genetic analysis to identify the presence/absence and density of channels specifically dedicated to Si transport.

Are the phytolith assemblages of the three studied species of C_4 comparable?

The phytolith assemblages of finger millet, pearl millet and sorghum differed in morphotype composition and concentration. The diversification among the three species emerged from the results of the generalised linear models (GLMs) tested. The phytolith assemblage of sorghum is characterised by elevated numbers of crosses, elongates sinuate and polylobates, present in high concentrations even in replications grown in water stressed conditions (WS), and by the almost total absence of saddles and rondels. This last characteristic was also mentioned in previous works by Novello and Barboni (2015) and Jenkins et al. (2020). Sorghum leaves produced a higher proportion of bilobates, bulliforms, stomata and elongates than finger and pearl millet leaves. In “young” leaves sorghum produced almost two times more trichomes than the other two millets species but pearl millet produces many more trichomes than sorghum in the oldest leaves and in chaff. Saddles and rondels are produced in high numbers in the leaves of finger millet, more abundant in WW conditions than in WS conditions but the production is comparable (by concentration) in young and old leaves. Bilobates, polylobates and crosses are almost absent in pearl and finger millets. Chaff of pearl millet, especially in WW conditions, is characterised by a very high concentration of trichomes (mostly forming large silica skeletons), which is the most abundant morphotype. In sorghum chaff, elongates sinuate are more abundant than in pearl millet. These results are in line with previous studies that indicate that short-cell concentrations are efficient in distinguishing species, e.g., crosses or saddles (Barboni and Bremond, 2009; Neumann et al., 2017), but they also highlighted that the concentration of trichomes and specific elongates can be equally species specifc.

The formation of silica skeletons also appears to be somewhat species-dependent. Silica skeleton size did not show any variation related to plant water availability and therefore might not be a reliable proxy in the studied landraces. This is in contrast to previous results on emmer wheat (Rosen and Weiner 1994). Sorghum seems to produce silica skeletons of larger dimension in respect to the remaining two millets, suggesting that high silica accumulators, as in the case of sorghum, have a higher chance to produce conjoined silica cell structures, which eventually can be preserved archaeologically. Pearl millet, on the other hand, as also mentioned above, produces large trichome skeletons in the chaff that have not been found in sorghum. Thus, pearl millet and sorghum chaff are distinguishable not only by the concentration of specific morphotypes but also by the characteristic formations with which they occur.

The archaeological implications of these results on phytolith assembly composition are varied. The “unbalanced” concentration of some morphotypes relative to others, depending on the species and the tissue, indicates that the abundance of specific species in the archaeological record cannot be derived from a comparison of the concentration of the respective phytoliths produced. Morphotype concentration depends on both environmental factors such as watering and taxonomic factors.

10.3 The environmental effect

Do water availability influence phytolith deposition?

The results from the experimental phytolith assemblages indicate that many of the morphotype concentration values for the three species can be predicted by the water treatment, demonstrating that phytolith assemblages can be used as proxies for water availability. In addition, preliminary isotopic analyses on individual phytoliths from sorghum leaves highlighted that bulliforms and elongates can discriminate water treatment on the basis of both δ^{30}Si and δ^{29}Si composition. The difference in the discrimination of the lighter ^{28}Si isotope and the two heavier cunterparts ^{30}Si and ^{29}Si, between the two treatments is bounded by 0.5‰ and 1‰ for both morphotypes and appears to have a significant negative relationship with transpired water. Bulliforms also increase their robustness (width + length + length of the beak) with increased levels of transpiration. Thus bulliforms are particularly suited to assess water availability, as this seems to affect their chemical composition, their concentration in the assemblage and their average size. So far, only bulliforms have been shown to change in size according to watering, consequently it seems appropriate to assume that, in general, as the silica supply changes, the concentration of most deposited phytoliths changes but not their dimensions (Schulz-Kornas et al., 2017). In this respect, with this work it was observed that some morphotypes increase their concentration in WW conditions while others in WS conditions, and that this depends on the species but also on the tissue where the morphotypes are deposited. This peculiarity highlights that to estimate water availability the complete morphotype assemblage should be observed and not a reduced group. Not all morphotypes proved to be equally efficient in predicting watering. The stepwise regression applied to select the most explanatory morphotypes for water treatment identification indicate acute bulbosus, bulliforms, elongates clavate, dentate, entire, and sinuate, crosses, polylobate, rondels and saddles as predictive and excluded the remaining morphotypes. It should be highlighted that the forms with the highest degree of significance are short cells, which are normally considered as fixed morphotypes, i.e., morphotypes for which the silicification is controlled by genetic factors and that in previous studies did not show changes in concentration in respect to water availability (Ermish and Boomgarden, 2022; Jenkins et al., 2016, 2020; Madella et al., 2009). Considering the results from the current study, it is possible to conclude that the concentration (per g of dry leaf in millions) of some morphotypes is influenced by water availability, but that not all of them (depending on the species) increase in concentration when transpiration rate rises. This leads to the conclusion that, since the mechanisms of phytolith deposition are complex (and in some way they still elude our understanding), in order to produce a predictive model based on the composition of phytolith assemblages originating from many different species, it is necessary to take into account all group of morphotypes, of all tissues, so that positive or negative variations to water availability in each are recorded. As a consequence of this, fixed and sensitive categories should be rethought, considering that while the concentration of some morphotypes increases with watering, others may be stimulated by water stress conditions (for further discussion see also the following section).

Is the ratio of sensitive to fixed morphotypes an efficient indicator of water availability in the C_4 species under study?

Even if phytolith deposition proved to have a direct relationship with water availability, a key conclusion of the current study on phytolith formation in finger millet, pearl millet and sorghum is that the sensitive *versus* fixed ratio approach (Jenkins et al., 2016, 2020, Madella et al., 2009) did not prove equally efficient in these three species. This result leads to two major considerations which emphasise that the deposition of morphotypes depends on both genetic and environmental factors, and that a more in-depth research on the physiological role of phytoliths needs to be addressed before classifying morphotypes as fixed or variable. The first consideration is about the way the ratio is calculated and what morphotypes are taken into consideration. The index includes stomata among the sensitive morphotypes but it does not include either bulliforms or trichomes. In view of the recent studies on the classification of morphotypes on the basis of their deposition mechanism (Hodson, 2019), and considering that silicified stomata in a still photosynthesising leaf could lead to the death of the tissue itself (Kumar et al., 2017b), it is suggested that stomata cannot fall into the sensitive category. Perhaps stomata belong to the class of phytoliths whose deposition is most probably genetically regulated, at least for the timing of deposition, which has to be later than leaf emergence (Motomura, 2004) in order not to cause a dysregulation of normal leaf development (Kumar et al., 2017b). For this reason, stomata should not be counted in either the sensitive or fixed category. In contrast, bulliform phytoliths form in cells with a function closely connected to water availability, and their deposition, composition and dimensions have been shown to be connected to water availability in the current and other studies (see Wang et al., 2019). Therefore, it is recommended that bulliforms should be included into the sensitive category. The same can be said of trichomes whose density has been proven to be higher in a xeric environment with low soil moisture (Olsen et al., 2013), and in our case, especially in pearl millet chaff and sorghum leaves, proved to be highly responsive to watering.

All recent studies, including this one (see discussion above), indicate that there is a strong correlation between Si deposition and taxonomy (Ball et al., 1996, 2016; Hodson et al., 2005; International Committee for Phytolith Taxonomy ICPT et al., 2019). Not only have different species shown different deposition capacities but some intra-specific variability has also been demonstrated (e.g., sorghum, Markovich et al., 2019). This seems to suggest that a “universal phytolith ratio” for exploring water availability in plants, which works for all species and in all growing environments, might be difficult to determine. The current study suggests an alternative approach by using a predictive model for water availability based on the phytolith concentrations. Such an approach has been successfully tested in three different millet species but the model must include all morphotypes produced by the plants because both elongated and short cells have demonstrated to be responsive to water availability.

10.4 The combined effect of taxonomy and environment

Do genetic or water treatment have the most significant effect on plant Silicon accumulation?

Species and genotypes predicted the amount of silica accumulated better than the water treatment alone (table 2 in chapter 6). Species and tissues predicted the production of most of the morphotypes better than the treatment (table 5 in chapter 8). It was already a fact that, in the plant kingdom, biosilica accumulation varies enormously within taxonomic groups growing in the same environment, suggesting that there is a strong genetic control on Si absorption and deposition (Barboni and Bremond 2009; Hodson et al., 2005; Katz et al., 2013; Naskar and Bera 2018; Neumann et al., 2017). This is supported by evidence even within the same genus where, for instance, there are large differences in biosilica accumulation among sorghum landraces. Thus, modelling the deposition of phytoliths in relation to water availability becomes complex, even more when considering that the analysis would be applied to archaeological assemblages. In the archaeological record, it is not always possible to reach inter-specific recognition of these three millets by phytolith morphometry and composition, and the intra-specific identification of varieties of the same species virtually impossible. Nevertheless, the creation of highly “regionalised” modern datasets composed of varieties traditionally in use in the area of interest and landraces, can offer useful insights. The use of landraces is particularly important as modern varieties may have undergone genetic variations that also affected the mechanism of Si absorption and deposition. According to the results presented in this thesis, different species and genotypes can display similar responses in phytolith production, related to environmental variations (see logistic regressions in chapters 7-8). An alternative to compose modern datasets for archaeological comparison by highly regionalized varieties may be to build an extended dataset with several species and with several varieties to include all the possible variability in biosilica accumulation. In this way representativity of specific biosilica accumulation mechanisms should be ensured by a broad spectrum of varieties analysed.

However, it should be mentioned that the highest variance in biosilica accumulation is explained by the interactive effect of genotypes and watering (table 2 in chapter 6), suggesting that both variables are significant. In this regard it can be speculated that the demand for biosilica depends on the physiological conditions related to water availability, which in turn depend on the efficiency of the species/genotype in responding to water availability. Consequently, it may be also hypothesised that some specific morphotype, possibly more efficient than others in performing structural or biochemical functions, might increase in response to drought or because their production is stimulated by other environmental events (abiotic or biotic stressors) with a strong impact on the physiology of the plant itself. The hypothesis is supported in this study as the morphotypes whose production increases in WS sorghum replications (acute bulbosus, crosses, elongates clavate and entire stomata in leaves and elongates sinuate and polylobates) could play a key structural role in response to hydric stress. Indeed, silicification of stomata could help in reducing transpiration by regulating water loss (Cooke and Leishman, 2016; Gao et al., 2020; Goto et al., 2003; Hosseini et al., 2017; Wang et al., 2021). Crosses according to Kumar et al., (2017a) could deposit to the side of minor veins,toughen them, and allowing xylem flow to continue (Kaufman et al., 1985). Elongates, arranged on the epithelial surface, could increase light inception (Cooke and Leishman, 2016) and would stiffen epidermal tissue (particularly fragile in WS conditions) and simultaneously prevent both pathogen attack and further crumpling of leaf blade (Meunier et al., 2017; Rodrigues et al., 2003; Yoshida et al., 1962). Trichomes, which have been proven to respond to pathogens attack (Daoud et al., 2018; Fauteux et al., 2005; Mateos-Naranjo et al., 2013; Oliva et al., 2021), have also demonstrated to be a consistent response to xeric environment (Olsen et al., 2013), and possibly help to maintain a film of moisture around the developing leaf or panicle. Another piece of evidence provided by this study that can support the hypothesis that the biosilica accumulation influences the species/genotype efficiency is given by the redundancy analysis (RDA, figure 6 in chapter 6). In chapter 6, it has been shown that biosilica (as a constraining variable) responds very closely to panicle production in the redundancy analysis (RDA), meaning that replications that produced more seeds are also those that accumulated more biosilica. This result opens up two possible interpretative options. Either the accumulated biosilica contributes to increasing the fitness of the species (contributing to plant hardening), to the implementation of the photosynthetic process or to water saving (as mentioned above), or more biomass corresponds to more Si accumulation. In the first case, the results are supported by Verma and colleagues (2022) who showed how higher levels of Si accumulation are associated with a better performance in water deficit conditions, as biosilica increases photosynthetic leaf gas exchange and improves plant production. In the second case, it must be mentioned that finger millet had a much higher average biomass production than sorghum, interrupting growth at the vegetative stage, which gave rise to a conspicuous production of leaf biomass. Despite the high biomass production rate, finger millet accumulated much less biosilica than sorghum and did not produce any panicles.

Heretofore, it has been discussed how taxonomy has a strong impact on biosilica accumulation and on phytolith production, which in turn respond to environmental factors such as watering, possibly increasing the ability to respond to both biotic and abiotic stresses from outside. Accordingly genetics and environment appear not to be two completely unrelated and independent factors in the accumulation of Si. Katz (2019) suggested, from an evolutionary ecological perspective, that if silica content is persistent in the plant kingdom from embryophytes onward (as demonstrated by Coskun et al., 2019), it has to confer some advantages to the plant fitness. All the results obtained from this work seem to support this same deduction, implying to a certain degree that Si content in plants could represent a functional trait. Functional traits have been defined as morphological, biochemical, physiological, structural, phenological, or behavioural characteristics that are expressed in phenotypes of individual organisms, relevant to the response of such organisms to the environment and/or affecting ecosystem properties (Díaz et al., 2013). The hypothesis is supported by different studies indicating that biosilica accumulation could be arranged in response to the plant internal demand (e.g., Hosseini et al., 2017; Li et al., 2020; Oliveira et al., 2022) by the regulation of the genes Lsi1-Lsi2-Lsi6 or orthologs expression (e.g., Mitani et al., 2011; Sun et al., 2017; Vatansever et al., 2017; Yamaji et al., 2008). It has been also established that biosilica deposition in plants react to environmental changes (e.g., Daoud et al., 2018; Hartley et al., 2015; Mandlik et al., 2020; Schaller et al., 2013), depends on the surrounding setting (e.g., soil composition) and simultaneously affects the Si circle on the ecosystem (e.g., Cornelis and Delvaux, 2016; de Tombeur et al., 2020; Grasic et al., 2020; Keller et al., 2021). If Si content is truly a plant functional trait (and it would need to be verified through specific ecological population studies), answering the question posed at the outset would be impossible. In fact, if Si content is a functional trait, the genetic and environmental effect would contribute in predicting biosilica accumulation by influencing each other. For this purpose, particularly important would be to test how the impact of the phenotypic variability in Si accumulation alters the role of organisms in the ecosystem.

10.5 Back to archaeology

What are the implications of this work in archaeology?

Having concluded that Si content is most likely a plant functional trait, on the one hand it complicates to some extent the interpretation of archaeological phytoliths assemblages, on the other hand it proposes a new prospective (and a new tool) with which to approach phytolith study for archaeological purposes. The importance of this work for archaeology mainly concerns the reliability and the level of approximation that can be assigned to phytoliths as proxies of the past. Studying modern plants and the manner in which they respond to watering by changing their phytolith content has shown which aspects are more trustworthy for archaeological interpretation.

One of the main archaeological applications of this study is the identification of a relationship between the concentration of phytoliths and water availability for the three species under investigation. The main result in this respect is that different species systematically increase or decrease production of specific morphotypes as water availability changes, depending on the tissue and plant growth stage. Thus, this study shows that phytoliths of different C_4 species are an informative proxy for past water availability. To observe these variations in phytolith assemblages consisting of more than one C_4 species, as in this case, the application of a predictive model based on logistic regressions was proposed, as an alternative to the use of the ratio of sensitive to fixed morphotypes current in use (Jenkins et al., 2016; Madella et al., 2009). The model allows for inter- and intra- species variability in phytolith production to be taken into account. In addition the model does not exclude all those differences in the production of both fixed and sensitive morphotypes in different plant tissues, showing great potential for archaeological studies.

To observe the change in watering levels, not only the general phytolith assemblage but also morphometry shows great potential. In fact, plants grown in well watered conditions show a distinct range of higher and larger bulliforms that are not produced in water-stressed plants. This means that by being able to identify the presence of the three species in the archaeological record, the results of this study can help formulate hypotheses on both palaeoenvironmental and past agricultural practices. The recognition of dimensional gradients in bulliforms size of finger millet, pearl millet and sorghum implies variations in water availability, either natural or anthropogenic. The same conclusion was hypothesised by Wang et al. (2019), after studying the morphometry of rice flabellate bulliforms, and sees an important implication in archaeology.

The great intra-specific variability both in physiological aspects and phytolith deposition underlines the importance of constructing modern interpretative models consisting of highly regionalised and specific varieties. Modern commercial variants are very unlikely to come close to the more primitive domesticated ones, consequently they may not respond equally to environmental variations for phytolith production. The information is of great interest in archaeology because it demonstrates how the environmental effects observable through the concentration of morphotypes cannot be detected independently of the species/genotypes. Nevertheless, this conclusion does not represent a limitation for archaeology but rather a new tool for observing the composition of phytolith assemblages (chapter 7). The model built on the basis of different African and Pakistani landraces allowed the interpretation of the archaeological records of these specific areas. Concurrently, its application is discouraged in different areas of the world where the same species are present, but different phytolith production strategies due to taxonomic differences may result.

A possible limitation to archaeological applications concerns the isotopic study of sorghum phytoliths. The results, while showing that the Si content of bulliforms responds to variations in water availability, also indicate that different morphotypes produced in the same organ (i.e., leaves) of the same replications discriminate differently between the light and heavy isotope of Si. Indeed, the results achieved show that the stage of the transpiration cycle where cells reside is not the only factor to be taken into account to justify the level of discrimination and that much more likely the transporters involved in silica intake for each cell have a substantial effect on the phytolith Si composition. This statement is supported by Hodson (2019) who argued how Si is only deposited in some cells once the transpirative function of the leaf has ceased. This conclusion leads to two important considerations. First, it hints at the difficulty in observing bulk isotopic content of archaeological phytoliths. Indeed, averaging between discriminations of different morphotypes from different organs of different plants could lead to biases in the interpretation of results because the composition of each morphotype may not depend entirely on environmental factors such as transpiration level. Second, the result opens new lines of investigation, indicating that the discrimination of the isotopic content of Si of phytoliths depends largely on the deposition process of each morphotype, which needs to be investigated in more detail. It must be said, however, that if bulliforms encountered can be attributed exclusively to sorghum, then water availability hypotheses can be developed from the Si isotopic composition of the bulliforms.

The results of this thesis show that phytolith deposition mechanisms in different millet species are complex and that the interpretation of the archaeological assemblages is not straightforward. This study showed that biosilica production is very different in the three species and that sorghum produces many more phytoliths than finger millet and pearl millet per gram of dry biomass. This observation indicates that the concentration of phytoliths does not correspond linearly with the abundance of the species which produced them. Similarly, the quantification of specific morphotypes is not directly related to the abundance of the original species as morphotype concentration might also depend on environmental factors. This conclusion may have an ecological implication and could be useful when environmental/species recognition indices are being developed based on the abundance of some shapes relative to others.

Another non-negligible fact, although marginal to this thesis, concerns the physiological results retrieved by the observation of the crop growth. The various landraces showed wide variability in the way they develop and reach maturity. Different levels of biomass production, seed production, flowering time, harvest index, transpiration efficiency and transpiration rate were recorded. These landraces are no longer widely in use today (supplanted by the more efficient modern hybrids) but somewhat approximate the archaeological varieties. This means that, based on observed physiological data, it could be possible to make assumptions about the use and handling of these millets in the past as well.

New research possibilities arise from the observations described above. First, is necessary to explore modern phytolith assemblages from a multi-proxy perspective, investigating the concentration of each morphotype but also the morphometry, the elemental content, and the anatomical arrangement within the tissues, in order to obtain useful information in as much detail as possible to be applied to archaeology. Second, this study has shown the importance of analysing phytoliths of millet leaves. Thus it is important to sample archaeological contexts where leaf may be more abundant. Archaeologists are normally more interested in targeting contexts that might yield phytolith assemblages related to the vegetative part of the plants as these offer more accurate information on the presence and use of crops. However, since leaf phytoliths can be very informative of the way these crops were cultivated it is of great interest to identify and study contexts that are not necessarily related to last stages of crop- or food-processing activities. Thus, sampling should be expanded to contexts where one would expect an abundance of leaf phytoliths, such as courtyards, pits, general habitation contexts and living areas (see for example Harvey and Fuller 2004; Weisskopf 2017).

The data generated in this study can be useful beyond archaeology. A more in-depth study of morphometry data may have a paleoenvironmental or phytolith classification application. For example, Barboni and Bremond proposed different studies on phytolith assemblages of tropical Africa where the associations between phytolith occurrence, taxonomy and growth environment are analysed in depth (Barboni and Bremond 2009; Barboni et al., 2007). In the future, it might be interesting to compare the results obtained from the short cells of this study on African landraces with those obtained by Barboni and Bremond. Morphometric indices have been proposed to relate the abundance of specific morphotypes such as bilobates/crosses and polylobates and their sizes to taxonomy (e.g., Fhamy 2008) and crop evolution (e.g. Rudall et al., 2014). Nothing precludes such studies from being conducted in the future on the samples extracted for this thesis as well, considering that the way the phytolith dataset was constructed allow for such taxonomic analysis.

Can this research contribute to a better understanding of agricultural water management in the past?

This research was developed within a project (RAINDROPS) that aims to analyse different archaeological sites scattered between East Africa (Mezber in Ethiopia and Al khyday in Sudan), Pakistan and India, where water management could play a key role in the development of societies inhabiting these areas of the world (see discussion in chapter 2). While the African archaeological samples are still being processed, those of the Pakistani and Indian archaeological sites have already been partly studied (although analyses at new sites are still in progress). During this thesis project, I was able to apply the model developed on the basis of modern phytoliths on samples derived from 4 archaeological sites located between southeastern Pakistan and northwestern India, all belonging to the Indus Valley Civilization. The assemblages compared to the modern dataset were recovered from layers dated to the Mature Harappan period (2500-1900 BCE) and come from four different archaeological settlements: Harappa, Kanmer, Shikarpur and Alamgirpur. Using C_4 phytolith samples from Mature Harappan period layers contributed to the open debate on the consumption and spread of millet crops throughout the Indus Civilization. During the Mature Harappan period, small millets represented anywhere from 3 to over 50% of the recovered cereals (Pokharia et al., 2014). However, their use is difficult to justify since their grain processing is very labour intensive (Arunachalam et al., 2005), apart from the fact that their productivity is lower when compared to that of C_3 cereals (such as barley and wheat) (Pearcy and Ehleringer, 1984). At present, there is also scarce information available on the presence and use of millets in this area and even less archaeological evidence of water management practices for crops (Petrie et al., 2017). We assume that a possible explanation could be directly related to water availability and management, this hypothesis was tested by applying the model developed. First of all, the model was applied on assemblages coming form contexts where the presence of the three species under study was confirmed by macrobotanical studies (e.g., caryopses). The presence of domesticated C_4 species has been reported at each of these settlements (Harappa: *Panicum* L. Sp. Pl.; Kanmer: *Pennisetum galucum* e *Setaria* Sp. P. Beauv.; Shikarpur: *Brachiaria ramosa* L. Stapf., *Coix lacryma-jobi* L., *Digitaria* Sp. Haller, *Eleusine coracana*, *Setaria* Sp. P. Beauv.; Alamgirpur: small millets in general) (Bates 2019; Bates et al., 2021). Secondly, the presence of C_3 wheat and barley was also attested (Bates and Petrie, 2016). Due to C_3 agronomic characteristics, these cereals, apart from needing a fair amount of water to grow (much more in comparison with C_4), were most probably grown during the winter period (winter type temperature) (Kalra et al., 2008) outside the rainy season. Therefore, the presence of C_3 cereals indicates that settlements had water available for agriculture, regardless of whether it was derived from more or less elaborate irrigation systems or riverine agriculture. Third, the model developed on the modern varieties could be applied because it was built on landraces typical of the area, grown in India during the dry season, and subjected to watering regimes plausible to those of the past (irrigation *versus* rainfed agriculture in drylands).

Once the model developed on modern phytoliths was applied to the archaeological record (see chapter 7) the outputs indicate that most of the plants grew in water scarcity conditions. All sites under analysis provided similar results, despite the fact that the water and climatic conditions are somewhat different among sites. Harappa and Alamgirpur were located near watercourses, with extensive *doabs* (tracts of alluvial lands between two possibly convergent rivers) that could possibly support irrigation systems and advantageous for agriculture (Kenoyer, 1998). In contrast, Kanmer and Shikarpur were most likely located in drier areas, with no access to substantial alluvial plains to exploit for agricultural purposes but/or served by *nullah* (watercourses) of small dimensions (Lancelotti, 2010) or, as the case of Kanmer, where artificials water basins (i.e., tanks) have been observed (Madella and Lancelotti 2022).

So far, ethnographic field surveys conducted by Madella and Lancelotti have revealed several strategies that could ensure a certain degree of water availability in riverine areas as well as more dry lands. Irrigation can be provided through basins or furrows (Madella and Lancelotti 2022). Basins are flat areas of land, usually not very large, surrounded by low bunds that prevent water from flowing to the adjacent piece of land. This technique is often used for the construction of paddy fields and less so for the cultivation of C_4 species that do not tolerate waterlogging. Furrow irrigation relies on small channels that carry water down land slopes between the crop rows. In the latter case waters of the rainy or flood season can be stored in simple or elaborate reservoirs and then directed to the fields when needed. Water harvesting is achieved mainly through two different strategies: the exploitation of river floods or the *gabarbands* (Madella and Lancelotti 2022). *Gabarbands* are terraces onto which the seasonal flood of nonperennial streams is directed, enabling the watering of the fields constructed behind the flood area. Although archaeological evidence is scarce, this information suggests that there may have been some degree of water availability at all four sites to ensure the growth of C_3 cereals and irrigation.

Based on this information, I proposed several hypothesis to explain the results achieved, which seem to show that the C_4 species grew on a low water regime in all the settlements. However, it must be mentioned that a multi-proxy approach would be needed and soils and seeds would also need to be tested for isotopic content (also and especially in C_3 remains) to support the hypothesis and verify it independently.

Firstly, the results obtained tend to exclude the option of multi-cropping (i.e., the growing of two or more crops together on the same land) in the same agricultural space. In fact, based on the result obtained from the model, there is about a 60% chance that C_4 cereals are grown with less than less than 300 mm of available water. This amount of water would not allow either C_3 cereals or legumes to grow, and advocates for alternative options to multi-cropping cultivation.

In areas with ample water availability (e.g., Harappa and Alamgirpur) there could be a system of double cropping (*rabi* or winter & *kharif* or monsoon season) with C_3 cereals (e.g., wheat and barley or rice) and vegetables grown under a water regime in winter while others species more resistant to drought, such as C_4 employed during the hot and less rainy period (Petrie et al., 2016). Therefore, a first hypothesis would imply that while during the cold season C_3 cereals were cultivated at a steady rate of water, in the hottest season, before the monsoon rains arrived, C_4 cereals (which are also very fast growing) were planted. This is supported by ethnographic evidence from the area, where millet crops are mostly rain-fed summer crops (Giosan et al., 2012; Madella and Fuller 2006). This strategy would also ensure the possibility that semi-nomadic populations could leave the millet field once sown and return only at harvest time, dedicating the season to pastoralism (Garcia-Granero et al., 2016). The option of double cropping (*rabi & kharif*) is, however, widely debated and several researchers argue that the archaeological evidence found to date cannot provide definite answers to the hypothesis of a double cropping system (see Petrie et al., 2017). To verify the existence of double cropping system it might be interesting to develop a “twin-model” based on the concentration of C_3 phytoliths and apply it to the archaeological assemblages retrieved from the areas. If the C_3 species were also found to be grown in water scarcity conditions then, perhaps, the option could be discarded. Although the results of the application of this model do not exclude the eventuality of a double cropping system, it is also true that there are more options to justify the presence of C_4 species grown in water scarcity. Another possible strategy, would include the cultivation of C_4 cereals alongside C_3 throughout the year, but in lands where irrigation/flooding/residual humidity of soils (due to distance from water sources such as rivers) was unfeasible. This would justify why the plants that compose the archaeological samples would have grown with some availability of water (perhaps due to rainfall) but not abundant.

The results of the model also do not rule out the hypothesis that the area included between southern Pakistan and northwestern India has experienced some form of drying and that people adapted to more consistent dry periods over time, as suggested by Wright et al. (2008). The authors suggested that after a millennium of riverine agriculture based on the overbank flooding, around 2800-2500 BCE, the rivers failed to deliver the usual balance of water and forced farmers to base their agricultural system on rainfall. The same study suggests that around 2000 BCE the reduction of rainfall could have been assessed on a level of about 240 mm/year. This information matches with the results obtained in this study, which hypothesised the possible presence of C_4 plants grown with less than 300 mm of available water. Whether the adoption of millets was due to a matter of progressive aridification, or whether there was an opportunistic cultivation of C_4 to exploit drier areas or that C_4 grew wild in arid parcels of the sites, what emerges from these findings is that most likely C_4 grew in water scarcity conditions.

In conclusion, the model has contributed in offering possible hypotheses related to the land use and agricultural development of the areas of interest and capable of answering a research question of a purely archaeological nature. These results do not allow us to lean more towards one of the hypotheses, but do suggest that there may be a certain intentionality in the choice of cultivation type of C_4 species being that in all the settlements analysed, they are found to have grown in water scarcity conditions.

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Chapter 11 - General conclusions

Archaeologically, much research has been devoted to the study of the long-term development of drylands, considered the cradle of the Old World social and agricultural system. The domestication of water in dry areas enabled the consolidation and spread of farming lifestyles that ignited an exponential population growth (Manning et al., 2011; Manning and Timpson, 2014). Arid regions are dominated by savannah and grassland biomes composed mainly by C_4 species. The study of C_4 millet crops, whose dispersion history in Africa, Asia and Europe has not been unravelled yet (Motuzaite-Matuzeviciute et al., 2016; Stevens et al., 2021; Winchell et al., 2018), has a key role in the archaeological debate on the amelioration trajectory on land use. The archaeological questions that are behind the study of millet remains, are not only related to the dynamics of consumption of the involved species, but also to the type of management of such species required (Briggs et al., 2006; Fuller, 2011; Stephens et al., 2019). C_4 crops management is closely related to the concept of water availability, as these plants are notorious for their ability to withstand extreme conditions, including high temperatures and water scarcity (Sage and Zhu, 2011; Yang et al., 2021). For this same reason, C_4 crops are thought to be difficult proxies' "producers", thought to be less sensitive to changes in precipitation or in temperature (Ghannoum et al., 2002). C_4 species capacity to keep low the transpiration trend constitute a real problem when the interest stands in observing which effects had the fluctuation of climatic variables on isotopical rate, phytoliths or yield. The C_4 specific pathway of CO_2 fixation predicts a leakage (Φ) defined as the rate of CO_2 which leaks out of the bundle sheath. This leakage is difficult to quantify and prevent the interpretation for paleoenvironmental reconstruction of $\delta^{13}C$ values of archaeobotanical remains.

To gain a crucial understanding of which type of environmental effects can be observed through micro- and macro-botanical remains of C_4 crops, the plasticity of their anatomy and biochemistry must be thoroughly studied (Ferrio et al., 2020). In this sense, the study of phytoliths has been indicated as an alternative to the evaluation of isotopic signatures in C_4 caryopses, as well as a possible indicators of watering. Phytoliths have several advantages over caryopses: they preserve well in the archaeological record and need neither charring nor water logging to survive taphonomic effects (Madella and Lancelotti, 2012; Strömberg et al., 2018) and they are produced by most of the cultivated cereals (Ball et al., 2016; Piperno, 2006).

Therefore, it was decided to further study on morphotypes assemblages to observe efficiency of phytolith produced by finger millet, pearl millet and sorghum as environmental indicators. By studying biosilica accumulation in this three different C_4 species, subjected to different water regimes, it has been shown that phytoliths can be good proxies for water availability, adding new species to those that had already been tested for the same purpose (Ermish and Boomgarden, 2022; Jenkins et al., 2016, 2020; Madella et al., 2009; Weisskopf et al., 2015). Through the comparison of the dataset with assemblages of phytoliths recovered from specific archaeological sites, hypothesis on the management of C_4 species in the past have been suggested, highlighting the potential of the results achieved applied to archaeological contexts.

Silicon deposition was investigated, promoting its potential as a water availability indicator and its issues related to taxonomic differences in biosilica accumulation. The results obtained support the hypothesis that phytolith deposition depends, at least to some extent, on environmental factors. Water availability influences phytolith deposition in finger millet, pearl millet and sorghum by affecting the concentrations and compositions of morphotypes assemblages. These differences, however, depend on the species and phytolith deposition tissues and are more evident in finger and pearl millet than in sorghum. More in detail, it has been shown that water availability plays a fundamental role in determining biosilica accumulation in finger millet and pearl millet, which seem to be more akin to passive accumulators. On the contrary, sorghum is seemingly characterised by a strong transporter-governed mechanism, which also determines a high variability among genotypes. Albeit the miscellaneous assemblage of landraces displayed different trends in phytolith production, the complete dataset allows to make predictions on the water availability. In connection with this consideration, it is evident that the genetics related to Si uptake should be better examined in the future, in light of the variations in biosilica accumulation among species, landraces and deposition tissues, uncovered during this study. It is advocated that widen the dataset of varieties analysed is fundamental, especially for archaeology, in order to approximate as closely as possible the assemblage recovered from ancient sediments. The concept of sensitive and fixed morphotypes should also be revised. The results presented here demonstrated that stomata can hardly belong to the sensitive category while bulliforms and acute bulbosus, which have not been taken into account so far, are. A key information that emerges from this study is that it is difficult to separate morphotypes whose deposition is controlled by genetics from those whose deposition is triggered by water availability, as both depend most possibly on an environment-dependent regulation.

All the results presented in this thesis seem to indicate that silica content is a functional trait. This deduction carries with it the idea that the abundance of certain morphotypes, strategic in this kind of studies, could increase in response to drought, especially in species such as sorghum where Si uptake is most probably regulated. However, the causes and the effects of silica deposition in the plants remain to be elucidated. A final crucial result of this study was that the method applied for growing the experimental plants has shown to be reliable and able to recreate field-like conditions while taking under control the physiological variables as transpiration. This is extremely important for producing material with the highest control on the growing variables and its standard use will facilitate the creation of fully comparable datasets in future studies.

11.1 Limitations of the present work

The development of this experimental PhD thesis has been a path full of learning trials, tentative and attempts. This last section identifies the limitations of this thesis and proposes alternative or additional analyses that could help to build a more complete framework to understand phytolith deposition in finger millet, pearl millet and sorghum species.

11.1.1 Experimental cultivation

Two experimental seasons at the same time of the year in two different years were planned for this research. The idea was to replicate the first experimental season, to build a dataset as reliable as possible. However, to encapsulate all the possible variability in Si deposition as environmental characteristics change, the experiment would have been more complete if the same varieties were grown also in different seasons. These additional fieldworks would also have enabled to observe whether environmental conditions such as temperature and relative humidity, or soil composition have a substantial effect on biosilica deposition, and whether this effect is stronger than watering. An attempt in this direction has been made, in fact at Pompeu Fabra University, we built an experimental field equipped with lysimeters to cultivate a small number of landraces (both a selection of the previous ones and of C_3) in summer and during winter. Unfortunately, the analysis of phytoliths from these additional experiments could not be included in this thesis. The main limitation in doing this was our capacity to process plant tissue samples and measure phytoliths, once purified. While the method we developed improved the quality of the data produced because of a higher degree of purity, silica skeletons preservation and counting for archaeological comparison, further efforts are needed to speed up the extraction procedure when working with such an amount of fresh samples.

11.1.2 Species and landraces under analysis

Three species, considered to be among the main food crops of drylands, were selected in this study and their physiological characteristics analysed. The aim was to build a dataset composed of different species and varieties. Sorghum, specifically, was selected also because it had already been studied for its phytolith production in relation to watering and was included to act as a control species. Likewise, additional C_3 species, which had already been proven to be sensible to water availability throughout the test of the ratio of sensitive to fixed morphotypes, should have been tested. With additional, previously tested species, an argument could have been made about the experimental set up developed, observing if different results would have arrived using different methodologies on the same species. Then, with more time and a larger number of cylinders available, it would have been ideal to also test more landraces from different climatic zones, to confirm that the environmental/genetic variable is crucial in explaining phytolith deposition.

11.1.3 Isotopical analysis

The methodology used to study the isotopic content of phytoliths is the fsLA-MC-ICP-MS. The procedure for analysing individual phytoliths with this technology is extremely experimental and required a number of trials before the complete and definitive step-up was made. However, it would have greatly benefited this study to have had independent proxies such as isotopes processed on all three species simultaneously to compare the results with those coming from phytolith assemblages. It would also have been of great use to test other types of isotopes on phytolith, such as oxygen isotopes to observe more fully the issue of phytolith composition. Later on, it would also have been interesting to analyse the isotopes of soils and water sources to observe the degree of discrimination between plant and external environment.

11.2 What is next? Further steps and solutions to problems left unsolved

The research answered the scientific questions it had set out to explore, by carrying out a comprehensive analysis that managed to converge crop science with archaeology. However, answering these questions led to many more. The main analyses that should be done to add a fundamental piece of knowledge, useful in both agronomy and archaeology, concern phytoliths as a phenotypic effect of silicon accumulation. What are the genetic factors that influence its production in these three species? And what functions does this element play within plant physiology? Answering these questions in the future would help to observe the deposition of the different morphotypes, with the aim of extracting two important pieces of information: environmental information that can be obtained from the composition of phytolith assemblages (archaeological implication); whether or not Si is very important element playing a role in the drought response (possible agronomic application). To this end, the isotopic analyses already underway on the silicon isotopes could be concluded in the future and oxygen isotopes added, accompanying the analysis with a more in-depth genetic one, aimed at testing the presence of Si content regulatory genes in the three species of interest. For this purpose, since for this thesis many more landraces have been cultivated than those analysed, it would be useful to test the missing ones in order to construct a more complete picture of possible inter- and intra-species variability. As far as isotopes are concerned, the data obtained on bulliforms are extremely promising, and I would suggest following up on these specific morphotypes, which have the advantage of being ubiquitous in C_4 and C_3.

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