UNIVERSITAT DE BARCELONA

Plant physiology and biotechnology for the study and improvement of Mediterranean japonica rice varieties

Irene Ferreres Contreras

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Irene Ferreres Contreras

Thesis presented by Irene Ferreres Contreras to obtain the Doctor degree from the *Universitat de Barcelona*. This thesis is framed within the Doctoral Program *Ecologia, Ciències Ambientals i Fisiologia Vegetal* corresponding to the period between 2017-2020 of the *Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals.*

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Pre-doctoral student	Thesis supervisor and tutor	Thesis supervisor
Irene María Ferreres Contreras	Dr Salvador Nogués Mestres	Dr Xavier Serrat Gurrera

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¡Cuando las cosas llegan a los centros,

no hay quien las arranque!

Federico García Lorca

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Abstract

Rice belongs to the *Poaceae* family and *Oryza* genus. The genus *Oryza* comprehends 24 species, being 22 wild and 2 cultivated. These two cultivated species correspond to *Oryza sativa,* originated in Asia, nowadays cultivated and consumed worldwide, and *Oryza glaberrima,* originated in Africa, but cultivated and consumed limitedly in West Africa, both are diploid (2n=24) (Bernis & Pamies, 2006; Wei & Huang, 2019).

Traditionally, *Oryza sativa* has been classified into two subspecies, indica and japonica. Molecular analyses, as well as biochemical and hybrid sterility analyses, point out at the separate domestication of *Oryza rufipogon* populations as the origin for these two subspecies (Garris et al., 2005; Rakshit et al., 2007; Wei et al., 2012), with a gene flow present from japonica to indica (Yang et al., 2012). Indica rice is usually long grained, less sticky and with a lower level of amylopectin than japonica rice, which is short grained and sticky. There are also phenotypical differences between these subspecies, for example, japonica plants are generally shorter, the leaves have a lighter color and shaper shape. Also, the tiller number is lower in japonica than in the indica plants (Wei & Huang, 2019).

The *O. sativa* plant is a semiaquatic annual monocot grass although rice can live as perineal in the tropics (McLean et al., 2013). The plant has a height that varies from 80 to 150 centimeters depending on the variety and growing conditions (Bernis & Pamies, 2006), or even less in modern varieties. Its morphology varies according to the development stage, this being the vegetative phase or the reproductive phase. The vegetative phase includes the germination, the seedling and the tillering stage. The reproductive phase comprehends panicle initiation, also called heading, and flowering stages.

As a food, rice contributes to the 20% of calories ingested worldwide (Kubo & Purevdorj, 2004), reaching half of the calories ingested daily in some areas (Counce et al., 2000). In terms of consumption, as well as production, Asia is the main continent (McLean et al., 2013). The rice production and consumption in Europe is minor compared to Asia. Despite that, rice holds an important sociocultural role since it is one of the basic foods of the Mediterranean diet. Some regions have developed famous rice dishes, like *risotto* in Italy or *paella* in Spain. The annual rice consumption *per capita* in Europe is 6-18

kilograms in the southern regions, and 3.5-5.5 kilograms in the northern regions (McLean et al., 2013). The main European producer is Italy, followed by Spain. These two countries hold more than 75% of the total rice production in Europe. Rice in Europe is also of ecological importance, due to the great biodiversity that inhabits and benefits from the paddy fields. Around 70-80% of rice cultivated in Europe are japonica varieties, and the rest indica (Bernis & Pamies, 2006; Ferrero, 2007). Agriculture is the most important bioeconomy sector in Spain, with a value of 43.8 million of euros in 2015 and coping the 50.9% employment of all bioeconomy sectors (Lániez & Periago, 2016). Rice mobilized 258.766 millions of euros in Spain in 2019 (MINECO, 2019). Thus, the studies dedicated to improving different aspects of the rice production in Spain are crucial.

This thesis has made a multidisciplinary approach, from genetics to the field, in order to study Mediterranean rice varieties considering its actual applicability for the Spanish rice industry through three experimental approaches: (i) an improvement of the anther culture protocol for two Mediterranean temperate japonica varieties and two tropical japonica varieties trough the addition of additives to the media; (ii) testing the salt-tolerance of *Saltol*-introgressed varieties to select those that will help to fight the apple snails plague in the Ebro Delta; and (iii) a study of genetic and physicochemical aspects of the rice pearl in five Mediterranean varieties, an important feature in gastronomical terms.

Experiment 1: Anther culture of two temperate and two tropical japonica varieties

Anther culture is a very useful technique that allows to obtain doubled haploid plantlets from haploid microspores. A two-step anther culture protocol for rice was first described by Niizeki and Ono (1968). In the first step, rice anthers are placed in an induction medium that contains stressing growth regulators that force the haploid microspores contained in them to follow the sporophytic pathway. The microspores induce calli, which are transferred into a regeneration medium as the second step, where plantlets develop (Mishra & Rao, 2016). Some of these regenerated plantlets are haploid, since they come from haploid microspores, but it is also very common the spontaneous chromosome doubling of the cells during anther culture, leading to the formation of doubled haploid (DH) plantlets (Davey, 2009).

The applications of DHs are wide. For example, in genetics DHs have been used from mapping genes to screening markers, as well as to locate whole quantitative trait *loci* (QTLs) (Forster & Thomas, 2010), many involved in agronomical traits of interest. In

terms of plant breeding, the first advantage is that DHs allow to shorten greatly the breeding period. Besides, DHs allow to obtain homozygous lines at any generation, which can be useful to reveal and fix interesting characters expressed by recessive genes. DHs can be commercialized themselves as cultivars, or be used as parents, which allows total knowledge of the progeny (Forster & Thomas, 2010).

Our study has improved an already established medium-grain, temperate japonica Montsianell-optimized anther culture protocol to be used for long grain tropical japonica (303012 and 303013) and long-grain temperate japonica (PL12) varieties through the addition of media additives, as well as improving the performance of Montsianell. Both Montsianell and PL12 are varieties cultured in Spain, and the tropical varieties, 303012 and 303013, had never been tested in anther culture protocols.

Regarding the induction medium, the staple medium (0D1) and a 150 mg· L^{-1} colchicine-supplemented one (150D1) were tested. The 150D1 medium significantly favored the calli induction for 303012, 303013, and Montsianell, inducing calli 20-fold more than in the 0D medium for this last variety.

Considering the regeneration step, three different media were tested: the staple XACRM medium described by Serrat et al. (2014); and two media derived from XACRM, named SARM and GERM, with different additives (sorbitol and Gelrite respectively) in order to increase osmotic stress and therefore to force regeneration. SARM allowed regeneration in all varieties, being the highest rate for the three long-grain varieties (PL12, 30312, 30103). GERM was the medium that caused the highest regeneration for Montsianell. Both supplemented media improved the performance of the XACRM medium, because the osmotic stress provoked by them allows a greater number of cells to differentiate, since it physiologically isolates the pre-embryonic cells by disrupting the plasmodesmatal connections between them (Wetherell, 1984).

Regarding the regenerated plantlets, Montsianell and 303013 were the only varieties that regenerated green doubled haploid plantlets from the calli induced at the colchicine-supplemented medium. Moreover, no regeneration of this kind of plantlets occurred in 0D1, which it is not hazardous since colchicine favors the green doubled haploid production (Chen et al., 2001). Colchicine had no effect on doubled haploid obtainment in PL12, as it only regenerated haploid and mixoploid plantlets. Since no diploidization occurred in this variety during the anther culture steps, a subsequent diploidization could be forced *in vitro* with the use of antimitotic agents. Hooghvorst et al. (2018) performed

diploidization in haploid plantlets proceeding from the anther culture of the temperate japonica variety Montsianell, improving the efficiency of their anther culture experiment. Our study has shown the importance of variety in the performance of rice anther culture in combination with medium additives. It has not only improved the previous anther culture protocol for Montsianell cultivar, but it has also performed for the first time an essay on long grain tropical and temperate varieties. Besides, it has proved that colchicine enhances the anther culture at both induction and regeneration steps, and the usefulness of osmotic stress for plantlet regeneration.

This work has been published (Ferreres et al., 2019), and contributes to the knowledge in japonica rice varieties anther culture, since few studies are yet published. Further investigations will help to elucidate the differential characteristics that anther culture of japonica rice varieties presents, meaning a substantial improvement in the obtaining of doubled haploids, and therefore, in breeding.

Experiment 2: Testing the *Saltol* region in hydroponic assays and in fields

In 2009 was reported for the first time an apple snail (*Pomacea sp.*) invasion focus in the drainage network of the left Ebro hemidelta (López, Quiñonero, & Tarruella, 2009). The apple snails are known for being extremely invasive (Mueck et al., 2018), due to their massive egg laying, reaching from 300 to 800 eggs at a time (Plaza & Galimany, 2013). Rice is one of their preferred targets, especially in its first phenological states, from germination to tillering (Plaza & Galimany, 2013). Massive apple snail ingestion of rice can cause from 60-90% harvest loss (MINECO, 2018). Since their first appearance, the apple snails have established as a permanent and aggressive plague in the left Ebro hemidelta, extending through 9,000 hectares of field and 400 kilometers of the drainage network.

Various strategies have been adopted to battle the plague expansion and try its eradication in the Ebro Delta. One of the most sustainable is the fields flooding with sea water, due to the apple snails sensitivity to salt. Apple snails present a high mortality with a salinity of 6.8‰ and almost total mortality above ≥13.6‰ (Ramakrishnan, 2007). The Mediterranean sea has an average salinity of 38‰ (Borghini et al., 2014). These sea water floods are kept 30 days at maximum, and then the fields are washed with fresh water. This solution is very effective, but involves a noteworthy problem: the residual salinity

left in the fields, since rice is one of the most sensitive cereal crops to salinity (Maas & Hoffman, 1977; Zhu et al., 2001).

Salinity causes two stress phases in the rice plant: (i) a first quick phase of osmotic stress, and (ii) a subsequent longer phase of ionic stress. The first phase of osmotic stress is provoked by the high solute concentration present in the soil. The immediate plant reaction is a reduction of the stomatal conductance, which leads to a decrease in the transpiration rate and CO_2 assimilation. Also, water uptake and plant growth drop in this initial response to salinity (Munns, 2002; Munns & Tester, 2008). It has been proposed that the reduction of growth would avoid further water use by the plant and, therefore, the moisture in the soil would be maintained, avoiding an increase of the salt concentration in it. If the stress is mitigated with this response, the plant will reach a certain recovery and gradually will restart its growth. If not, ion stress mechanisms will activate. An excess of Na^+ in the plant aerial parts can lead to chlorosis, senescence and necrosis. Therefore, as a ionic-stress response, plant mechanisms make the Na^+ ions migrate from shoots to roots through apoplastic (passively) and symplastic (actively) pathways (Frouin et al., 2018; Negrão et al., 2011), being the apoplastic via the preferential (Ranathunge et al., 2004). This Na^+ influx to the roots activates ABA-dependent responses that prevent the further uptake of Na^+, due to the activation of selective ion transporters with high affinity for K^+ and Ca^+ ions and low affinity to Na^+ ions. This also stops the movement of Na^+ from roots to shoots. Therefore, the Na^+/K^+ ratio in shoots is a good measure of salt tolerance (Frouin et al., 2018). The lower the ratio, the better the salt resistance, since it indicates the success of the regulatory mechanisms that lower Na^+ concentration in shoots.

Many efforts have been put in to discover and characterize genes or QTLs that confer salt stress tolerance in rice. The most relevant QTL involved in salt resistance, is known as *Saltol*, placed in the short arm of chromosome 1 (Platten et al., 2013). It has been proved to explain from 64.3% to 80.2% of phenotypic variation and 43% of variation in Na^+/K^+ ratio in shoots (Bonilla et al., 2002),

The NEURICE project, in which this thesis takes part, is a Horizon 2020 project funded by the European Union. Its main objective has been to develop new commercial rice varieties by incorporating salt tolerance alleles into Mediterranean varieties in order to face climate change and the present apple snail plague in the Ebro Delta (NEURICE comes from **N**ew commercial **EU**ropean **RICE**). Two elite salt-sensitive temperate japonica varieties provided by *Càmara Arrossera del Montsià*, the long-grain PL12 and

the short-grain PM37, were crossed with two *Saltol*-carrying indica donors: FL478, a salt tolerant recombinant inbred line (RIL) obtained from the Pokkali x IR29 cross (Thomson et al., 2010) and IR64-*Saltol*, a salt tolerant nearly isogenic line (NIL) obtained from the FL478 x IR64 cross (Ho et al., 2016). The cross between PL12 and FL478 has been named LP, and the cross between PM37 and IR64-*Saltol* has been named MS.

This thesis has focused in evaluating salt-tolerance in lines coming from these crosses and selected upon seed availability, percentage of parental returning and *Saltol*-region integrity. BC3F5 LP varieties have been assayed in both hydroponic and field assays, BC3F4 MS varieties have been evaluated in field assays and BC3F5 MS varieties have been evaluated in hydroponic assays.

Regarding the hydroponic assays, three analyses were performed at 80 mM NaCl; a SPAD analysis to measure the leaf relative chlorophyll content and therefore, the plant integrity; a standard evaluation system (SES) analysis used as a visual scale of the phenotypical salinity damage and a Na^+/K^+ ratio analysis. It was statistically proven that the SES score and SPAD measurements correlate in a significant, strong and negative way. Furthermore, the Na^+/K^+ ratio measurements and SES score correlate in a significant and positive way. The top five best performing LP varieties in the hydroponic assays according to the Na^+/K^+ ratio, SES and SPAD values were LP9, LP7, LP28, LP18 and LP16. The top five best performing MS varieties following the same criteria are MS2, MS20, MS11, MS4 and MS23. For the field assays, the Na^+/K^+ ratio was also measured. The best performing varieties in the field, according to this parameter, were LP29, LP30, LP24, LP26 and LP22 for the LP family, and MS28 and MS14 for the MS family.

These tested salt-resistant varieties will tolerate the residual salinity derived from the fields treated with saline water to eradicate the apple snails' plagues, thanks to the successful breeding of the *Saltol* introgression into them.

Furthermore, in a global context of climate change, salinity-tolerant varieties, especially those grown near coastal areas, will be increasingly demanded due to the sea level rise accelerated by climate change (Mimura, 2013).

Experiment 3: Studying the rice pearl in Mediterranean varieties by different approaches

The rice pearl is a limited and enclosed opaque zone found in the rice grains of some varieties (Bernis & Pamies, 2004). The presence of pearl is generally considered a defect in most of the rice-consuming countries, especially in Asia, since its cooking and eating

qualities do not match their standards (Cheng et al., 2005). On the other hand, the rice pearl is considered an added gastronomical value in Spain and Italy, since it absorbs the flavour of the ingredients that it is cooked with (Bernis & Pamies, 2004). This is directly related to the rice recipes prepared in these countries such as *paellas* and *risottos*, where the rice is the main ingredient, and not consumed as a side dish, like in most Asian cuisines.

The rice pearl has not been studied as profusely as other characters due to its lack of value in most of the rice consuming countries. Besides, some of the published studies and reviews confuse the concepts of rice pearl and rice chalkiness (the chalky grains are completely opaque), or study mutants of non-pearled varieties instead of pearl varieties *per se*. Furthermore, most of the few existing studies focus on Asian indica or japonica varieties, and research made with Mediterranean varieties of interest for Spain or Italy are practically inexistent. Hence, the rice pearl is a complex character to dissect.

Regarding genetics, the presence of pearl has not been linked with a single gene for all japonica and indica varieties. Instead, many QTLs and some genes have been associated with the pearl and also with chalkiness (Sreenivasulu et al., 2015). Other studies have focused on physicochemical aspects that might be related to the rice pearl, like amylose and protein content, crystallinity degree or the microscopic structure.

Considering the previous knowledge, this thesis has performed a multidisciplinary approach to the rice pearl in Mediterranean varieties, by studying three pearled varieties, (Montsianell, Bomba and Carnaroli) and two crystalline varieties (PL12 and Guadiamar), along with chalky grains of each variety, in order to shed some light on the possible causes or characteristics of the rice pearl.

Although a big part of our study has focused on a physicochemical approach to the pearl character, an experiment was also performed at a genetic level. A sequencing study was made to check the affirmation made by Li et al. (2014) that the presence of pearl can be monogenically explained in indica varieties by the high expression of the gene *Chalk5*. This was due to the presence of two polymorphisms in the promotor region that allowed the binding of promoter elements. The sequencing performed in our five japonica varieties confirmed that, at least, the expression of this gene is not regulated in the same way as in indica varieties. Consequently, the single expression of the *Chalk5* gene is not determinant to the presence of pearl in our studied varieties.

Representative pictures were taken to normal and chalky grains of our varieties trough scanning electron microscopy (SEM). The pearl and chalky areas of our varieties

presented irregular and round-shaped amyloplasts and loose starch granules, with spaces between them and an irregular distribution. The crystalline fractions and grains presented the starch granules perfectly arranged into the amyloplasts, with polyhedral shapes. Protein bodies and their slots were visible between the limits of the amyloplasts. Our SEM observations match those made by various authors in other pearled/chalky rice grains (Kim et al., 2000; Li et al., 2014; Lin et al., 2014, 2016; Wang et al., 2010; Xi et al., 2014; Zhu et al., 2019).

In order to determine the degree of crystallinity of each variety, X-ray diffraction was used since is the most widespread technique to study starch structure. All diffractograms of each variety and grain type followed an A-type pattern, with peaks close to 15º, 17º, 18º and 23º, as expected for cereal starches (Li et al., 2014; Wani et al., 2012). Regarding our obtained crystallinity percentages, their range seemed to vary more depending on the variety than if the variety is pearled or crystalline. A variation on the crystallinity percentage for different rice varieties had already been reported (Iturriaga et al., 2004; Patindol & Wang, 2003). The percentage of crystallinity in chalky grains also did not follow a fix pattern, being for some varieties higher than the normal grain's (Bomba, PL12) and lower than the values from the normal grain (Montsianell, Carnaroli, Guadiamar).

The amylose content was also measured in our varieties, with results that showed non-significant differences in the pearled grains fractions (whole grain, pearl part or crystalline part). The pearl fraction presented a slightly higher amylose content than in the crystalline or whole grain parts for the Montsianell and Bomba varieties. Lin et al. (2016) reported that the amylose content did not relate to the presence of pearl *per se*. A clear pattern was found in the percentage of amylose in all varieties' chalky grains, presenting significant lower values. This fact matches the results obtained by various authors when measuring the amylose content in normal and chalky grains of various rice varieties (Kim et al., 2000; Patindol & Wang, 2003; Singh et al., 2003). This points out to a different explanation for the formation of chalky grains and pearled grains. Patindol and Wang (2003) proposed that there is a mechanism related to chalky grains that favors glucan branching over elongation.

The relative content of the storage proteins was also measured in the different fractions and chalky grains of the five varieties. The distribution pattern of the proteins was heterogeneous in our varieties with some exceptions for the chalky grains. The prolamins content in the chalky grains of all varieties (except for Bomba) resulted the lowest of all

fractions. Lin et al. (2016) found a lesser quantity of prolamins in the pearl fraction than in the crystalline fraction of a pearled mutant. Prolamins are contained in protein bodies (PBs) (Kawakatsu & Takaiwa, 2018), so a measurement of the quantity of PBs in the different fractions, as performed by Li et al. (2014) would be an interesting next experiment to perform. Our results showed also a higher level of pro-glutelins or glutelins precursors in the chalky grains of Montsianell, Bomba, Guadiamar and PL12, so a further study of the protein trafficking and its structures might help elucidate the differences between the origin of chalky and pearled grains.

In conclusion, our study has set a first approach to dissect the causes of the rice pearl in Mediterranean japonica varieties, first, pointing out to differences of the pearled grains and the chalky grains; and second, evaluating the existing studies making a clear difference of both grain types for the discussions. Due to the differences in the amylose and protein measurements found between the chalky and pearled grains, we can conclude that their origin or causes are not the same. On the other hand, some premises have been established in order to continue the study of the rice pearl in Mediterranean japonica varieties. The next step should be to perform a complete genomic study with RILs obtained from Mediterranean japonica varieties. This should be made considering the already detected QTLs and also, genes or QTLs related to the differences detected in our study, such as those involved in protein synthesis and trafficking. With the data obtained from our study, further experiments could be designed focusing in elements of interest, like expression analysis, cloning certain genes, transmission electron microscopy observations, PB studies, immunofluorescence analyses, and so on. At the end, this could lead to the creation of a marker profile useful for the genotyping and breeding of rice varieties.

This thesis has been developed in a global context that presents challenges as the increase of the population, and therefore, the food demand, as well as the emergence of climate change and the need of competitiveness of the economy. These must be faced through the empowerment of science and technologies in connection with private and public entities. Concerning Spain, agriculture is the main bioeconomy sector, thus, the research dedicated to improving different aspects of the production of agricultural products in the country is very relevant.

Regarding this thesis, the results of the anther culture experiment can be applied to the improvement and shortening of the breeding processes of Spanish rice varieties. The

tested *Saltol*-introgressed varieties can be used to effectively battle the apple snail plague in the Ebro Delta. Their salt tolerance will be also very useful when facing climate change effects such as the increase of the sea level that will affect the crops grown near the coastal areas. The study of the rice pearl has set a path to further study this valued gastronomical character in the Mediterranean area, whose knowledge could potentiate its positioning as an added value in an increasingly demanding global market.

The experiments performed in this thesis have contributed to expand the knowledge and/ or to study for the first time aspects related to Mediterranean japonica rice varieties trough plant physiology and biotechnology, with direct applicability in terms of production and market competitiveness.

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Abbreviations

2,4-D: 2,4-dophenoxy acetic acid

ABA: abscisic acid

BAP: benzyl amino purine

BC: backcrossed hybrids

BSA: bulked segregant analysis

DH: doubled haploid

DNA: deoxyribonucleic acid

EST: expressed sequence tag

HKT: high-affinity potassium transporter

HRR: head rice recovery

IRRI: International Rice Research Institute

KASP: competitive allele specific PCR

kDa: kilodaltons

LP: lines derived from the cross PL12 x FL478

MS: lines derived from the cross PM37 x IR64-*Saltol*

NAA: naphthalene acetic acid

NIL: near-isogenic line

PB: protein body

PCR: polymerase chain reaction

PSV: protein storage vacuole

QTL: quantitative trait locus

RCC: relative chlorophyll content

RIL: recombinant inbred line

SEM: standard error of the mean / scanning electron microscopy

SES: standard evaluation system

SSR: simple sequence repeats

TF: transcription factor

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1.Introduction

1.1 Rice characteristics and relevance

1.1.1 Rice origin and domestication

Rice belongs to the *Poaceae* family and *Oryza* genus. The genus *Oryza* comprehends 24 species, being 22 wild and 2 cultivated. These two cultivated species correspond to *Oryza sativa,* originated in Asia, nowadays cultivated and consumed worldwide, and *Oryza glaberrima,* originated in Africa, but cultivated and consumed limitedly in West Africa, both are diploid (2n=24) (Bernis & Pamies, 2006; Wei & Huang, 2019).

It is believed that *Oryza rufipogon* (also called *Oryza nivara* when referring to an *Oryza rufipogon* ecotype) is the progenitor species of *Oryza sativa*, domesticated approximately 8000 years ago, while *Oryza barthii* would be the *Oryza glaberrima* ancestor, later domesticated, approximately 3500 years ago (Furuta et al., 2015). This is supported by many phylogenetic analyses as well as morphological, distributional and sterility evidences (Wei & Huang, 2019).

Classically, *Oryza sativa* has been classified into two subspecies, indica and japonica. Molecular analyses as well as biochemical and hybrid sterility analyses point at the separate domestication of *Oryza rufipogon* different populations as the origin for these two subspecies (Garris et al., 2005; Rakshit et al., 2007; Wei et al., 2012), with a gene flow present from japonica to indica (Yang et al., 2012). Indica rice is usually long grained, less sticky and with a lower level of amylopectin than japonica rice, which is sticky and short grained. There are also phenotypical differences between each subspecies, for example, japonica plants are generally shorter, the leaves are of lighter color and of shaper shape. Also, the tiller number is lower in japonica than in the indica plants (Wei & Huang, 2019). More differential characteristics are reflected in Table 1.

Table 1. Some physiological characteristics differential in japonica and indica varieties. Adapted from Bernis and Pamies (2008).

Characteristic	Japonica	Indica
Low temperature resistance	High	Low
Drought resistance	Low	High
Lodging resistance	High	Low
Nitrogen response	High	Low
Competitive ability	Low	High
Minimum temperature to germinate	Low	High
Grain shelf life	Short	Long

Phylogenetical analyses such as a neighbor-joining tree constructed with chloroplast markers and short sequence repeats (SSRs) have led to a finer classification of the *Oryza sativa* subspecies into five groups: indica, aus, aromatic, temperate japonica and tropical japonica (Garris et al., 2005; Huang et al., 2012; Kovach et al., 2007) (Figure 1). Further single nucleotide polymorphisms (SNPs) analyses even divided the indica group into indica I and indica II groups (Xie et al., 2015).

This thesis will focus in the study of the *O. sativa* species, which will be called rice indistinctly from now on.

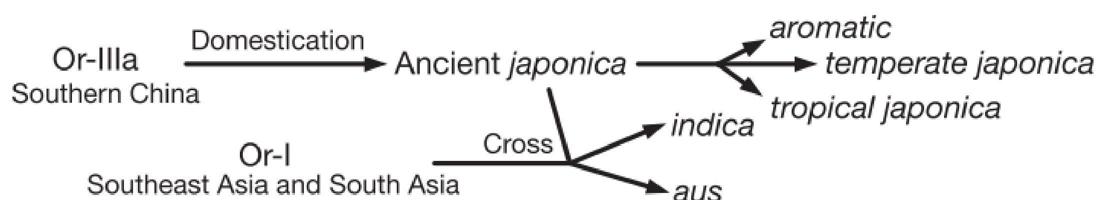


Figure 1. Huang et al. (2012) analyzed the genome of more than 400 accessions of *O. rufipogon*, clustering it into three groups: Or-I, Or-II and Or-III. They proposed the origin of japonica subgroups was the domestication of the Or-III subgroup, and that indica subspecies originated from crosses with ancient japonica and group Or-I of wild rice.

1.1.2. Rice morphology and life cycle

The *O. sativa* plant is a semiaquatic annual monocot grass, although rice can live as perineal in the tropics (McLean et al., 2013). The plant has a height that varies from 80 to 150 centimeters depending on the variety and growth conditions (Bernis & Pamies,

2006), or even less in modern varieties. Its morphology varies depending on the development stage, this being the vegetative phase or the reproductive phase. The vegetative phase includes the germination, the seedling and the tillering stage. The germination happens with the emergence of the radicle or coleoptile from the embryo of the seed or rice grain. The seed consists mainly of the embryo and the endosperm (Figure 2).

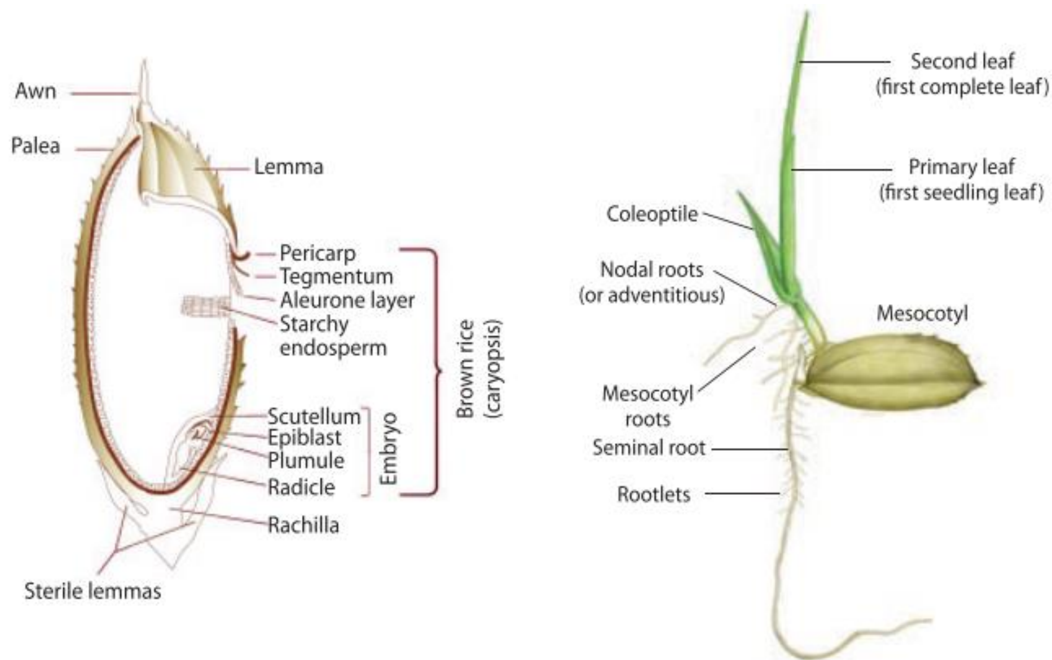


Figure 2. On the left, a hulled rice grain. The seed surface has various differentiated layers that enclose the embryo and the endosperm. On the right, the germination of a rice grain is represented. In aerobic conditions, the seminal root emerges first and later the coleoptile. Under anaerobic conditions, the coleoptile is the first to emerge, and once it has reached an aerial environment, the roots begin to develop. Retrieved from McLean et al. (2013).

After the germination, the seedling or pre-tillering phase takes place. The leaves and lateral roots develop, consuming the nutrients present in the endosperm. When the stem grows, it divides in internodal sections (internodes) limited by nodes. The internodes have varying length depending on developmental conditions, although it usually increases in the upper part of the plant. The number of nodes of a developed rice plant vary from 13 to 16. Each node has an inserted leaf sheath that extends to the immediately next superior node. These superior nodes have a leaf and a bud, which can develop as a tiller (Figure 3).

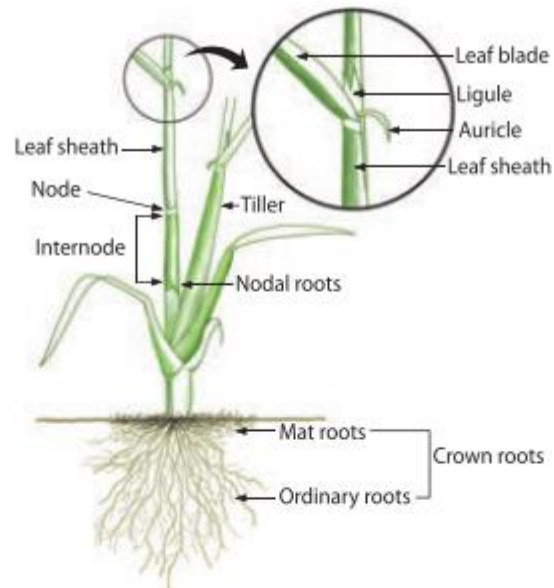


Figure 3. Parts of the stem and roots in a growing rice plant. Retrieved from McLean et al. (2013).

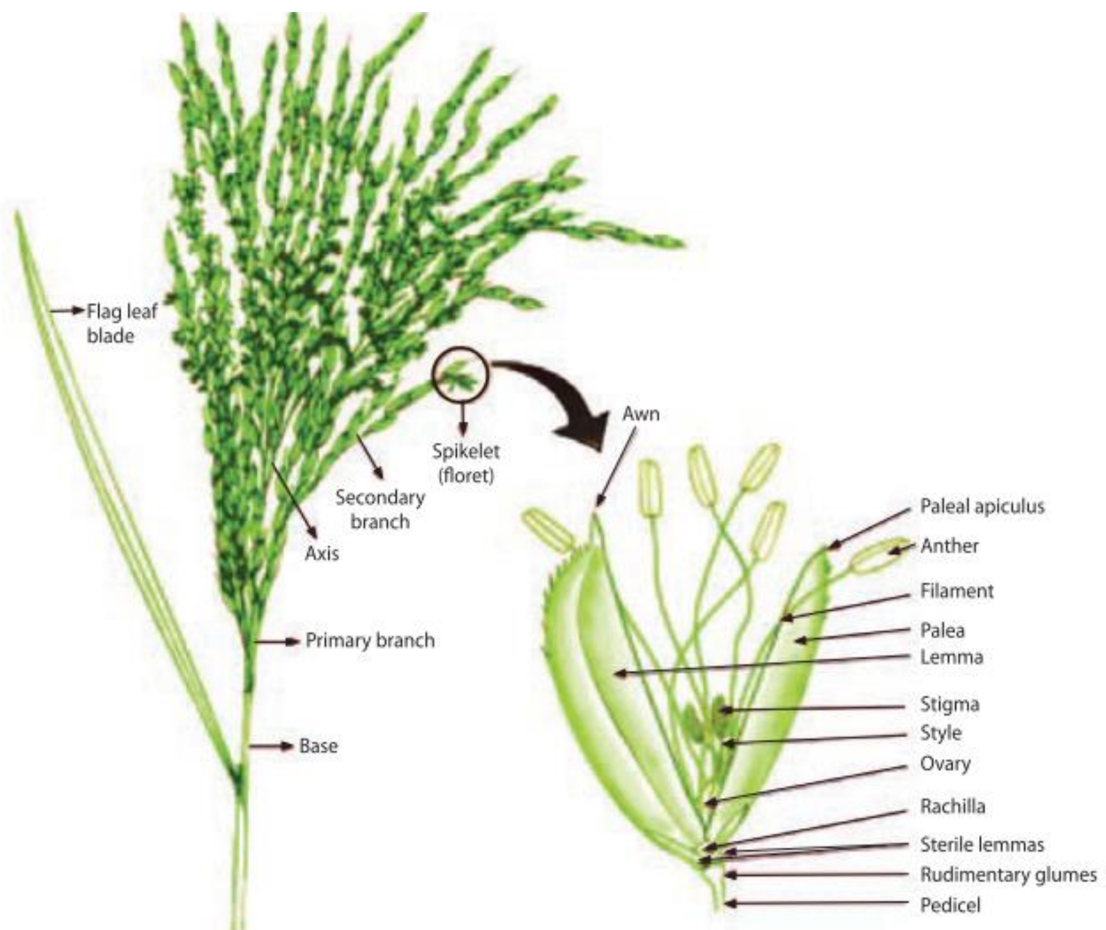


Figure 4. Panicle components, including a dissection from the spikelet or floret. Retrieved from McLean et al. (2013).

The tillering stage begins with the emergence of the first tiller from an axillary bud at a node placed in base of the plant, usually when the seedling has five leaves. Subsequently, tillers will keep emerging from upper nodes.

The reproductive phase comprehends panicle initiation, also called heading, and flowering stages. It takes place when the plant is about to reach the maximum number of tillers or when it presents its maximum tillering activity. The panicle initiation is marked by the emergence of the panicle primordium. The components of the panicle can be observed in Figure 4.

The panicle soon protrudes from the leaf sheath, in a process called heading, and can last from 10 to 14 days. At the same time, the flowering takes place, with the beginning of the anthesis. Due to the simultaneity of both processes, this phase is called indistinctly heading or flowering stage.

The anthesis initiates with the protrusion of the first anthers, that contain pollen, from the terminal spikelet or floret in the panicle. Subsequently, the self or crossed pollination and fertilization of the floret takes place. A grain begins to develop from the fertilized egg in the ovary. The rice grain endosperm is liquid in the forming seeds, and it solidifies during the ripening. This process can take from 30 to 65 days to give a spikelet full of mature grains depending on the region and temperature. Seed ripening takes place from the upper to the lower part of the spikelet, which acquires a yellowish color when maturing (Bernis & Pamies, 2006; Chang & Bardenas, 1965; McLean et al., 2013).

1.1.3. Global rice production and consumption

Rice contributes to the 20% of calories ingested worldwide (Kubo & Purevdorj, 2004), even meaning half of the calories ingested daily in some areas (Counce et al., 2000) (Figure 5).

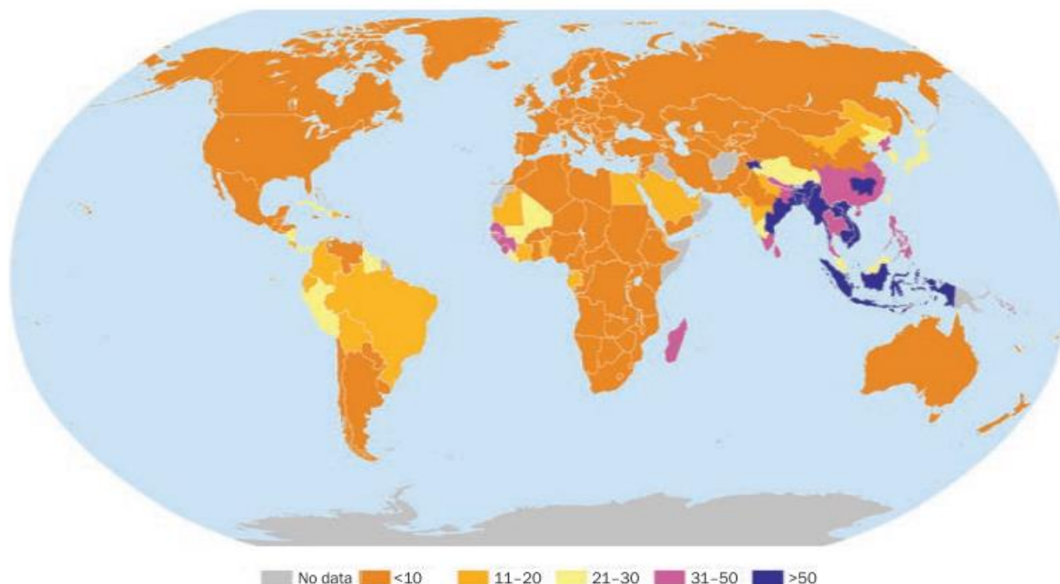


Figure 5. Percentage of calories supplied by rice in the diet. Retrieved from McLean et al. (2013).

In terms of consumption, as well as production, Asia is the main continent (McLean et al., 2013) (Figure 6). In the regions of South Asia and Southeast Asia, indica varieties are mainly cultured due to the abundance of submerged regions. In contrast, japonica

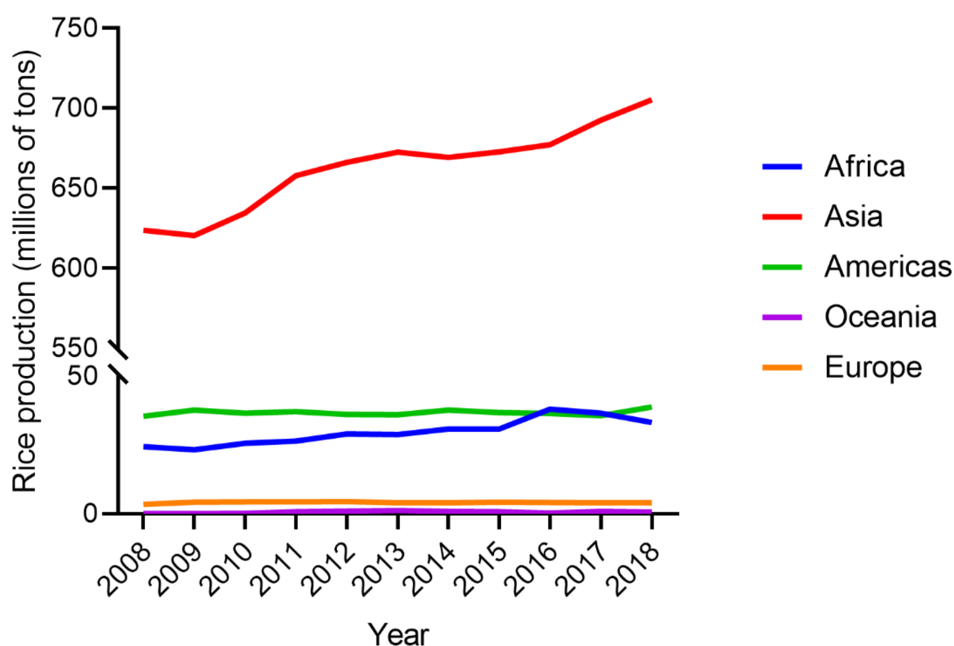


Figure 6. Rice production (paddy) worldwide in 2008-2018. Data retrieved from FAOSTAT database online.

varieties are grown in areas with less water, like northern latitudes of East Asia, elevations in South Asia and upland areas in Southeast Asia (Wei & Huang, 2019).

China and India are the main producing countries worldwide. Although India possesses more hectares of rice fields than China, China is the main producer due to its higher yield. This is because China has almost all fields irrigated in opposition to India, whose irrigation only gets to half of the paddy fields (McLean et al., 2013). In 2018, approximately 782 million tons of paddy rice were produced worldwide (FAOSTAT, 2020), with the top seven producers placed in Asia and accumulating 80% of the production (Figure 7).

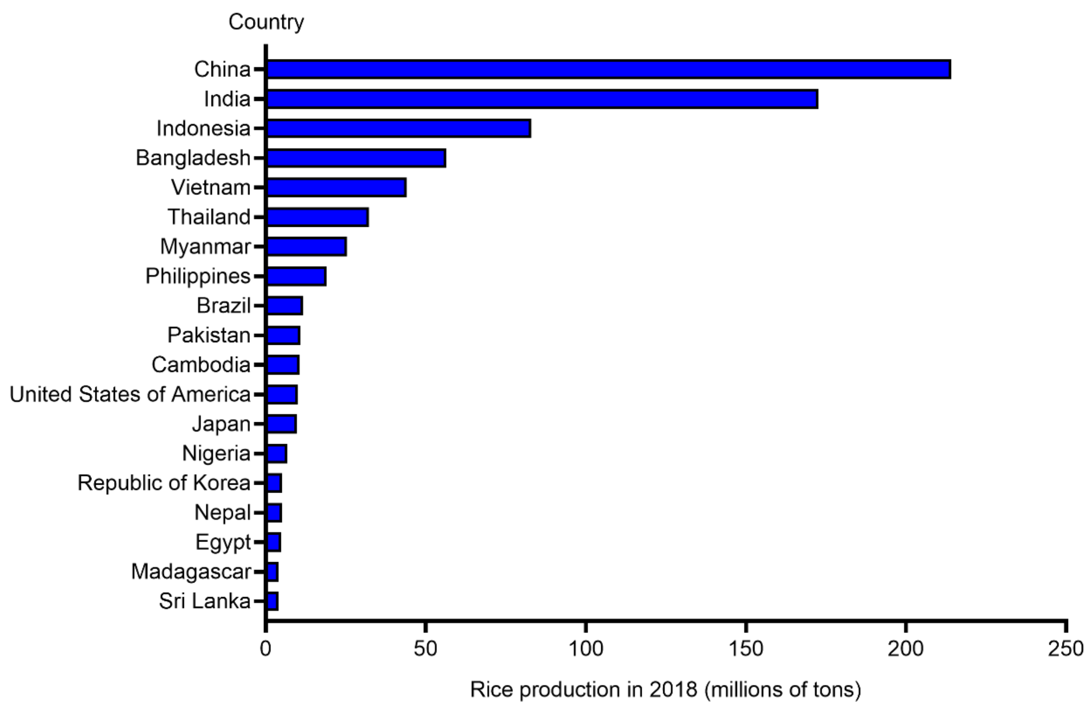


Figure 7. Top 20 paddy rice-producing countries in 2018. Spain would be placed at the 42th position with 0.80 million tons produced in 2018. Data retrieved from FAOSTAT database online.

1.1.4. Rice in Europe

The rice production and consumption in Europe is minor compared to Asia. Despite that, rice holds an important sociocultural meaning since it is one of the basic foods of the Mediterranean diet. Some regions have developed famous rice dishes, like *risotto* in Italy or *paella* in Spain. Rice in Europe is also of ecological importance, due to the great biodiversity that lives and benefits from the paddy fields.

The annual rice consumption per capita in Europe is 6-18 kg in the southern regions, and 3.5-5.5 kg in the northern regions (McLean et al., 2013). Only a few southern countries produce rice (Figure 8).

Figure 8. Main rice-producing regions in Europe. Retrieved from Kraehmer et al. (2017).

In Italy, fields are placed in the Po valley, in the regions of Piedmont, Venetia, Lombardy and Romagna. Other regions that contribute in lesser quantity to the production are Tuscany, Latium or Sardinia. Regarding Spain, the main producing regions are Andalusia, Extremadura, Valencia, the Ebro Delta and Navarra. Greece production is focused in Thessaloniki. Portugal production comes from three regions, Coimbra, the Tagus plain, and the Sado and Guadiana valleys. France obtains rice from the Rhone delta, placed in the Camargue region. Bulgaria produces rice in the Plovdiv and the Pazardzhik regions, while Romania cultures rice in the counties of Ialomiţa, Brăila, Olt and Dolj. In Hungary, paddy fields are placed in the Great Hungarian Plain (Kraehmer et al., 2017; McLean et al., 2013).

The main European producer is Italy, followed by Spain (Table 2). These two countries hold more than 75% of the total rice production in Europe. Around 70-80% of rice cultivated in Europe are japonica varieties, and the rest indica (Bernis & Pamies, 2006; Ferrero, 2007). Spain has a relative higher production of indica rice in comparison with the European average. The average yield of japonica varieties ranges between 6.5-6.7 t/ha, meanwhile is 7.1-7.8 t/ha for indica varieties (Kraehmer et al., 2017). This fact could explain why Spain holds the highest yield of all the European producing countries. Another explanation for this could be the variety improvement that has been taken place

for the past years, along with the fields treatment to avoid fungal plagues (Bernis & Pamies, 2006).

Table 2. Rice production (paddy), area harvested and yield in 2018 in Europe. Data retrieved from FAOSTAT database online.

Country	Production (t)	Area harvested (ha)	Yield (hg/ha)
Italy	1,512,241	229,545	65,880
Spain	808,167	105,012	76,959
Greece	222,690	30,350	73,374
Portugal	160,648	29,350	54,735
France	73,306	13,383	54,775
Bulgaria	63,415	11,004	57,629
Romania	43,355	8,251	52,545
Hungary	11,971	3,022	39,613

The most popular rice varieties cultured in Spain are Puntal, Bomba, Balilla x Sollana, Montsianell, Gleva, Guadiamar, J. Sendra, Argila, and Sirio. For Italy, the main cultured varieties are Arborio, Carnaroli, Roma, Baldo, Thaibonnet, Loto, Augusto, Sant' Andrea, Luna, Balilla, Centauro, Vialone nano, Padano, Lido, Crono, Sole and Selenio.
This thesis will specially focus in the study of relevant European rice varieties.

1.2 Rice menaces in the Mediterranean basin

1.2.1. Salinity, a major abiotic stress affecting rice productivity

After drought stress, salinity stress is one of the most severe abiotic stresses that decreases rice yield (Shankar et al., 2016), affecting spikelet number per panicle, grain yield and delaying panicle emergence and flowering (Negrão et al., 2011). In comparison to other cereals such as barley or wheat, rice appears to be the most sensitive crop to salinity (Maas & Hoffman, 1977; Zhu et al., 2001), although there are some landraces that have been grown in natural salinized areas worldwide that present some degree of salt tolerance (Platten, Egdane, and Ismail 2013). Salinity impact seems to vary depending on the stage of development, being the reproductive and the seedling stages more sensitive, while tillering and grain filling stages are less susceptible to salt stress (Frouin et al., 2018;

Shankar et al., 2016). Salinity in rice causes two stress phases in rice: (i) a first quick phase of osmotic stress, and (ii) a subsequent longer phase of ionic stress.

The first phase of osmotic stress is provoked by the high solute concentration present in the soil. The immediate plant reaction is a reduction of the stomatal conductance, which leads to a decrease in the transpiration rate and CO_2 assimilation. Consequently, water uptake and plant growth drop in this initial response (Munns, 2002; Munns & Tester, 2008). It has been proposed that the reduction of growth would avoid further water use by the plant and therefore, the moisture in the soil would be maintained, avoiding an increase of the salt concentration in it (Munns & Tester, 2008). It is believed that this phase is regulated by root-shoot communication routes (Frouin et al., 2018; Negrão et al., 2011) and a change of abscisic acid (ABA) concentration (Zhang et al., 2006). If the stress is mitigated with this response, the plant will reach a certain recovery and gradually will restart its growth. If not, ion stress mechanisms will activate.

An excess of Na^+ in the aerial parts of the plant can lead to chlorosis, senescence and necrosis. Therefore, as a ionic-stress response, plant mechanisms make the Na^+ ions migrate from shoots to roots through apoplastic (passively) and symplastic (actively) pathways (Frouin et al., 2018; Negrão et al., 2011), being the apoplastic via the preferential (Ranathunge et al., 2004). This Na^+ influx to roots activates some ABA-dependent responses that prevent the further uptake of Na^+, due to the activation of selective ion transporters with high affinity for K^+ and Ca^+ ions and low affinity to Na^+ ions. This also stops the movement of Na^+ from roots to shoots. Therefore, the Na^+/K^+ ratio in shoots is a good measure of salt tolerance (Frouin et al., 2018). The lower the ratio, the better the salt resistance, since it indicates the success of the regulatory mechanisms that lower Na^+ concentration in shoots.

1.2.2. The Saltol region, a major QTL for salinity tolerance

Many efforts have been put in to discover and characterize genes or quantitative trait *loci* (QTLs) that confer salt stress resistance, a complex trait. Mapping studies have been conducted over crosses previously made to search for other characters, usually japonica x indica, or after crossing known traditional salt resistant varieties (Nona Bokra, Pokkali, or varieties derived from them) with susceptible varieties (Thomson et al., 2010).

Many salt resistance QTLs have been detected this way, significantly in chromosomes 1, 4, 6 and 7, and in less quantity in chromosomes 2, 3, 5, 9, 10 and 12 (Negrão et al., 2011). The most relevant QTL involved in salt resistance is known as *Saltol*, placed in the short

arm of chromosome 1 (Platten et al., 2013). The *Saltol* QTL explains from 64.3% to 80.2% of phenotypic variation and 43% of variation in Na^+/K^+ ratio in shoots (Bonilla et al., 2002). Various authors differ in the exact position of the QTL within chromosome 1, but the most recent publication, of Nutan et al. (2017), places *Saltol* between the simple sequence repeats (SSR) markers RM1287 and RM6711 with an approximate length of 9.3-16.4 Mb.

This region contains the *OsHKT1;5* gene (Ahmadi et al., 2011), previously named *SKC1* when discovered by crossing the salt-tolerant indica variety Nona Bokra with a salt-sensitive japonica variety, Koshihikari (Ren et al. 2005), and also known as *OsHKT8* before a nomenclature consensus was established (Platten et al. 2006). Ren et al. (2005) noted that the substitution of four amino acids is what makes the gene functional in Nona Bokra when comparing it with its homologous genetic region in Koshihikari.

The *OsHKT1;5* gene encodes for a Na^+-selective transporter that keeps the Na^+/K^+ homeostasis under salt stress by unloading Na^+ from the xylem (Frouin et al., 2018; Thomson et al., 2010). The *OsHKT1;5* gene is orthologous to the *TaHKT2;1* gene from wheat (Wang et al., 1998), previously known as *TaHKT1* (Platten et al., 2006); and to the *AtHKT1;1* gene in *Arabidopsis thaliana* (Berthomieu et al., 2003; Uozumi et al., 2000), previously known as *AtHKT1* (Platten et al., 2006). A study carried on the *AtHKT1;1* gene suggests that this high-affinity potassium transporter (HKT) has eight transmembrane domains and four pore regions (Kato et al., 2001).

Another gene localized in the *Saltol* QTL is the *Sal*T gene, believed to be involved in osmotic adjustment (Claes et al., 1990), that contains a lectin-like domain (Haq et al., 2010). A pectinesterase gene has also been found within the *Saltol* region (Kumar et al., 2015; Thomson et al., 2010).

Not only genes but other coding elements such transcription factors (TFs) have been localized in the *Saltol* QTL, the so-called SalTFs, which present relevant expression changes under salt stress conditions (Nutan et al., 2017).

1.2.3. The apple snails, a major rice pest

The *Pomacea sp.* comprises the so-called apple snails. Apple snails can measure up to ten centimeters large, presenting a brownish or golden yellow shell (GISD, 2019). They are considered “amphibian” gastropods, since they have both pulmonary and gill respiration. Their egg-laying are terrestrial and massive, reaching from 300 to 800 eggs. These eggs present a characteristic appearance, with a bright pink or red color (Plaza & Galimany, 2013) and forming a clutch (Figure 9). Eggs hatch within 7-15 days, and clutches are laid every few weeks (GISD, 2019).

Figure 9. Specimen of Pomacea insularum next to a cluster of its eggs. Retrieved from CABI (cabi.org).

Apple snails are herbivores, preferring submerged environments and succulent plants (Plan et al., 2008). This is why rice is one of their preferred targets, especially in its first phenological states, from germination to tillering (Plaza & Galimany, 2013). Massive apple snail ingestion of rice can cause from 60-90% harvest loss (MINECO, 2018).

Although their wide natural habitats cover the tropical and subtropical areas of Africa, Asia and America, at least five apple snail species have been introduced to non-native areas of these continents, along with Australia and Europe since the 1980s (Yang et al. 2018). *Pomacea canaliculata* and *Pomacea maculata* (also known as *Pomacea insularum*) are known for being two extremely invasive species (Mueck et al., 2018),

included among the list of the worst 100 invasive alien species by the International Union for Conservation of Nature (IUCN, 2001).

Regarding the Iberian Peninsula, in 2009 was reported, for the first time, an apple snail invasion focus in the drainage network of left Ebro hemidelta (López, Quiñonero, & Tarruella, 2009). Since then, it has established itself as a permanent and aggressive plague in this left hemidelta, extending through 9,000 hectares of field and 400 kilometers of the drainage network. Still, in the right delta hemisphere, which takes up 11,000 hectares, some small focuses are localized every year, but they are neutralized or limited at the end of the harvest (MINECO, 2018).

At first, due to the morphological similarity of the apple snail species, the Delta-invading snail was believed to be *P. canaliculata,* but after a molecular analysis, it was identified as *P. maculata* (López et al. 2010).

Various strategies have been adopted to battle the plague expansion and try its eradication in the Ebro Delta. A first approach was the installation of metallic meshes and other structures to prevent the discharge of snails into the irrigation network and also avoid the reinfestation of the fields trough the drainage network, although it was proved insufficient. The agricultural machinery cleaning was also applied, being mandatory nowadays. The fields drainage during the winter was a strategy adopted during the first years of the plague, and it seemed to highly reduce the snail populations in the field, killing 65-99% of individuals, although the biggest specimens buried themselves and survived. Since 2016 this measure is optional, due to the associated costs since the farmers do not perceive the grants given by European Union for keeping the fields flooded during the winter, in order to maintain the migratory birds population (MINECO, 2018).

Another strategy was the fields flooding with sea water, due to the *P. maculata* sensitivity to salt, presenting high mortality with a salinity of 6.8‰ and almost total mortality above ≥13.6‰ (Ramakrishnan, 2007). The Mediterranean sea has an average salinity of 38‰ (Borghini et al., 2014). These sea water floods were kept 30 days at maximum, and then the fields were washed with fresh water. Although being very effective at first, farmers complained about the problems that caused the residual salinity in their fields due to the rice sensitivity to salt.

The application of phytosanitary products has also been used to fight the apple snail. The most used is a molluscicide called saponin, which does not eradicate the plague but minimizes its negative impact in the harvest (MINECO, 2018). Saponins are pentacyclic

triterpenoids that disrupt the snail respiration along with destroying their immune system, causing their death (Yang et al. 2018).

This plague damages rice fields worldwide, resulting in losses of tens of billions of euros a year (Balsells, 2010). The invasive apple snails that rapidly spread through north-east Spain, as mentioned, mean a threat to the main European rice production areas. Two years ago it was detected for the first time in France (Lac St. Esprit, Fréjus) (EPO, 2018), just 100 km away from Ventimiglia (Italy), being Italy the main rice producing country in Europe. The European Food Safety Authority (EFSA) considers it as one of the worst gastropod crop plagues introduced in the European Union in the recent time (EFSA, 2012, 2016), which could lead to unprecedented damages to Europe's wetlands biodiversity and rice production.

1.2.4 The NEURICE project

The NEURICE project, in which this thesis takes part, has been a Horizon 2020 project funded by the European Union. Its main objective was to develop new commercial rice varieties by incorporating salt tolerance alleles into Mediterranean varieties in order to face climate change and the present apple snail plague in the Ebro Delta (NEURICE comes from **N**ew commercial **EU**ropean **RICE**).

As it was mentioned before, the flooding of rice fields with sea water is a very efficient way to minimize the apple snail plague into the field and watering and drainage channels, although after this treatment, some degree of yield loss had been observed due to residual salinity in the following season. Considering the existing knowledge of salt tolerance QTLs, it was proposed to introduce the *Saltol* QTL into elite Mediterranean commercial varieties sown in the Ebro Delta. This way, it would be possible to easily face the apple snail plague by flooding the fields with sea water without losing rice yield, since these new varieties would tolerate the residual salinity conditions thanks to the *Saltol* region.

Two elite salt-sensitive temperate japonica varieties provided by *Càmara Arrossera del Montsià*, the long-grain PL12 and the short-grain PM37, were crossed with two *Saltol*-carrying indica donors: FL478, a salt tolerant RIL obtained from the Pokkali x IR29 cross (Thomson et al., 2010) and IR64-*Saltol*, a salt tolerant NIL obtained from the FL478 x IR64 cross (Ho et al., 2016). Both varieties were provided by the International Rice Research Institute (IRRI). The cross between PL12 and FL478 has been named LP, and the cross between PM37 and IR64-*Saltol* has been named MS.

To introduce the *Saltol* region into the elite Mediterranean varieties, a marker assisted breeding program was carried on. This program had the objective to obtain homozygous *Saltol*-carrying individuals with the highest parental return.

After the initial *Saltol*-carrying x recurrent parental hybridization, multiple backcrosses with the recurrent parental were performed to obtain the desired parental genetic load besides carrying *Saltol*. Selection of the best individuals was carried on with a polymerase chain reaction (PCR) analysis to select the *Saltol*-carrying individuals in all crosses, and a competitive allele specific PCR (KASP) analysis with validated indica/japonica markers was performed in each generation after the first backcross to check the parental genetic return. Once achieved a high recurrent parental return, a series of self-pollinations were carried out in order to obtain homozygous lines for the *Saltol* salt tolerance allele and multiply seeds for further hydroponics and field assays. The breeding program is shown in Figure 10.

This thesis focuses in evaluating salt-tolerance with the BC3F5 LP varieties in both hydroponic and field assays, and for the MS varieties, the BC3F4 varieties evaluation in field assays and the BC3F5 evaluation in hydroponic assays.

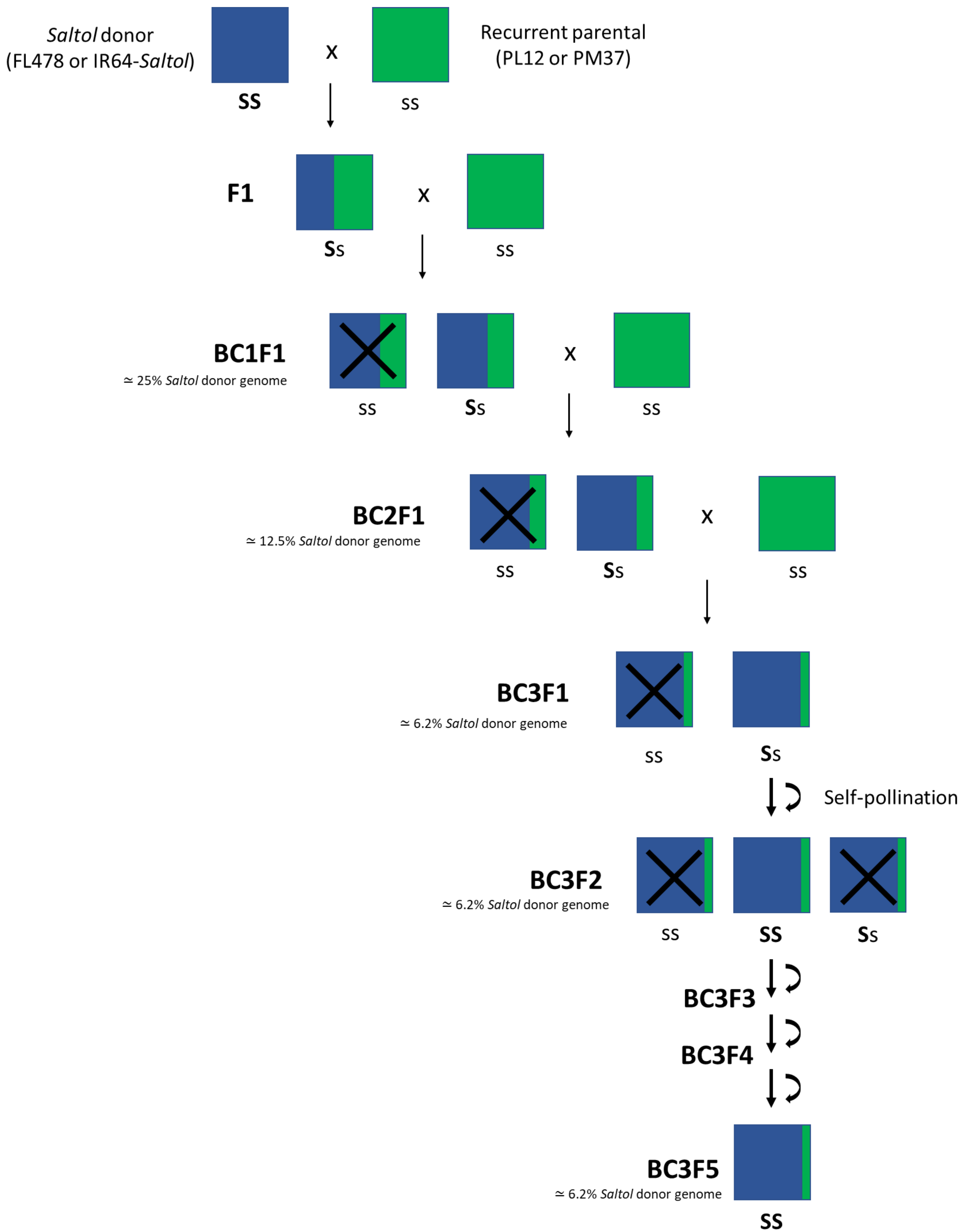


Figure 10. Breeding map for the NEURICE project. Upper case “S” refers to the *Saltol* allele, and lower case “s” refers to the absence of Saltol. Blue color represents the genome of *Saltol*-donor varieties and green color represents the genome of the recurrent parentals.

1.3 The rice pearl, a misunderstood Mediterranean character

1.3.1 Rice grain classifications and the relevance of the rice pearl

The rice grain can be classified according to different criteria. In terms of morphological measurements, the IRRI and the European Union have established grain type classifications attending the same criterion (length and the length/width ratio), but with different terminology. Both systems are shown in the following Tables 3 and 4.

Table 3. Rice grain classification attending length and ratio length/width established by IRRI (IRRI, 2002).

Grain size	Length (mm)	Grain shape	Length/Width
Short	< 5.50	Bold	≤ 2.0
Medium	5.51 to 6.60	Medium	2.1 to 3.0
Long	6.61 to 7.50	Slender	> 3.0
Very long	> 7.50		

Table 4. Rice grain classification attending length and ratio length/width established by the European Union (Faure & Mazaud, 1995).

Grain type	Length (mm)	Length/Width
Short grain	< 5.2	< 2
Medium grain	< 6.0	< 3
Long A	> 6.0	< 3
Long B	> 6.0	> 3

Another classification can be established by the visual appearance of the grain. There are completely translucent grains called crystalline, semi-opaque grains called pearled grains, and fully or almost fully opaque grains called chalky (Figure 11).

Figure 11. From left to right, a crystalline grain, a pearled grain and a chalky grain.

There is a common misconception in many scientific publications that refer to the pearled grains and to the chalky grains as chalky indistinctly (Bernis & Pamies, 2004). In this thesis, the chalky grain definition given by the European Commission will be followed. This definition establishes that chalky grains are only those whose opaque area covers at least three quarters of the grain surface (Council Regulation (EC) No 1234/2007 of 22 October 2007, 2007). Chalky grains are considered defective and unprofitable because of their undesirable appearance and low palatability (Chun et al., 2009).

We will refer to as pearl to the limited and enclosed opaque zone in the rice grains (Figure 12), as mentioned by Bernis & Pamies (2004). Depending on the position of the pearl within the grains, these can be white-core, when the pearl is placed at the centre of the grain (Kang et al., 2005), or white-belly, when the pearl is placed touching a lateral surface of the grain (Lin et al., 2016).

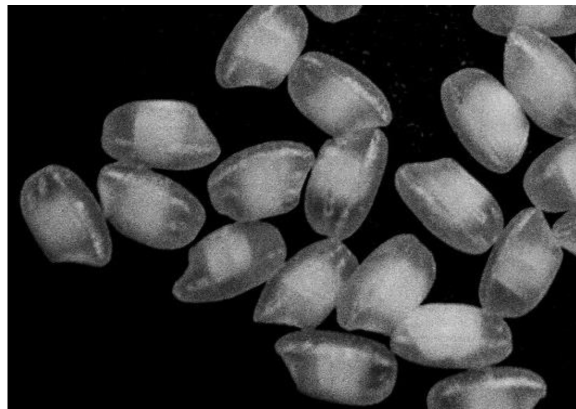


Figure 12. Pearled grains from the Montsianell variety.

The presence of pearl is considered a defect in most of the rice-consuming countries, especially in Asia, since its cooking and eating qualities do not match their standards (Cheng et al., 2005). Also, the milling process is not adapted for pearled grains, reducing the head rice recovery (HRR) (Del Rosario et al., 1968; Sreenivasulu, 2019). An exception is the use of pearled varieties for the sake production in Japan (Wan et al., 2005; Yoshida et al., 2002). Nevertheless, the rice pearl is considered an added value in Spain and Italy, since it absorbs the flavour of the rest of the ingredients (Bernis & Pamies, 2004). This is directly related to the rice recipes prepared in these countries such as *paellas* and *risottos*, where the rice is the main ingredient. If we think of how rice is consumed in India, unflavoured white rice as base to add sauces, or Japan, as a side dish, we can understand why the pearl is disregarded in these gastronomies, since the flavour of the preparation relies on the sauces and other ingredients.

1.3.2 Genetic and environmental factors associated to the rice pearl

The rice pearl has not been studied as profusely as other characters since it is not considered of interest for breeding purposes. Besides, some of the published studies and reviews confuse the concepts of rice pearl and rice chalkiness, or study mutants of non-pearled varieties instead of pearl varieties *per se*. Furthermore, most of the studies focus on Asian indica or japonica varieties, and research made with Mediterranean varieties of interest for Spain or Italy are practically inexistent. Hence, the rice pearl is a complex character to dissect.

In terms of environmental factors, it has been proved that high temperatures increase the average number of pearled and chalky grains of crystalline varieties (Sreenivasulu et al., 2015). Tsutsui et al. (2013) studied the yield of Koshihikari rice, a japonica variety, at Niigata, Japan, during 2009 and 2010. In 2009, the average ripening temperature was 24.4 ºC and especially hot, 28 ºC, during 2010. These authors found that the number of pearled grains increased significantly in 2010 in comparison with 2009 due to the temperature raise. Also, Li et al. (2011) subjected the indica cultivar 9311 to three different temperatures during the daytime ripening period, obtaining a 10% more of chalkiness in the cultivar subjected to the highest day temperature in comparison with the other two sets of temperatures.

Regarding genetics, the presence of pearl has not been linked with a single gene for all japonica and indica varieties. Instead, many QTLs and some genes have been associated with the pearl and also with chalkiness (Sreenivasulu et al., 2015). Various studies have found QTLs in diverse chromosomes with varying correlation percentage to the presence of pearl. Gao et al. (2016) identified 19 QTLs in chromosomes 1, 4, 6, 7, 9 and 12 in indica recombinant inbred lines (RILs) coming from the cross PA64s x 9311 (indica x indica-like varieties) that explained the presence of pearl with percentages ranging from 5.1% to 30.6 % of correlation. From a cross with the same varieties, Zhou et al. (2009) detected two QTLs: qPGWC-6, placed at chromosome 6, explaining the presence of pearl in a 19.18%, and qPGWC-7, placed at chromosome 7, and explaining it in a 28.18%. Liu et al. (2012) studied RILs coming from a japonica x japonica cross (Koshihikari x C602) and found three QTLs placed in chromosomes 5, 8 and 10 that, altogether, explained the presence of pearl in a 50.8% of correlation. Fine mapping has allowed to establish concrete genes associated with the presence of pearl, being many of these mutants of metabolic enzymes. Woo et al. (2008) found that a mutation in the UDP-glucose pyrophosphorylase 1 gene of the Korean japonica variety Hwacheongbyeo caused male

sterility and chalky endosperm. Mutations of the pyruvate kinase have been associated with the presence of pearl in various studies. Cai et al. (2018) found that a mutation in the plastidic pyruvate kinase isoform caused white core in the grains of the japonica variety Koshihikari. A mutation in another isoform of the pyruvate kinase was detected to cause white core in the grains of the japonica variety Zhonghua 11 by Cai et al. (2018). Kang et al. (2005) detected that the knockout of the pyruvate orthophosphate dikinase gene produced white core phenotype in the grains at lines coming from Dongjin and Hwayoung varieties. Wang et al. (2008) pointed out that the mutation of the cell-wall invertase in Zhonghua 11 caused a pearled phenotype. In the review of Sreenivasulu et al. (2015), mutations in the starch metabolism, branching and debranching and their relationship with pearl and chalkiness have been collected (Figure 14). Li et al. (2014) fine mapped the *Chalk5* gene in a study with an indica x indica cross of H94 x Zhensan97, and associated its expression with the presence of white core. *Chalk5* encodes for a vacuolar H+-translocating pyrophosphatase (V-PPase). A high expression of the gene in the grain endosperm cells causes the disruption of the pH homeostasis. This leads to an alteration of the endomembrane trafficking system, reducing the biogenesis of protein bodies (vesicular structures that carry storage proteins) and increasing the presence of small vesicle-like structures, causing ultimately the formation of a white core within the grain. A more detailed explanation can be seen in Figure 13.

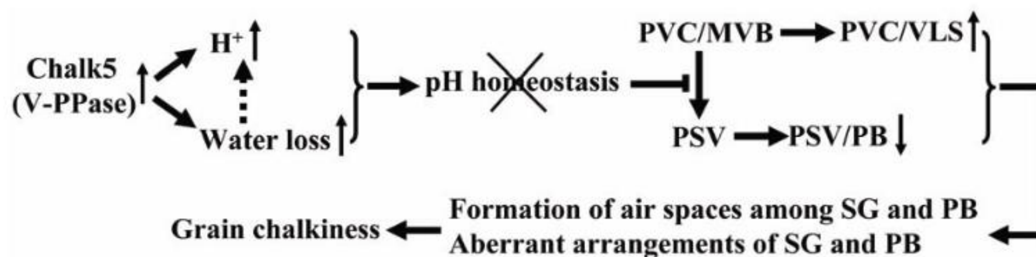


Figure 13. Mechanism through which the high expression of *Chalk5* causes the presence of white core (called chalkiness by the authors) in the rice grains. Retrieved from Li et al. (2015).

The authors also found that the expression of *Chalk5* in indica varieties varied depending on the nucleotide polymorphisms of the positions -721 and -485 in the gene promoter. The presence of a cytosine (C) and an adenine (A) in those positions, respectively, allowed for two cis elements to bind the promoter and increase the expression of the gene,

causing the white core phenotype. If both positions were occupied by a thymine (T), the cis elements were not able to bind the promoter and subsequently not promote the gene expression, causing a crystalline phenotype.

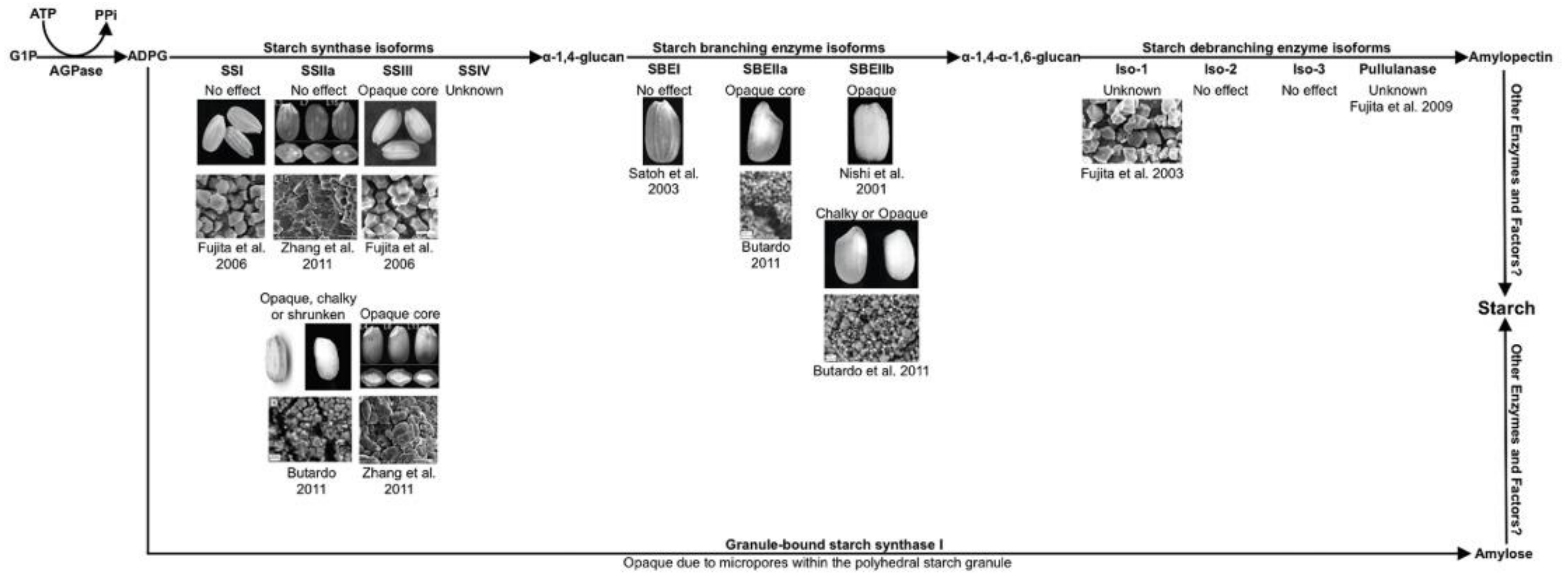


Figure 14. Mutations in the starch synthases, starch branching and starch debranching enzymes and their effects in the grain phenotypes. Retrieved from Sreenivasulu et al. (2015).

1.3.3 Physicochemical factors associated to the rice pearl

In order to expand the knowledge about the pearl character, many studies have also focused in differences in the chemical composition and physical factors between pearl parts and crystalline parts of pearled varieties, or between pearled and crystalline varieties.

Regarding the physical parameters of the grain, the degree of crystallinity has been studied in relation with the grain chalkiness. Patindol and Wang (2003) studied chalky and crystalline grains of six varieties, and found that the chalky grains had a significantly higher mean of crystallinity degree than the crystalline grains.

In terms of the rice grain composition, starch is the main component of milled rice, taking up 90% of it (Bao & Bergman, 2018; Lin et al., 2014). The starch is contained in the amyloplasts, membranous structures that occupy almost the totality of the endosperm cells. Starch is composed by linear chains of glucose molecules joined by α(1→4) bonds, called amylose; and chains of amylopectin, highly branched, composed by glucose chains with α(1→6) bonds (Zhou et al., 2002) (Figure 15).

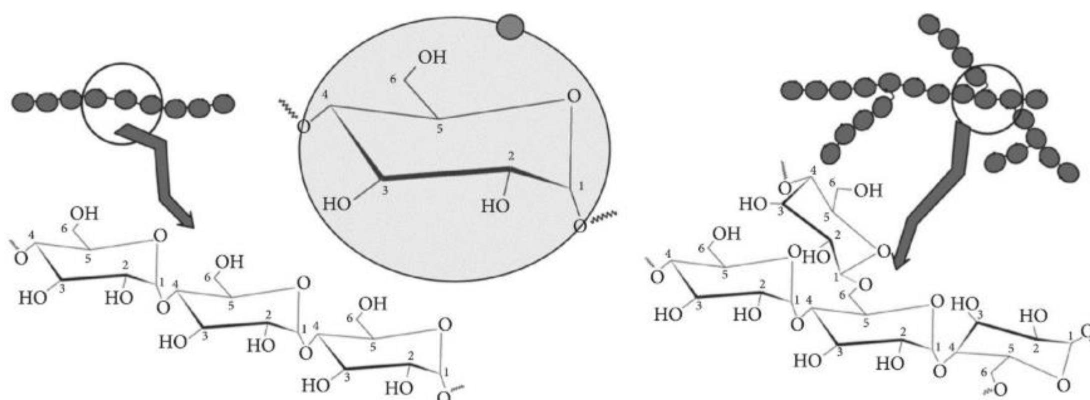


Figure 15. At the centre, circled, a molecule of glucose. At the left, amylose, and at the right, amylopectin. Retrieved from Alcázar-Alay & Meireles (2015).

Lin et al. (2016) discussed that in some studies that compare pearled/chalky grains with crystalline ones, the amylose content in the first group was usually lower, while no significant differences were found in amylose or amylopectin contents in other studies, when comparing both groups. Probably, these discrepancies are given by the already explained pearl/chalkiness confusion, and the different methodology applied. *i.e.* it is not the same to study a mutant of a crystalline variety, a chalky grain of a crystalline or pearled variety, or a pearled variety *per se* in comparison with crystalline varieties. These authors also studied the starch composition between the pearl part and the crystalline part

of a white-belly mutant form the variety DY1102 and found that the pearl fraction showed a higher amylose content than the crystalline fraction. Liu et al. (2010) compared the crystalline variety Asominori with the CSSL50-1 variety, a pearled near isogenic line derived from it. They found that the amylose content was higher in the NIL than in the parental variety. Other studies have focused in comparing the starch fractions of crystalline and chalky grains from the same variety. Patindol & Wang (2003) analysed six different cultivars and found that the amylose content was lower in the chalky grains of all the cultivars in comparison with the crystalline grains. This is consistent with the results obtained by Singh et al. (2003), that made the same comparison with other three varieties, and found that, as previously mentioned, amylose content was higher in crystalline grains than in chalky ones.

Proteins, which take up approximately a 7% of the rice grain, are also a component studied in relation to the pearl. Most of these proteins concentrated in the grain endosperm are storage proteins, like glutelins, prolamins, globulins and albumins. They are not free in the endosperm but contained in particles called protein bodies (PBs), further classified in type 1 (PB-I) and type 2 (PB-II). PB-Is measure from 1-2 µm, come from the rough endoplasmic reticulum and contain mostly prolamins. PB-IIs, also called protein storage vacuoles (PSV) measure from 2-4 µm, are vacuoles, and contain glutelins and α-globulins (Kawakatsu & Takaiwa, 2018) (Figure 16). Li et al. (2014) found that 20-days old grains of the crystalline variety H94 had more PB-I and PB-II than the same-age grains of the pearled variety Zhensan 97, while this last variety presented more vesicle-like structures, precursors of PB-II. Lin et al. (2016) found that the pearl fraction of the mutant DY1102,

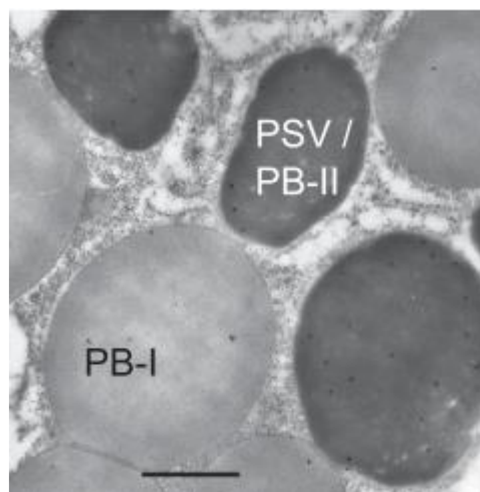


Figure 16. Both types of protein bodies (i.e. type 1 (PB-I) and type 2 (PB-II)) can be found in rice endosperm. PB-IIs are also called protein storage vacuoles (PSV). Bar: 1 µm. Retrieved from Kawakatsu & Takaiwa (2018).

in comparison with the crystalline fraction of the same grain, showed an increased content of globulins and reduced content of prolamins.
Considering all the knowledge available about the rice pearl, a section of this thesis will focus in delve into the differential parameters that Mediterranean pearled varieties have in comparison with Mediterranean crystalline varieties, opening a new line of research for European rice varieties.

1.4 Anther culture, a biotechnological tool for fast breeding

1.4.1 Doubled haploids and their applications in genetics and breeding

Doubled haploid (DH) plants are those who come from haploid cells, usually gametic cells (Davey, 2009; Forster & Thomas, 2010). These DH individuals are completely homozygous and true breeding. Therefore, they offer a wide range of applications in genetics and breeding fields.
Since the 1990s, DHs have been used in genetic studies, due to the advances in their production techniques. At first, small DH populations helped to map single major genes with pleiotropic effects that possess commercial interest and to study the segregation of some markers in barley, as a model cereal. During the 90s, DHs were also proved useful when performing the recently described bulked segregant analysis (BSA), which allowed to screen genetic markers associated with a phenotypic trait. DHs allowed to be repeatedly tested and to easily map the locus of interest, being very efficient in the location of monogenic traits (Forster & Thomas, 2010). For example, the rice yellow mottle virus resistance gene was identified by BSA thanks to two DH populations (Ghesquière et al., 1997). Nowadays, DH populations are widely available and the standard to produce genetic maps in diverse plant species. Deoxyribonucleic acid (DNA) is extracted from the DH population and screened with molecular markers in order to elaborate the genetic map. Another application of DHs in mapping is their use as homozygous parental lines, simplifying the subsequent analyses.
DHs have not only allowed to identify monogenic traits, but also to map QTLs thanks to the availability of genetic maps. A great advantage that DHs offer in this kind of studies is their replicability, as QTLs expression is influenced by the environment and requires several trials. This reproducibility also reduces greatly the timescale to map QTLs (Forster & Thomas, 2010). In rice, DHs have allowed to locate many QTLs related to

yield, grain quality, disease resistance, agronomic traits, abiotic stresses tolerance and morphology (Fan et al., 2005; Jiang et al., 2004; Kim et al., 2004; Kim et al., 2017; Lee et al., 2016; Li et al., 2005; Liu et al., 2010; Liu et al., 2007; Lu et al., 1997; Ma, Bao, et al., 2009; Ma, Yang, et al., 2009; Ren et al., 2016; Swamy et al., 2018). Thanks to expressed sequence tags (ESTs) the identification of genes co-located with QTLs coming from DH populations has become easier and more precise, entailing an advance in genomics. These studies have led to fine map rice genes involved in drought response (Wang et al., 2005), resistance to blast (Wang et al., 2001) or the target enzyme for glyphosate, the 5-enolpyruvylshikimate 3-phosphate synthase (Xu et al., 2002).

In terms of plant breeding, DHs are of interest due to the possibility to obtain homozygous lines at any generation (Hooghvorst et al., 2020). This line purity is relevant in rice since it often presents hybrid weakness (Chen et al., 2014; Mishra & Rao, 2016; Sun et al., 2017), meaning that, in contrast to a lot of plant species, hybrids present poorer agronomic traits than the pure lines. Also, as it has been exposed previously, DHs allow to develop markers that can be associated with specific agronomical traits in the interest of the breeder. In some specific cases like ornamentals, seeds from DHs can substitute vegetative propagation, since those seeds will be completely identical genetically speaking.

Traditional pedigree breeding cycles take from 10-15 years. Since the F1 generation is completely heterozygous and unique, it is impossible to carry out a selection of the best individuals until F2, and subsequent generations must be generated in order to keep the selection and test them with reproducibility. The immediate reproducibility in trials with DHs allow to shorten greatly the breeding timeline. This means a huge advantage when adapting to the rapidly changing market demands.

Besides the rapid generation of lines suitable for commercialization, DHs allow to reveal and fix interesting characters expressed by recessive genes. DHs can be commercialized themselves as cultivars, or be used as parentals, which allows a total knowledge of the progeny (Forster & Thomas, 2010). Mishra and Rao (2016) have gathered a list of rice DHs released as cultivars (Table 5).

Table 5. DH rice varieties commercialized as varieties. Retrieved from Mishra and Rao (2016).

Varieties	Characteristic	Country	Reference
Huayu I, Huayu II, Xin Xiu, Late Keng 959, Tunghua 1, Tunghua 2, Tunghua 3, Zhonghua 8, Zhonghua 9, Huahanzao, Huajian 7902, Tanghuo 2, Shanhua 7706, Huahanzao 77001, Nanhua 5, Noll, Hua 03	High yielding varieties with superior grain quality; resistant to blast and bacterial blight diseases	China	Zang (1980); Hu and Zeng (1984); Chen (1986); Loo and Xu (1986); Yang and Fu (1989)
Guan 18	Early maturity; good quality and disease resistance	China	Zhu and Pan (1990)
Huayu 15	Resistant to lodging and diseases; good quality	China	Shouyi and Shouyin (1991)
Milyang 90	Good grain quality; resistant to brown planthopper and stripe virus disease	China	Chung (1987)
Hwacheongbyeo, Joryeongbyeo, Hwajinbyeo	Resistant to brown planthopper, rice stripe tenuivirus, blast and bacterial blight	South Korea	Lee et al. (1989)
Bicoll (IR51500AC11-1)	Salt tolerant	Philippines	Senadhira et al. (2002)
Parag-401	Superior grain quality and resistant to iron chlorosis	India	Patil et al. (1997)
Risabell	High milling and cooking quality; resistant to blast	India	Pauk et al. (2009)
Janka	Drought tolerance; good grain quality	India	Pauk et al. (2009)
Abel	Cold tolerance at early stage	India	Pauk et al. (2009)
CR Dhan 10 (CRAC2221-43), Satyakrishna	Resistant to neck blast, sheath-rot and yellow stem borer	India	CRRI Annual Report (2008)
CR Dhan 801 (CRAC2224-1041, IET18720), Phalguni	Resistant to leaf blast, gall midge; moderately resistant to sheath rot, rice stripe tenuivirus, yellow stem borer, brown spot and sheath blight	India	CRRI Annual Report (2010)

1.4.2. Doubled haploids production: anther culture in rice

Doubled haploid plants are obtained from haploid cells or individuals that experiment spontaneous or forced duplication with antimitotic agents. DHs can be produced both *in vivo* and *in vitro*. The *in vivo* method is called wide crossing. It consists of pollinating an individual with pollen coming from related but different species. This way, the fertilization takes place, but at some point of the early development, due to the genetic incompatibility, the male chromosomes are eliminated and therefore a haploid embryo develops. These embryos are cultured *in vitro* and artificially doubled. Regarding the *in vitro* methods, they are based in forcing the development of female gametes (gynogenesis) or male gametes (androgenesis) under a determinate pathway. Gynogenesis consists of *in vitro* culture of ovules or ovaries, although this technique is less extended than androgenesis due to its lower efficiency. Androgenesis consists of *in vitro* isolated microspore culture or *in vitro* anther culture (Davey, 2009; Forster & Thomas, 2010).

Under normal conditions, the microspores (the first generation of male gametic cells) contained in the anthers follow a gametophytic development. In this pathway, the microspore is programmed to produce a vegetative cell and a generative cell, and after that, the generative cell gives rise to two male gametes encased in the vegetative cell. This is the mature pollen, able to carry out fertilization. The androgenesis techniques make the microspore develop under a sporophytic/embryogenic pathway instead of the gametophytic pathway when placed under stress conditions in early development stages, when the gametic cells are yet totipotent. This implies the re-programming of the microspore, which experiments a proliferation process leading to embryogenesis. The haploid embryo is developed *in vitro*, originating haploid or diploid individuals (Figure 17) (Davey, 2009; Maraschin et al., 2005; Rodríguez-Serrano et al., 2012; Serrat et al., 2014).

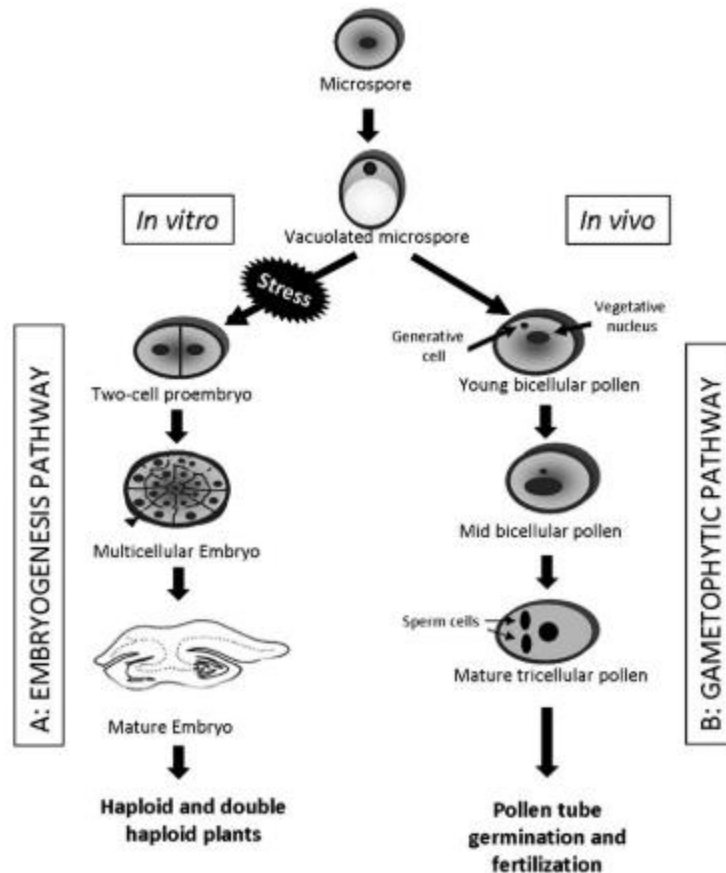


Figure 17. Embryogenesis or sporophytic pathway and gametophytic pathway that a microspore can follow. Retrieved from Rodríguez-Serrano et al. (2012).

Anther culture is the preferred and more efficient method to obtain DHs in rice. A two-step anther culture protocol for rice was first described by Niizeki and Ono (1968). In the first step, the anthers are placed in an induction medium that contains stressing growth regulators that force the haploid microspores to follow the sporophytic pathway. The microspores induce calli, which are put in a regeneration medium as the second step, where plantlets develop (Mishra & Rao, 2016). Some of these regenerated plantlets are haploid, since they come from haploid microspores, but it is also very common the spontaneous chromosome doubling of the cells during anther culture, leading to the formation of DH plantlets (Davey, 2009). The rate of spontaneous doubling in rice can reach 30-40% (Mishra & Rao, 2013). In order to increase the efficiency of anther culture, the haploid plantlets can be treated with antimitotic agents to obtain DHs (Davey, 2009; Germanà, 2011; Hooghvorst et al., 2018).

A great advantage of obtaining DHs by anther culture in rice is that the whole process takes up approximately one year, reducing drastically the duration of the breeding processes as explained previously (Serrat et al., 2014; Szarejko & Forster, 2007). Despite

this, anther culture also presents some specific problems such as a high rate of albinism in the regenerated plantlets, which is a lack of chlorophyll pigments or incomplete chloroplast membranes. Albino plants do not perform photosynthesis and eventually die before reaching maturity (Kumari et al., 2009). Some authors hypothesize that albinism is caused by a programmed cell death activated during the embryogenesis of the microspore, resulting in damage to the chloroplast DNA (Davey, 2009). The incidence of albinism is determined by the genotype, pre-treatment and culture conditions, ranging from 5% to 100% in some cases (Talebi et al., 2007). Albinism especially appears when performing anther culture in indica rice varieties (Chen, Tsay, & Huang, 1991; Silva, 2009), but also in temperate japonica ones (Serrat et al., 2014).

1.4.3. The benefits of pretreatment and media additives in rice anther culture

The donor plant material highly determines the efficiency of anther culture protocols (Mishra & Rao, 2016), therefore, they are currently optimized for each rice line. A first parameter that influences in the efficiency of anther culture is the developmental stage of the pollen. Many studies have been performed on this issue, and an extended consensus is that uninucleate and early-bicellular pollen grains are the most efficient to perform androgenesis (Germanà, 2011).

Cold pretreatment of the donor plant material has been proved to boost androgenesis, and is employed routinely in rice anther culture (Dwivedi et al., 2015; Germanà, 2011). Some of the explanations for the effectiveness of cold treatment are that it delays wall senescence, increases the symmetric divisions of pollen grains due to the reorientation of the spindle axis and causes the releasement of molecules that help androgenesis (Baenziger & Meredith, 1985; Kaushal et al., 2014; Mishra & Rao, 2013). An adequate period of cold pretreatment can reduce the incidence of albinism (Islam & Tuteja, 2012) and increase the rate of spontaneous chromosome doubling (Kasha et al., 2001).

Regarding the *in vitro* culture, N6 medium (Chu, 1981) is the most commonly used medium for anther culture, especially in cereals (Germanà, 2011). A carbon source, usually sucrose, is also necessary, due to its osmotic and nutritional effects (Powell, 1988). In some species, maltose has been reported as a better carbon source than sucrose (Singh & Singh, 2011). It also has been proved that the addition of sorbitol enhances the effectivity of the regeneration medium (Yoshida et al., 1994). Growth regulators such as auxins and cytokinins are frequently used in anther culture due to its intervention in differentiation processes. Auxins such as 2,4-dophenoxy acetic acid (2,4-D) and

naphthalene acetic acid (NAA) are frequently used in the induction media to help the formation of calli. Cytokinins like kinetin and benzyl amino purine (BAP) are useful in terms of plant regeneration (Mishra & Rao, 2016). The addition of amino acids to the media, which are related to the degeneration of wall tissues (Maheshwari et al., 1982), enhance green plant regeneration rates (Germanà, 2011; Ogawa et al., 1995). It has been proved that the presence of colchicine, an antimitotic agent, in the calli induction medium may increase the amount of regenerated doubled haploid green plantlets in some crops such as maize (Obert & Barnabas, 2004), triticale (Ślusarkiewicz-Jarzina, Pudelska, Woźna, & Pniewski, 2017) or rice (Alemanno & Guiderdoni, 1994).

Stresses can also help improving green doubled haploid plantlet regeneration (Germanà, 2011), being osmotic stress one of them (Tsukahara, 1992). It allows a greater number of cells to differentiate, since it physiologically isolates the pre-embryonic cells by disrupting the plasmodesmatal connections between them (Wetherell, 1984). The presence of high amounts of gelling agents helps to impose this stress, being Gelrite the most suitable since it does not release impurities to the medium (Calleberg & Johansson, 1996).

Another chapter of this thesis will focus on improving the anther culture technique for varieties of interest in the Mediterranean region besides performing a first try into the anther culture of some tropical varieties. This experiment has been recently published (Ferreres et al., 2019).

2. Objectives

The main objective of this thesis is to improve the performance of biotechnological techniques for Mediterranean rice varieties, as well as broaden the knowledge related to the yield and gastronomical qualities for these varieties, mainly focusing in those of interest for the Ebro Delta region.

This objective is carried on within three research areas, with the following specific objectives:

1. To improve the efficiency of the anther culture technique for two Mediterranean japonica varieties as well as taking a first approach to the anther culture of two tropical japonica varieties, by
   i. Testing the influence of colchicine in the induction media in the regeneration step and in the spontaneous duplication rate of the haploid individuals.
   ii. Testing three different regeneration media with varying concentrations of additives and its effect in the regeneration rates.
2. To select salinity-tolerant accessions of Mediterranean varieties cultivated in the Ebro Delta to use them in an integrated strategy for controlling the apple snail plague in the mentioned delta, by
   i. Performing hydroponic assays in normal and salinity conditions and collect data related to salt tolerance.
   ii. Performing field assays in normal and salinity conditions and collect data related to salt tolerance.
3. To characterize physicochemical and genetic aspects associated to the rice pearl present in Mediterranean varieties of gastronomical interest, by analyzing pearled and non-pearled Spanish and Italian varieties.

3. Materials and methods

3.1 Experiment 1: Anther culture of two temperate and two tropical japonica varieties

3.1.1 Plant material and growth conditions

Four japonica rice varieties (*Oryza sativa* spp. japonica) were used in this research. Two long grain tropical japonica cultivars coded as 303012 and 303013, one long grain Mediterranean temperate japonica coded as PL12 and a medium grain Mediterranean temperate japonica coded as Montsianell. All the seeds were provided by *Càmara Arrocera del Montsià SCCL* (Amposta, Tarragona, Spain).

The plant material was cultivated in controlled greenhouse conditions at the *Servei de Camps Experimentals* (*Universitat de Barcelona*). Four-liter flowerpots were used, filled with a specific rice substrate composed by peat moss (Floratorf Vertriebs, Oldenburg, Deutschland): vermiculite (2:1 v/v), Osmocotte Exact Standard (1 g · substrate L^{-1}) that contains 15-9-12 (N-P-K) + 2MgO + micronutrients (The Scotts Company, Washington, USA) and calcium carbonate (1 g · peat moss L^{-1}). This high amount of calcium carbonate is used to adjust the substrate pH around 6. The temperature in the greenhouse was maintained in a controlled range between 18-35 ºC. The irrigation system was automatic, and plants were manually fertilized using a growth fertilizer twice a week to avoid leaching problems.

3.1.2 Cold pretreatment

Seeds of the four parental varieties were sown weekly for two months, in order to continuously obtain tillers at the desired developmental stage. Tillers at booting stage, in which the distance from the flag leaf to the penultimate leaf auricle was between 5 and 12 cm, were daily collected from 8:00 to 9:30 a.m. (Chen et al., 1991; Serrat et al., 2014) for three months. Selected tillers were disinfected by soaking them into ethanol 70% for one minute and rinsed in distilled water. Disinfected tillers were introduced into polystyrene bags and pre-treated at 10ºC and obscurity for nine days, as suggested by Hooghvorst et al. (2018). Tillers from different varieties were maintained in separate bags.

3.1.3 Calli induction

After cold treatment, tillers were disinfected again using ethanol 70% for one minute followed by one rinse with sterile distilled water for another minute in sterile conditions. The spikelets were extracted from the foliar sheaths and selected following the criteria of Afza et al. (2000). The selected spikelets were subjected to a third disinfection process using a solution compounded by sodium hypochlorite 10% (v/v), 30 drops · L^{-1} Tween 20 and hydrochloric acid 1N (50 drops · L^{-1}) for three minutes as described by Serrat et al. (2014). Afterwards, they were rinsed three times for one minute in sterile distilled water. The spikelets were placed in sterile paper and carefully cut, dividing the filaments from the anthers. The upper part of the flower containing the anthers was held and slightly pressed using sterile tweezers, to subsequently bump it against the 90 mm Petri dish (Sterilin Limited, Newport, Wales) containing the calli induction media. Performing this precise beating, approximately a hundred anthers were sown in each Petri dish. Finally, the Petri dishes were sealed using Parafilm (Bemis, Wisconsin, USA) and incubated during 6-8 weeks in darkness at 24°C.

Two different calli induction media were assayed in this experiment: (i) a colchicine-free medium coded as 0D1 (Serrat et al., 2014) and (ii) a colchicine-supplemented medium coded as 150D1 (Hooghvorst et al., 2018). The 0D1 medium consisted of 3.957 g · L^{-1} N6 salts & vitamins (Chu, 1981), 30 g · L^{-1} sucrose, 0.25 g · L^{-1} L-proline, 1 g · L^{-1} casein hydrolysate, 1 mg · L^{-1} naphthaleneacetic acid, 2 mg · L^{-1} kinetin (kin), 0.5 g · L^{-1} 2-(N-morpholino) ethanesulfonic acid and 2.5 g · L^{-1} Gelrite. In the 150D1 medium, the same reagents and concentrations were used, but supplemented with 150 mg · L^{-1} colchicine. In both cases, the pH was adjusted to 5.8. The anthers sown in 150D1 were transferred to 0D1 after 24 hours. The anthers were maintained in the 0D1 induction medium for a maximum of three months with a two-week medium change.

3.1.4 Plantlet regeneration

When a callus of *ca.* two millimeters in diameter emerged from a microspore, it was transferred to a regeneration medium and the anther forming the callus was removed to avoid data overestimation. This way a unique callus was obtained from each anther. A similar amount of calli from each variety was transferred to each regeneration medium. Those calli were protected from light by using filter paper for 24 hours to reduce the initial light stress shock. After this period, calli were settled at 24 ± 1°C and 50-70 µmol · m^{-2} · s^{-1} fluorescent tube light intensity under a 18h/16h light/darkness photoperiod. Every four

weeks, calli were transferred to the corresponding fresh regeneration medium for up to three months.

Three different plantlet regeneration media were assayed in this step: (i) a reference regeneration medium named XACRM, previously defined by Serrat et al. (2014), (ii) a sorbitol-supplemented XACRM medium (which was named SARM) and (iii) a Gelrite-enriched XACRM medium (which was named GERM). The reference XACRM medium was composed by 3.957 g · L^{-1} N6 salts & vitamins (Chu, 1981), 30 g · L^{-1} sucrose, 0.25 g · L^{-1} L-proline, 1 g · L^{-1} casein hydrolysate, 1 mg · L^{-1} naphthaleneacetic acid, 2 mg · L^{-1} kinetin, 0.5 g · L^{-1} 2-(N-morpholino) ethanesulfonic acid and 3 g · L^{-1} Gelrite. SARM was supplemented with 20 g · L^{-1} sorbitol and GERM 4.5 g · L^{-1} Gelrite. Finally, the pH was adjusted to 5.8 in all media.

Green little plantlet masses regenerated from calli were transferred to hormone-free MS based medium (Murashige & Skoog, 1962) described by Serrat et al. (2014) known as root medium (RM). All regenerated albino plantlets were discarded in this study. The composition of the RM medium was 4.4 g · L^{-1} MS salts & vitamins (Murashige & Skoog, 1962), 30 g · L^{-1} sucrose, 0.5 g · L^{-1} MES and 2 g · L^{-1} Gelrite, with the pH adjusted to 5.8. A plantlet propagation was carried out 21 days after the transference to RM tubes to avoid any chimeric plantlet having two or more ploidy levels. Ploidy analyses were performed for each separate plantlet.

3.1.5 Ploidy determination by flow cytometry

Bead beating trituration procedure was used to extract nuclei from approximately 10 mg of fresh leaf and sample preparation was carried out as proposed by Hooghvorst et al. (2018). Every sample was analyzed by using a GalliosTM flow cytometer (Beckman Coulter, Indianapolis, United States) provided by *Centres Científics i Tecnològics de la UB* (CCiTUB). This cytometer was equipped with a high-throughput sampler (HTS) and a 488 nm laser that excited the propidium iodide. Samples with a defined peak in the channel number as the reference diploid samples were defined doubled haploids, whereas those producing half the fluorescence were defined as haploids.

3.1.6 Statistical analysis

To determine the efficiency of the different anther culture steps, some parameters were calculated for each variety, such as the percentage of induced calli (number of anthers producing calli · 100 · number of sown anthers $^{-1}$), percentage of regenerated plantlets (regenerated plantlets · 100 · number of total calli $^{-1}$), ratio calli regenerating green

plantlets / calli regenerating albino plantlets (number of calli regenerating green plantlets / number of calli regenerating albino plantlets), percentage of green plantlets regeneration (number of green plantlets · 100 · number of induced calli $^{-1}$) and percentage of green doubled haploid plantlets (number of green doubled haploid plantlets · 100 · number of non-contaminated green plantlets^{-1}). To determine significant differences in the first two parameters, a chi-squared test (χ^2 test) was assayed with 95% confidence with Minitab 17 Statistical Software (Minitab Inc., State College, PA, United States).

3.2 Experiment 2: Testing the *Saltol* region in hydroponic assays and in fields

3.2.1 Plant material

PL12 and PM37 temperate japonica rice varieties (*Oryza sativa* spp. japonica) provided by *Càmara Arrossera del Montsià SCCL* (Amposta, Tarragona, Spain) were previously introgressed with the *Saltol* salt-tolerance chromosomic region described by Thomson et al. (2010) using FL478 and IR64-*Saltol* (*Oryza sativa* spp. indica) rice varieties respectively as trait donors, coming from the IRRI seed bank. LP varieties are those derived from PL12-*Saltol* introgressed lines, while MS varieties are derived from PM37-*Saltol*-introgressed lines.

For the timepoint assay, two parental varieties (PL12, FL478) and two PL12-*Saltol* introgressed varieties selected upon seed availability (LP5, LP8) were employed.

For the *Saltol*-introgressed varieties testing experiment, parental varieties from both introgression lines were used (PL12, FL478, PM37, IR64-*Saltol*) along with the maximum number of LP and MS varieties, selected upon the *Saltol* region integrity, return to the recurrent parent and seed availability. Nineteen MS varieties (MS2, MS3, MS4, MS5, MS6, MS7, MS8, MS9, MS11, MS12, MS14, MS16, MS17, MS18, MS20, MS21, MS23, MS25, MS26) and 23 LP varieties (LP1, LP2, LP3, LP4, LP6, LP7, LP9, LP10, LP11, LP12, LP14, LP15, LP16, LP17, LP18, LP20, LP21, LP23, LP24, LP26, LP28, LP29, LP35) were assayed.

3.2.2 Hydroponic assays

Rice seeds were dehulled manually and submerged in ethanol 70% for 1 minute, rinsed, and submerged for 30 minutes in sodium hypochlorite 30% with 8 drops of Tween 20 · L^{-1}, both processes in agitation, to sterilize the seeds. Then, the seeds were rinsed 3 times using sterile distilled water in sterile conditions, and after, they were cultured in Petri

dishes with sterile wet filter paper. The dishes were left for a week in a culture chamber at 25 ± 1 °C under a 18h/16h light/darkness photoperiod, with a 100 -130 µmol · m^{-2} · s^{-1} fluorescent tube light intensity to make the seeds germinate.

The timepoint assay was performed at *Centre for Research in Agricultural Genomics* (CRAG, Bellaterra, Barcelona, Spain), and the testing of the *Saltol*-introgressed varieties at *Servei de Camps Experimentals* at the University of Barcelona (Barcelona, Spain). The assays were performed in greenhouse conditions (28 ± 2 °C during the day and 24 ± 2 °C during the night), and a 14h/10h light/darkness photoperiod. Plants were placed randomly in 10-liter containers filled with modified Yoshida solution (Yoshida et al., 1976) (Table SM 3). They were allowed to grow for 7 days in the nutritive solution. After that, the Yoshida solution was renewed in the control containers, while 80 mM NaCl-supplemented Yoshida solution was added to half of the containers. For the timepoint establishment experiment three replicates for each variety and condition were collected. A replica consisted in 50 mg of fresh leaves from different plants. The timepoints established to collect the leaves were 1, 2, 4 and 7 days after salinization. At the following hydroponic assays replicates were designed the same way, and the leaves were collected at the optimal timepoint established.

3.2.3 Relative chlorophyll content analysis

The relative chlorophyll content at the hydroponic assays was measured at 1, 3, 5 and 7 days after salinization of the media with a Chlorophyll Meter SPAD-502Plus (Konica Minolta, Tokyo, Japan). The oldest leaf of each plant was selected for the measurement, and five replicates per variety and treatment were analyzed.

3.2.4 SES score evaluation

The standard evaluation system (SES) established by IRRI (2002) was used to evaluate salt tolerance (Table 6). This visual evaluation was performed 1, 3, 5 and 7 days after the salt treatment, with at least five replicates per variety.

Table 6. Standard evaluation system (SES) established by IRRI, which determines salt tolerance with a ranging score based on the visual damage in the rice plant.

Score	Symptom/observation	Degree of tolerance
1	Normal plant growth, only the old leaves show white tips with no symptoms on young leaves	Highly tolerant
3	Near normal growth, but only leaf tips burn, a few older leaves become partially whitish and rolled	Tolerant
5	Growth severely retarded, most old leaves severely injured, a few young leaves elongating	Moderately tolerant
7	Complete cessation of growth, most leaves dried, only few young leaves still green, some plants dying	Sensitive
9	Almost all plants dead or dying	Highly sensitive

3.2.5 Field assays

Field assays were conducted at the experimental fields from non-salinized *Càmara Arrossera del Montsià* field (40°42'18.6"N 0°38'15.1"E), and salinized Vascos field (40°45'28.2"N 0°47'17.6"E) (Figure SM 1), both placed at the Ebro Delta.

The varieties assayed were selected upon previous salinized and non-salinized hydroponic assays, field trial assays and seed availability. Parental varieties for both introgression lines were included (PL12, FL478, PM37 and IR64-*Saltol*). For the LP family, 24 varieties were assayed (LP1, LP2, LP3, LP4, LP5, LP6, LP7, LP8, LP11, LP12, LP13, LP14, LP16, LP17, LP20, LP21, LP22, LP24, LP25, LP26, LP27, LP28, LP29, LP30, LP31) and 8 varieties for the MS family (MS3, MS12, MS14, MS17, MS21, MS23, MS25, MS28).

Plants from all the varieties were germinated in multi-pot trays and then transplanted into the fields at four leaves stage. In both salinized and non-salinized fields, plants were arranged in lines, with 20 plants from the same variety per row. Four replicates of each variety were randomly placed in each field. The separation between rows was 50 cm, while the distance between plants in the same row was 20 cm. Four samples of each variety were collected 15 days after field transplantation, each sample consisting of the tip of three leaves coming from one plant. These collected samples weighed an average of 100 mg approximately.

At the end of the season, yield data of these varieties was also collected and expressed as kg production / hectares.

3.2.6 Na^+/K^+ analysis

The collected samples from the hydroponic and field assays were dried at 70 °C for 3 days at an oven. Dried samples were introduced in 2 mL microcentrifuge tubes along with 3 crystal balls, and grinded at 120 Hz at the TissueLyser II (QIAGEN, Venlo, Netherlands) for 2 minutes to obtain a fine powder. Each sample was weighed and placed in a Teflon reactor previously cleaned with HNO_3. Then, 2mL of HNO_3 and 1 mL of H_20_2 were added to each reactor. Afterwards, the reactors were hermetically closed and placed at an oven overnight at 90 °C. The next day, 30 mL of ultrapure H_2O were added to each reactor and the content was homogenized by agitation. Ten mL of this solution were placed in tubes to be analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) with an Optima™ 8300 (PerkinElmer, Massachusetts, United States) by *Centres Científics i Tecnològics de la UB* (CCiTUB) (Barcelona, Spain).

3.2.7 Statistical analysis

All the statistical analyses were performed with Minitab 17 Statistical Software (Minitab Inc., State College, PA, United States). In order to determine significant differences between the SPAD scores and between the Na^+/K^+ ratio of control conditions and salinized conditions, the normality of the data was assumed, and a t-student test was assayed with 95% of confidence. A one-way ANOVA was assayed with a 95% confidence in order to determine if the control and saline SPAD measurements were variety dependent. The Spearman test was applied to analyze the correlation between the measured parameters under salinity conditions for the Na^+/K^+ ratio measurements in both LP and MS varieties. The Pearson correlation coefficient was calculated to establish the relationship between yield and Na^+/K^+ ratio measurements.

3.3 Experiment 3: Studying the rice pearl in Mediterranean varieties by different approaches

3.3.1 Plant material

In this study, dehulled and polished rice grains from five different Mediterranean japonica rice varieties (Montsianell, PL12, Guadiamar, Bomba and Carnaroli) were used along with chalky grains from each variety. The first four varieties were provided by *Càmara Arrossera del Montsià* (Amposta, Spain), while the last was provided by *Società Italiana de Sementi* (San Lazzaro di Savena, Italy). Montsianell, Bomba and Carnaroli present

pearled grains, meanwhile PL12 and Guadiamar present crystalline grains. Three biological replicates of each group were used in all experiments except for the physical measurements.

3.3.2 Physical measurements

Weight, area, perimeter, length, width and L/W relation were determined by examining 100 grains of each variety. To determine the average weight, the samples were weighted in a precision scale in groups of 10. To determine the rest of measurements, the grains were photographed in groups of 20 over a translucent surface with a light source using a mounted camera. The images were processed at the Image Analysis department at *Centres Científics i Tecnològics de la UB* (CCiTUB) (Barcelona, Spain).

3.3.3 X-ray diffraction

For this experiment, whole grains and chalky grains form each variety were used. In the case of pearled varieties, they were classified in three groups according to the pearl area results obtained in the physical parameters study: i) grains with small pearl, ii) grains with large pearl and iii) chalky grains. The small pearl grain was defined as having between a 10-25% of pearl area and a large pearl grain having a 30-50% of pearl area, depending on the variety studied. For the crystalline varieties only two groups were made: whole grains and chalky grains.

X-ray diffraction patterns were obtained with a PANalytical X'Pert PRO MPD Θ/Θ powder diffractometer (Malvern Panalyitical Ltd, Malvern, United Kingdom) equipped with a copper anode X-ray tube. The diffractometer was used in a convergent beam configuration with a focalizing mirror and a transmission geometry of approximately cylindrical capillary samples. The diffractometer was operated 40 mA and 45 kV and scanned over a diffraction angle (2θ/θ) range of 4-50º, at a step size of 0.039º and a step time of 350 seconds per step. Whole grain samples were placed on the goniometric head of the capillary sample stage, acting as a capillary.

Crystallinity indexes of the samples were calculated using the data analysis software HighScore (Degen et al., 2014) on the 7º-28º area of the different X-ray diffractograms. Each sample crystallinity index was calculated as the proportion of crystalline peak area to the total diffraction area. The crystalline peak area was established using the automatic constant background determination tool with a bending factor and granularity parameters of 0 and 30, respectively. The total diffraction area was found using the manual constant

background function, maintaining the default points only at both edges of the diffractogram.

3.3.4 Scanning electron microscopy (SEM)

Normal and chalky grains of each variety were cut transversally in half with a scalpel and coated with silver under vacuum conditions. The grains of each variety were observed with a JSM-7001F Scanning Electron Microscope (JEOL, Japan) at an accelerating voltage of 5 kV at *Centres Científics i Tecnològics de la UB* (CCiTUB) (Barcelona, Spain).

3.3.5 Amylose content determination

Amylose content determination was performed using flour coming from different grain fractions depending on the variety. The flour was obtained by pulverizing the samples in a mill, the Tissue Lyser II (QIAGEN, Venlo, Netherlands) at 120 Hz for 60 seconds.

For the pearled varieties, the quantification was performed in four different fractions: whole grains, chalky grains, the grain crystalline part and the pearl one, these last two obtained by cutting the grains with a scalpel. For crystalline varieties, since they present uniform grains with no distinguishable parts, only whole grains and chalky grains were used.

Amylose content of the different samples was determined using a colorimetric method adapted from Hovenkamp-Hermelink et al. (1988). This method is based on the extraction of starch from rice flour and the determination of a chromophore formed between amylose and a I_2-KI. Starch extraction was carried out by gently mixing 100 mg of a rice flour sample with 1 mL of 95% (v/v) ethanol and 9 mL of 1 N NaOH (v/v) in a 100 mL volumetric flask. Fifty mL of distilled water were then added to the mixture and left resting for 30 minutes. The uncovered flask was heated at 85-90 ºC for 5 minutes in a water bath with manual agitation and cooled down for 20 minutes at room temperature. Finally, the flask was filled up to 100 mL with distilled water and mixed three times by inversion.

To perform the colorimetric determination, 250 µL of the flask solution were transferred to tubes filled with 2.6 mL of distilled water. Then, 50 µL of 1 M acetic acid, 100 µL of I_2-KI (0,2% (w/v) and 2% (w/v), respectively and 2 mL of distilled water were added. The solution was mixed by inversion three times and incubated 2 hours at room temperature under dark conditions. After that, the sample was analyzed at a Cecil Aquarius CE 7460 spectrophotometer (Cambridge, United Kingdom) to determine its

absorbance at 550 and 618 nm. Blanks for the colorimetric determination were prepared as the rest of the samples without the addition of flour.
Finally, the percentage of amylose content was calculated using the following equation:

$$\%Amylose = \frac{33.2 * A618 - 22.8 * A550}{1.29 * A550 - 0.68 * A618}$$

3.3.6 Total protein extraction

For this experiment, the same flour fractions used for the amylose determination experiment were used, three replicas per variety and fraction. Protein extraction from the samples was performed following an adapted method from the one described by Lin & Wang (2016).
In a 50 mL tube, 500 mg of sample flour were mixed with 140 µL 40 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mL of extraction buffer, composed of 0.7 M sucrose, 0.5 M Tris-base, 50 mM EDTA (pH 8.0), 0.1 M KCl (w/v), 5 mM HCl (v/v) and 0.238 M β-mercaptoethanol in miliQ water, and then vortexed during 1 minute. Next, the mixture was centrifuged at 15,000 rpm (4 ºC) for 20 minutes, and the supernatant transferred to another fresh tube. Three times the volume of cold 0.1 M NH_4OAc, 10 mM β-mercaptoethanol and methanol (precipitation buffer) was added to the recovered phenol phase and, after being mixed by inversion, it was left incubating overnight at -20 ºC to precipitate the proteins. The next day, the samples were centrifuged at 15,000 rpm (4 ºC) for 20 minutes and the supernatant was discarded. The pellet was washed twice with 1.8 mL precipitation buffer, recovering the pellet by centrifugation at 8,000 rpm for 5 minutes. Another rinse using 1 mL of cold 100% acetone with 0.7 µL of 14.3 M β-mercaptoethanol was performed, also recovering the pellet by centrifugation at 8,000 rpm for 5 minutes. Then, the pellet was dried in an Eppendorf 5301 Concentrator SpeedVac system (Eppendorf, Hamburg, Germany) for about 2 or 3 minutes and resuspended in 1.5 mL of lysis buffer, made of 9.5 M urea, 40 mM tris-base and 15 mM DTT in distilled water. Finally, the protein concentration of these extracts was calculated using the Bradford method.

3.3.7 Protein separation and determination

Protein separation was performed by SDS-PAGE gels using an adapted method from the one originally described by Laemmli (1970). The polymerization support (Bio-Rad

Laboratories, Hercules, CA, United States) was cleaned with 70% ethanol and assembled with the glasses. Then, the reagents for the stacking (6% acrylamide) and resolving (15% acrylamide) gels were mixed except for the polymerizing agent (TEMED). Eight µL of TEMED were added to the resolving gel mixture and 4 mL of it were added to the polymerization support. While the gel was polymerizing, 500 µL of isopropanol were poured over it to ensure its polymerization in a regular and leveled way. Once polymerized, the isopropanol was removed by decantation. After adding TEMED to the stacking gel mixture, it was poured to the support until it reached the end of the glasses and the comb was carefully inserted to ensure no bubbles remained in the wells. When the stacking gel solidified, the comb was removed, and the glasses with the gels taken from the support and placed in the electrophoresis tray. Next, the tray was filled with enough running buffer.

The samples, three per variety and fraction, were mixed 1:1 with the loading buffer (buffer and β-mercaptoethanol in a 95:5 proportion). The volume of each sample was determined according to its concentration, so the same amount was loaded in each lane (*ca.* 20 µg). They were then heated at 95ºC for 5 minutes and immediately placed on ice. Finally, the samples were loaded in the wells and the gel ran at a constant amperage of 40 mA until the front reached the end of the gel. Then the gel was placed in a container with enough staining solution to cover the whole gel (50% methanol, 10% acetic acid, 0.25% (w/v) Coomassie blue R-250 in MiliQ water) and left in agitation for about 2 hours. Then, the solution was discarded and replaced with destaining solution (30% methanol, 10% acetic acid in MiliQ water) leaving the gel in agitation overnight. The destaining solution was renewed the next day during a few hours until the gel turned translucent and the bands could be clearly seen. Finally, the gels were photographed.

Quantification of SDS-PAGE bands was performed using ImageJ (Schneider et al., 2012). The previously obtained gel images were subjected to a color inversion and the integrated density of each relevant band and the whole lanes was calculated. The percentage content of each protein fraction was calculated as the proportion between that fraction integrated density and the whole lane integrated density.

3.3.8 Chalk5 promoter analysis

Seeds of each variety were sown in multi-pot trays at the greenhouse. When the plants reached the fourth leaf stage, two-centimeter pieces were collected and placed in 2 mL microcentrifuge tubes along with three glass balls. These tubes were frozen by

submersion in liquid nitrogen and grinded using a TissueLyser II (QIAGEN, Venlo, Netherlands) at 120 Hz for one minute to obtain a fine powder.

The extraction buffer (100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% MATAB, 1% PEG 6000 and 0.5% sodium sulphite) was added (0.9 mL per each microcentrifuge tube). The tubes were incubated during one hour at 74°C in a dry bath incubator (Lan. Technics, Navarra, Spain) placed at an orbital shaker Unimax 1010 (Heidolph, Schwabach, Germany) at 80 rpm to maintain agitation. The tubes were allowed to cool and 0.9 mL of chloroform:IAA (24:1) were added. Tubes were centrifuged at 13,000 rpm for 5 minutes. Each supernatant was transferred to fresh 2 mL microcentrifuge tubes, and 5 µL of RNAse A were added. The tubes were incubated at 37 °C for 30 minutes. Afterwards, 1 mL of chloroform:IAA was added, and the tubes were centrifuged at 13,000 rpm for 5 minutes. Each supernatant was transferred to fresh 1.5 mL microcentrifuge tubes and 0.6 mL of isopropanol were added. The tubes were placed in the freezer for twenty minutes and centrifuged (13,000 rpm for 5 minutes), and the supernatant was discarded by inverting the tube. Then, 0.4 mL ethanol 70% and 10 µL sodium acetate 3M were added. The tubes were again centrifuged at 13,000 rpm for 5 minutes and the supernatant was discarded by inverting them. The pellet was dried overnight, and the DNA was resuspended in 100 µL of sterile miliQ water in agitation for two hours. Quantity and purity of the extracted DNA was checked with the NanoDrop One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Massachusetts, United States).

In order to sequence the promoter region of the studied varieties to search for polymorphisms in the -721 and -485 positions, two primers were designed to amplify a region that included both positions with Genome Complier v.2.2.88 using the Zhensan97 *Chalk5* sequence (NCBI accession KJ363317.1). The forward primer sequence was CTTACCACAGGAAGCAAAACC and the reverse primer sequence was TTGAGCACTGAACAAACCAT. The predicted amplicon size was 849 pb. PCR was carried out by using PAQ5000 polymerase (Agilent, Santa Clara, CA, United States) following the manufacturer instructions. PCR protocol was 94 °C for 2 minutes, followed by 35 cycles of 94 °C for 25 seconds, 58 °C for 30 seconds and 72 °C for 35 seconds and a final extension at 72 °C for 7 minutes. The PCR products were purified with the ExoSAP-IT Express PCR Product Cleanup (Thermo Fisher Scientific, Massachusetts, United States) and sequenced by Sanger's method by the Genomic platform of the CCiT-UB (Barcelona, Spain). The amplicon sequences obtained were aligned with the reference

Zhensan97 sequence by using T-Coffee (Notredame, Higgins, & Heringa, 2000). The alignment was represented with the use of ESPript 3.0 (Robert & Gouet, 2014). From three to five sequences of each variety were analyzed to obtain a consensus sequence at the studied positions.

3.3.9 Statistical analysis

All statistical analyses for physical measurements, X-ray diffraction and amylose and protein determination was performed with Minitab 17 Statistical Software (Minitab Inc., State College, PA, United States). Data sets were subjected to a one-way analysis of variance (ANOVA) with 95% confidence, and significant differences were established by using a Tukey post-hoc analysis. Data sets lacking variance homoscedasticity were subjected to a Welch ANOVA with 95% confidence and significant differences were established by using a Games-Howell post-hoc analysis.

4. Results

4.1 Experiment 1: Anther culture of two temperate and two tropical japonica varieties

4.1.1 Anther sowing and calli induction

For Montsianell and PL12 temperate japonica varieties, 6,029 and 4,470 anthers were sown respectively. Regarding tropical japonica varieties, 10,866 anthers were sown for 303012 and 8,814 for 303013 varieties, respectively. A different number of calli was obtained depending on the assayed varieties and media. Calli induction number and percentages depending on variety are shown in Table 7.

Table 7. Total anther culture parameters. IC: induced calli, RP: regenerated plantlets, CRGP: calli regenerating green plantlets, CRAP: calli regenerating albino plantlets, RGP: regenerated green plantlets, RDHGP: regenerated doubled haploid green plantlets.

Variety	Sown anthers	No of IC (%)	No of RP (%)	Ratio CRGP/CRAP	No of RGP (%)	No of RDHGP (%)
Montsianell	6029	334 (5.54)	243 (72.75)	0.42	1000 (2.99)	313 (37.53)
PL12	4470	71 (1.59)	22 (30.99)	0.29	78 (1.10)	0 (0.00)
303012	10866	42 (0.39)	2 (4.76)	1.00	28 (0.67)	7 (0.43)
303013	8814	35 (0.40)	5 (14.29)	0.25	0 (0.00)	0 (0.00)

Regarding the induction media, in temperate japonica varieties, 0.35% of the Montsianell anthers induced calli in 0D1 medium, while 7.57% of them induced in 150D1 medium. For PL12 variety, 4.32% of the anthers were induced in 0D1 while 0.94% induced calli in the 150D1 medium. For the tropical japonica varieties, 303012 induced calli from 0.12% of the anthers in 0D1 medium and 0.47% in 150D1 medium. Similarly, in 303013, 0.14% anthers induced in 0D1 medium and 0.49% in 150D1 medium (Figure 18). Statistical analysis showed that there were significant differences in calli induction in relation to the media ($p=0.000$) (Table SM 1).

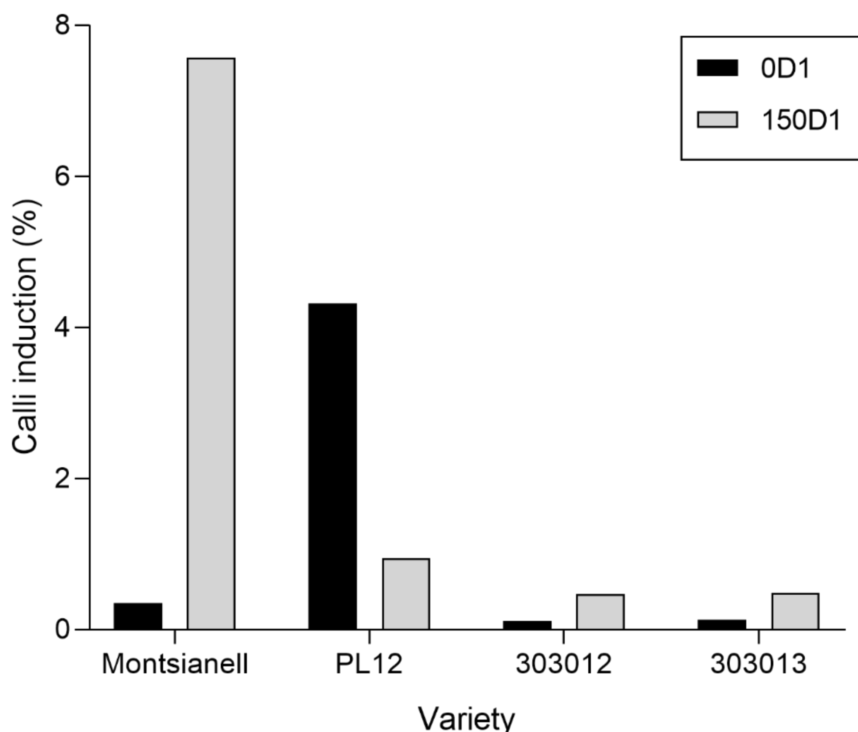


Figure 18. Percentage of calli induction for each variety when using the non-supplemented colchicine medium (0D1, black columns) and the colchicine-supplemented one (150D1, grey columns).

4.1.2 Plantlet regeneration

The calli obtained from each variety in both induction media were distributed equally in three plantlet regeneration media (XACRM, SARM and GERM). The effect of the different media in the regeneration of plantlets was evaluated for each variety. Plantlet regeneration percentages depending on variety are reflected in Table 7.

Attending to the regeneration media, for PL12 and Montsianell temperate japonica varieties plantlet regeneration percentages were 24% and 46.85% in XACRM, 28.57% and 68.14% in GERM and 32% and 64% in SARM respectively. Regarding the 303012 tropical japonica variety, the only regeneration value obtained was 16.67% in SARM. Finally, in the 303013 variety, plantlet regeneration percentage was 8.33% in XACRM, 15.38% in GERM and 20% in SARM (Figure 19). Statistical analysis showed significant differences in plantlet regeneration depending on the regeneration media (p=0.000) (Table SM 2).

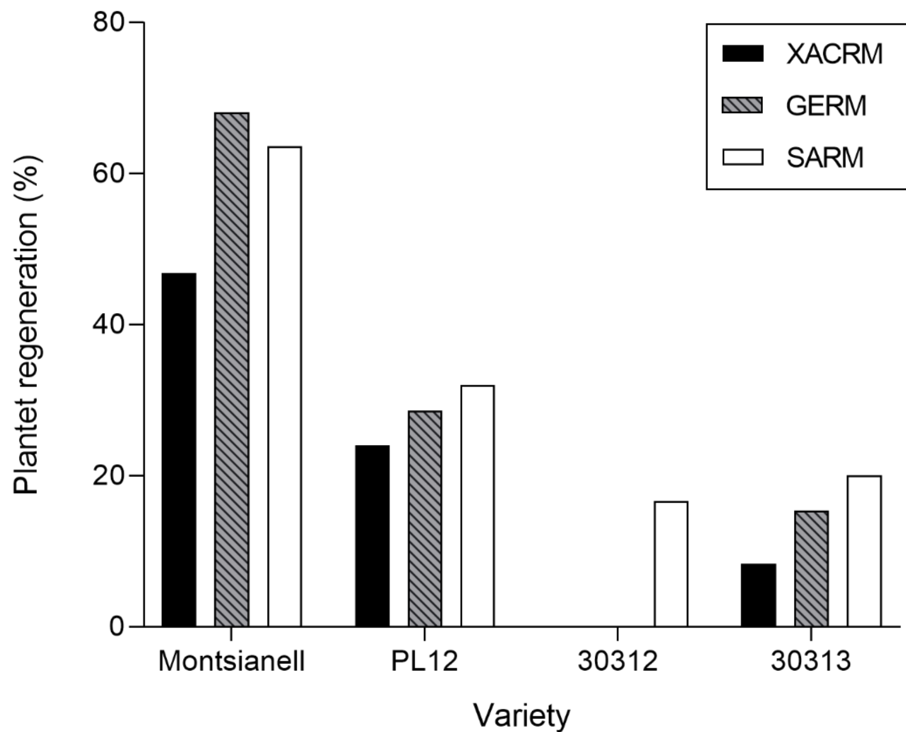


Figure 19. Percentage of calli regenerating plantlets for each variety in the regeneration media assayed, XACRM (black columns), GERM (dark grey striped columns) and SARM (white columns).

4.1.3 Green plantlets and ploidy determination

In this experiment, a total amount of 1,106 green plantlets, 194 albino plantlets and 46 chimeras (*i.e.* stripped albino-green) plantlets were successfully regenerated in the root medium after their extraction from the calli. Regarding the green plantlets, 1,000 and 78 plantlets were regenerated for Montsianell and PL12 temperate japonica varieties respectively. Contrary, for tropical japonica varieties, 28 plantlets were obtained from 303012, whereas no green plantlet regeneration and survival was observed from 303013. Green plantlet percentage as well as albinism rates depending on variety are reflected in Table 7.

After the cytometry analysis, different ploidy levels were detected in the regenerated green plantlets of each variety. For PL12, no doubled haploids were regenerated. More than 97% of the regenerated green plantlets were haploid whereas 2.78% had a ploidy other than doubled haploid, e.g.– triploid, tetraploid and chimeras in this case. Regarding Montsianell, 61.27% of the regenerated green plantlets were haploid, 37.53% were doubled haploid and 1.20% had other kind of ploidy. Finally, regarding 303012, 69.57%

of the regenerated green plantlets were haploid and 30.43% were doubled haploid. No other ploidy was detected for the green plantlets regenerated in this variety (Figure 20).

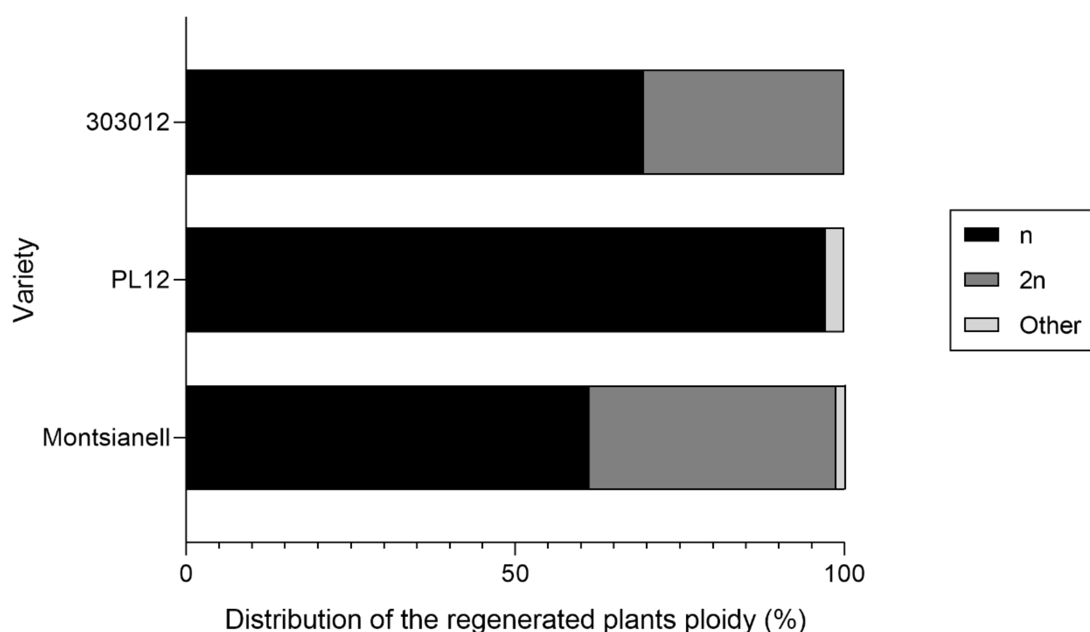


Figure 20. Distribution of the regenerated green plantlets ploidy for each variety. Haploid plantlets (n) are represented in black, doubled haploid (2n) in dark grey and other kind of ploidy (other) in light grey.

The percentage of doubled haploid green plantlets of each variety was calculated (Table 7) and also in detail for each induction and regeneration medium (Table 8). In the case of the temperate japonica varieties, no green doubled haploid plantlets were obtained for the PL12 variety. Montsianell produced green doubled haploid plantlets from the calli originated in the 150D1 medium in all regeneration media. However, no green doubled haploid plantlets were regenerated from calli coming from the 0D1 medium. For the 303012 tropical japonica variety, green doubled haploid plantlets were only obtained in SARM regeneration medium from the calli induced in 150D1.

Table 8. Percentage of green doubled haploid plantlets according to the calli induction media and the regeneration media.

	Temperate japonica				Tropical japonica	
	Montsianell		PL12		303012	
	0D1	150D1	0D1	150D1	0D1	150D1
XACRM	-	51 (28.98)	-	0 (0.00)	-	-
GERM	-	89 (40.64)	0 (0.00)	0 (0.00)	-	-
SARM	-	173 (39.41)	0 (0.00)	-	-	7 (30.43)

4.2 Experiment 2: Testing the Saltol region in hydroponic assays and in fields

4.2.1 Na^+/K^+ ratio measurement timepoint assay on hydroponics

The Na^+/K^+ ratio was determined at a hydroponic assay for the two parental varieties (PL12 and FL478) and for two LP varieties derived from them, in days 1, 2, 4 and 7 after salinization of the media with 80 mM NaCl. The Na^+/K^+ ratio was also determined for control samples (*i.e.*those grown in non-salinized media), at the same timepoints (Figure 21). Statistics are presented in Table SM 4 and Table SM 5.

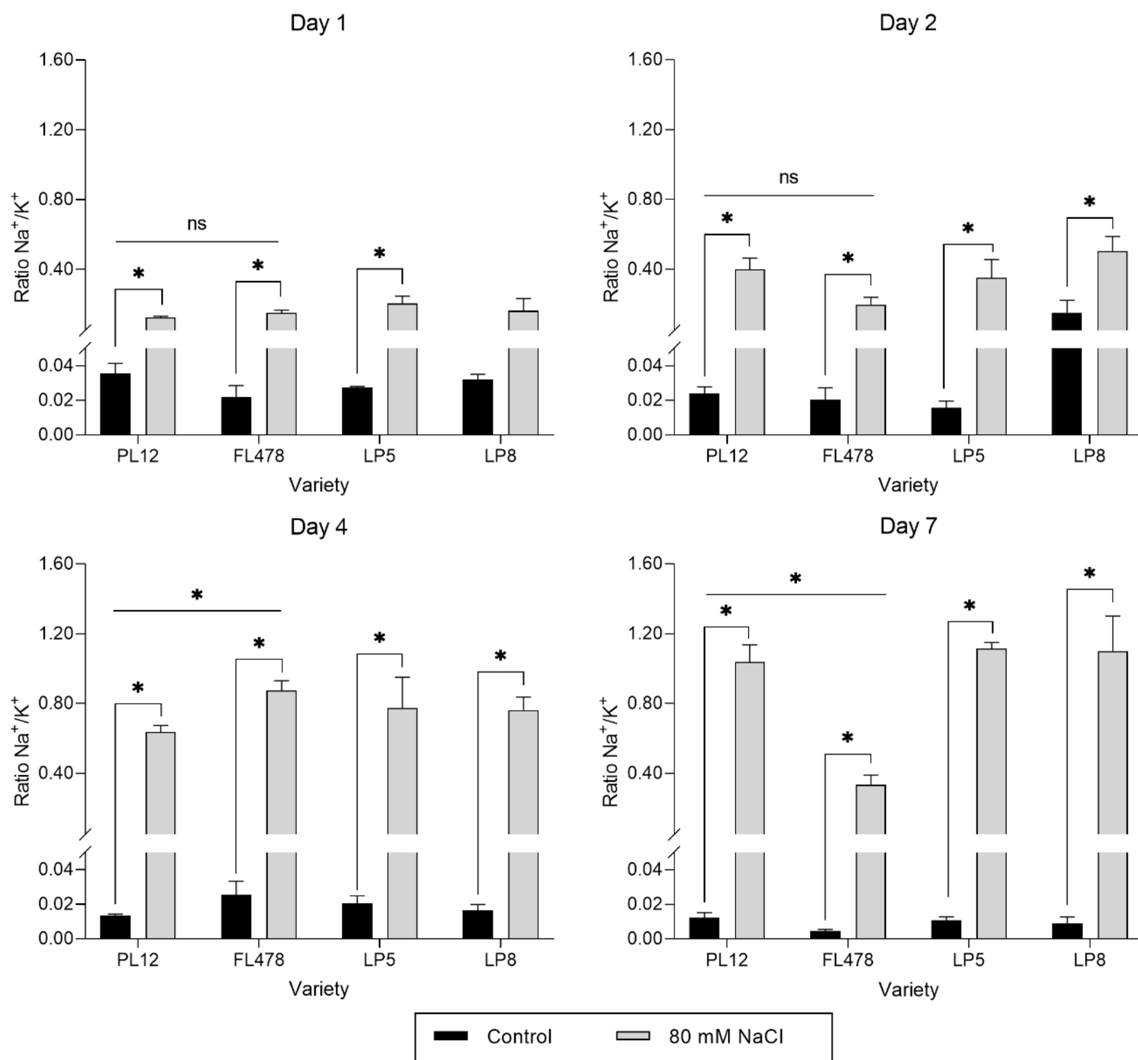


Figure 21. Na^+/K^+ ratio determined for control samples (black bars) grown in a non-salinized media, and for samples grown in a salinized media of 80 mM NaCl (grey bars) collected in the days 1, 2, 4 and 7 after salinization of the media. Each bar is represented along with the SEM. Significant differences between varieties per treatment are shown, as well as the significance of the comparison of the parental salt treatment values ($p < 0.05$). ns: non-significant differences.

The Na^+/K^+ ratio of the parentals is the reference data to evaluate salt resistance in the hybrid lines. Therefore, the ideal timepoint to collect the samples for the measurement would be (i) that in which the differences in the Na^+/K^+ ratio between the control and the salinity-treated samples from the parental varieties would be significant and (ii) that in which the Na^+/K^+ ratio of the salt-sensitive parental (PL12) is significantly different (and higher, since a high ratio means a poor salt-resistance) than the salt-resistant (FL478) parental under salinity conditions. The only timepoint that matched these two conditions was the day 7 after salinization. Thus, the 7th day after salinization timepoint was established as the ideal to measure the Na^+/K^+ ratio in the following hydroponic assays.
The LP varieties were included as extra material in the experiment and were not relevant to establish the ideal timepoint, although their Na^+/K^+ ratio in the 7th day would look more like a salt-sensitive line than a salt-resistant one.

4.2.2 Relative chlorophyll content on hydroponic assays

After the timepoint experiment, hydroponic assays with LP varieties, MS varieties and their parentals were performed. The relative chlorophyll content was measured at days 1, 3, 5 and 7 after salinization with a SPAD meter in both control and salinized individuals (Figures 22 and 23). Statistics are presented in Table SM 6 and Table SM 7.
The relative chlorophyll content (RCC) is also referred to as SPAD score. Salinity damage in the rice plants lowers the RCC since it compromises the functionality of the photosystems.
For the LP varieties, the SPAD measurements showed a similar score the first day after salinization in both control and salinized samples. At the 3rd day after salinization, both PL12 and FL478 parentals, showed a similar SPAD score in saline conditions. There were some varieties that presented a lower SPAD score than the sensitive parental (PL12), like LP3 or LP24, although they did not show significant differences with their control samples. In general terms, the control plant RCC values tended to be higher than the salinized RCC values. During the 5th day after salinization, a clearer response to salinization was observed in the measurements. The RCC values of the salinized PL12 variety were clearly lower than the FL478 salinized values. This was expected since FL478 is resistant to salinity, and therefore its RCC values would not decrease as heavily with salinity. Besides, the almost equal value in control and salinized conditions for FL478 reinforces the idea of its salt tolerance. It is also clear that some varieties did not show high RCC values in salinized conditions such as LP4, that according to this

measurement, seems to be severely affected by salinity. There were also other varieties such as LP9, LP14, LP15 or LP21 whose RCC values were closer to the sensitive parental in saline conditions than to the resistant one. However, varieties such as LP6 and LP7 showed RCC values almost equal to FL478, the resistant parental, in saline conditions. On the 7th day after salinization, the differences between control and salinized RCC measurements seemed to be the largest. PL12 was significantly affected when comparing its values in control and salinized conditions. LP4, which already presented a low SPAD score on the 5th day, kept its low values and equaled them to the PL12 values. FL478 still presented a high RCC value in both control and salinized conditions, even increasing it in comparison with the 5th day. Almost all varieties presented an intermediate value between the PL12 and FL478 values in saline conditions, with some closer to the sensitive parent values (LP24, LP26) and others closer to the resistant parental values (LP7).

Regarding the MS varieties and their SPAD scores, on the 1st day after salinization both control and salinized measurements were similar in all varieties. The IR64-*Saltol* values, that belong to the resistant parental, were lower from the beginning than the values from the sensitive parental, PM37. The 3rd day after salinization, a slight decrement in the salinized values in comparison with the day 1 was observed, remaining most of them close to the control values, except for the IR64-*Saltol* variety. At the 5th after salinization the differences of the values for both treatments were more evident in the parental varieties as well as in MS5, MS7, MS14, MS17, MS18, MS20 and MS25 varieties. There were some measurements in saline conditions whose values were lower than the sensitive parental, PM37, such as MS7, MS17 or MS25. However, varieties such as MS2 showed a much higher RCC value than PM37, indicating a stronger tolerance to salinity than the sensitive parental. On the 7th day after salinization, more varieties showed significant differences on their values between both treatments, including the parentals. MS17 and MS18 varieties showed an RCC value of 0, meaning all the plants from these varieties lacked green parts (they had died). Nevertheless, there were varieties that presented higher RCC in salinity conditions than PM37, like MS2 especially, but also MS4, MS6, MS11, MS16, MS20 or MS23 varieties.

A one-way ANOVA with a 95% confidence assay was conducted to determine if the control measurements and the salinity measurements depended on the variety. In the case of the LP varieties, for the 1st day after salinization, it was determined that control measurements did not depend on variety (p-value= 0.106), and that the salinity measurements also did not depend on variety (p-value= 0.278). For the 3rd day after

salinization, it was determined that control measurements depended on variety (p-value= 0.047), and that the salinity measurements did not depend on variety (p-value= 0.697). For the 5th day after salinization, it was determined that control measurements depended on variety (p-value= 0.020), and that the salinity measurements did not depend on variety (p-value= 0.416). For the 7th day after salinization, it was determined that control measurements did not depend on variety (p-value= 0.793), and that the salinity measurements also did not depend on variety (p-value= 0.944). In the case of the MS varieties, for the 1st day after salinization, it was determined that control measurements did not depend on variety (p-value= 0.062), and that the salinity measurements depended on variety (p-value= 0.001). For the 3rd day after salinization, it was determined that control measurements did not depend on variety (p-value= 0.083), and that the salinity measurements also did not depend on variety (p-value= 0.063). For the 5th day after salinization, it was determined that control measurements depended on variety (p-value= 0.002), and that the salinity measurements also depended on variety (p-value= 0.001). For the 7th day after salinization, it was determined that control measurements did not depend on variety (p-value= 0.292), and that the salinity measurements depended on variety (p-value= 0.010). Statistics for both LP and MS varieties are presented in more detail in Table SM 8 and Table SM 9.

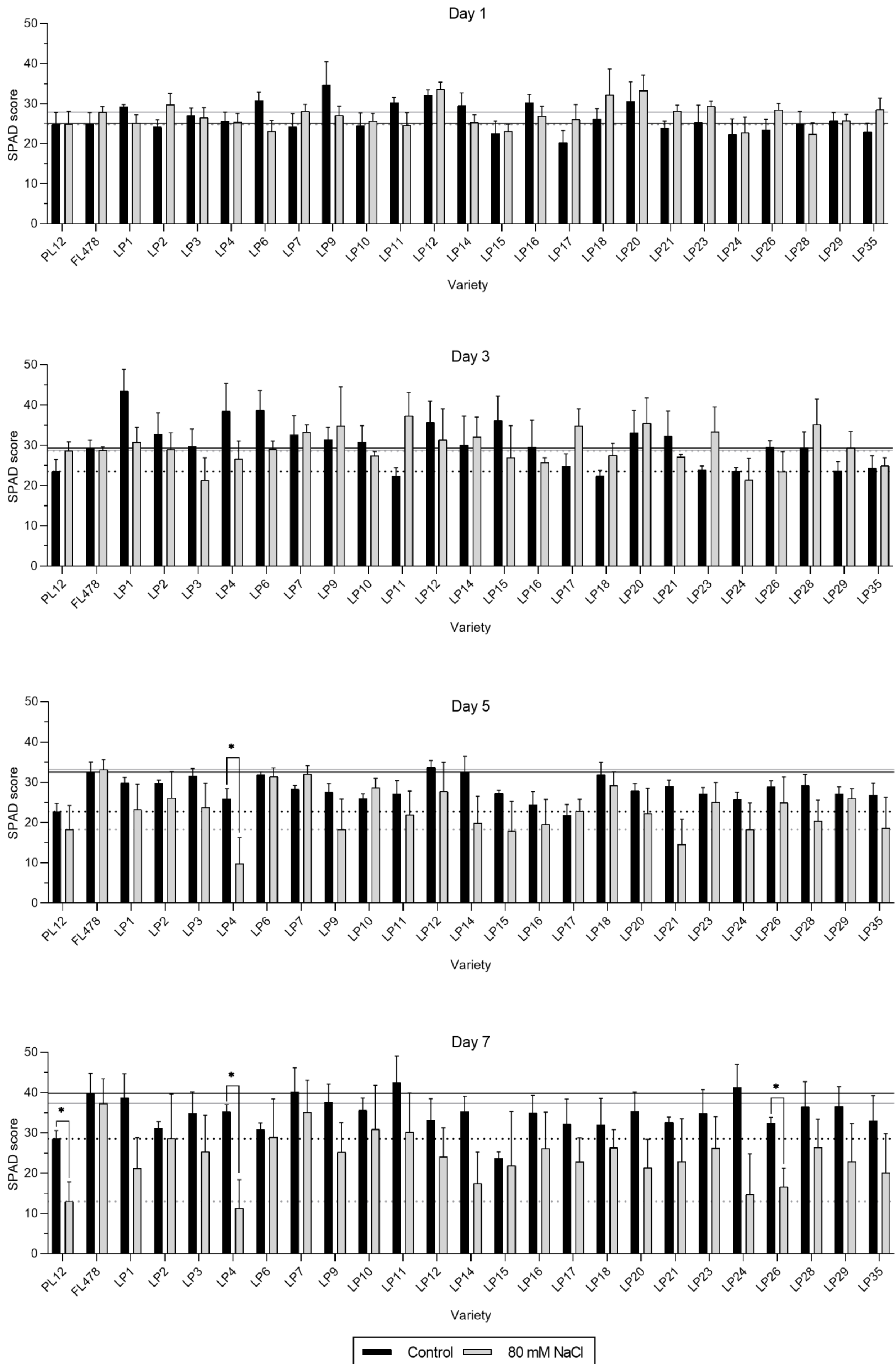


Figure 22. SPAD score measured with LP varieties for control samples (black bars) and for salinized samples (grey bars) in days 1, 3, 5 and 7 after salinization of the media (80 mM). Dotted lines represent PL12 control (black) and salinity (grey) values and straight lines represent FL478 control (black) and salinity (grey) value. Each bar is represented along with the SEM. Significant differences between varieties per treatment are shown ($p < 0.05$).

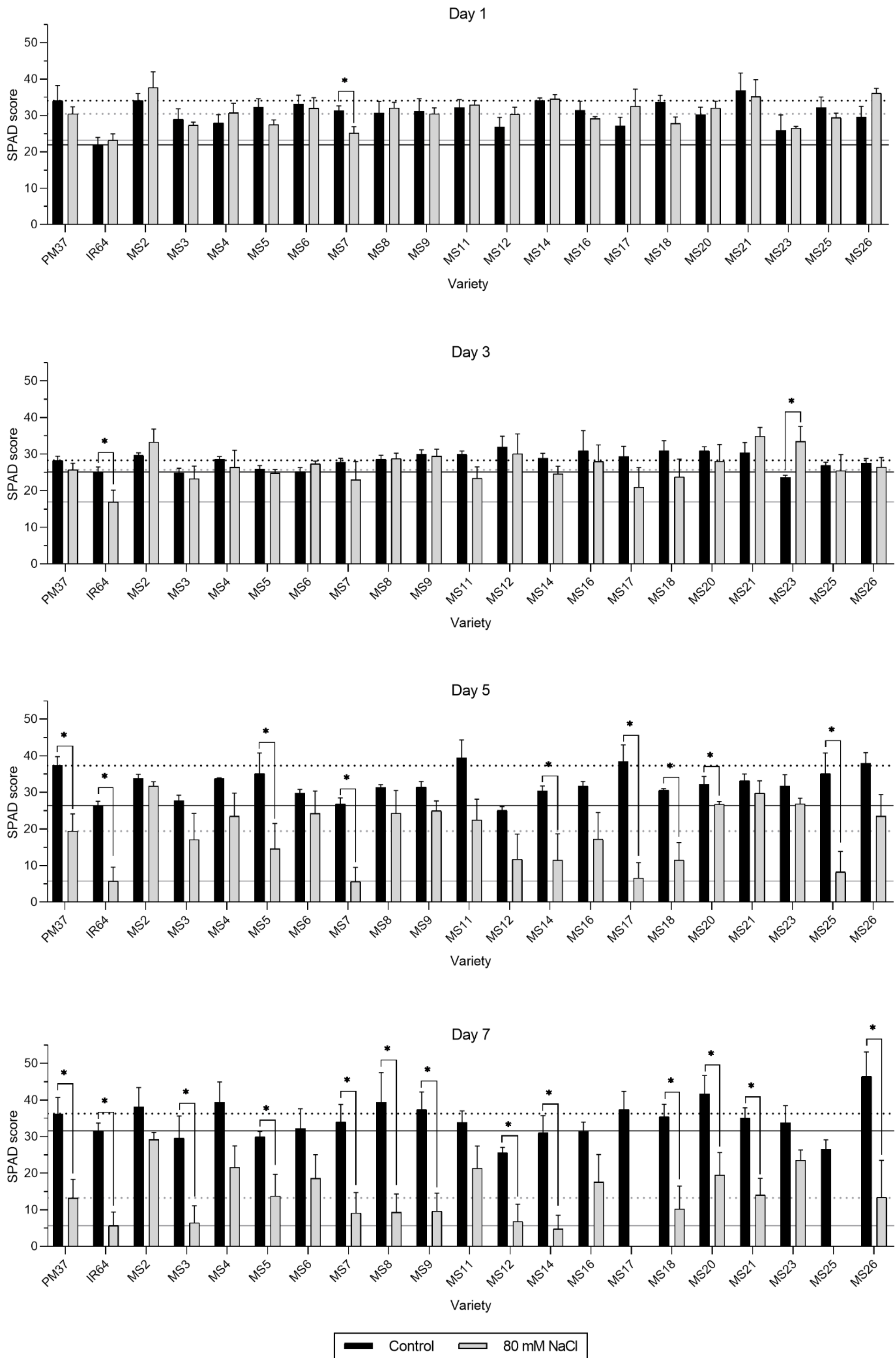


Figure 23. SPAD score measured with MS varieties for control samples (black bars) and for salinized samples (grey bars) in days 1, 3, 5 and 7 after salinization of the media (80 mM). Dotted lines represent PM37 control (black) and salinity (grey) values and straight lines represent IR64-*Saltol* control (black) and salinity (grey) value. Each bar is represented along with the SEM. Significant differences between varieties per treatment are shown ($p < 0.05$).

Also, the percentage of reduction or increase in the SPAD score between the days 1 and 7 of measurement was calculated for each variety and treatment (Tables 9 and 10).

Table 9. Percentage of reduction or increase of the SPAD score between the days 1 and 7 of the measurements for both control and salinity treatments on LP varieties. No symbol before the percentage indicates an increase of the SPAD score, and a minus symbol before the percentage indicates a reduction of the SPAD score.

Variety	% control	% salinity
PL12	14,88	-47,93
FL478	58,88	33,79
LP1	32,44	-15,90
LP2	28,55	-3,77
LP3	28,95	-4,59
LP4	37,59	-55,38
LP6	-0,06	24,59
LP7	65,51	25,00
LP9	8,37	-6,65
LP10	45,51	20,59
LP11	40,46	22,60
LP12	3,18	-28,33
LP14	19,16	-30,85
LP15	4,79	-5,53
LP16	15,73	-3,12
LP17	58,13	-12,42
LP18	21,95	-18,22
LP20	15,59	-35,84
LP21	36,38	-18,62
LP23	37,94	-10,88
LP24	84,45	-35,49
LP25	51,93	6,09
LP26	37,82	-41,52
LP28	45,77	17,57
LP29	42,19	-11,09
LP35	43,22	-29,74

Table 10. Percentage of reduction or increase of the SPAD score between the days 1 and 7 of the measurements for both control and salinity treatments on MS varieties. No symbol before the percentage indicates an increase of the SPAD score, and a minus symbol before the percentage indicates a reduction of the SPAD score.

Variety	% control	% salinity
PM37	5,66	-56,50
IR64	43,87	-75,59
MS2	11,59	-22,53
MS3	2,07	-76,26
MS4	40,69	-29,65
MS5	-7,13	-49,93
MS6	-2,83	-41,84
MS7	8,75	-63,67
MS8	28,56	-70,85
MS9	19,73	-68,33
MS11	5,15	-35,38
MS12	-4,54	-77,46
MS14	-9,13	-86,01
MS16	-0,51	-39,61
MS17	37,88	-100,00
MS18	5,41	-63,34
MS20	37,97	-39,14
MS21	-4,72	-59,92
MS23	30,53	-11,09
MS25	-17,46	-100,00
MS26	57,16	-62,89

Regarding the LP varieties, all the percentages of the control plants were positive, showing an increase of the RCC content, and presumably, plant growth. This percentage changed depending on the variety. There was an exception with LP6, with an irrelevant decrease of a 0.06%. As for the salinity treated plants, there were some varieties that experimented an increase of the SPAD score and some that showed a reduction of the score, and presumably, a decrease of plant growth. The varieties that increased their RCC content the most were FL478, LP7, LP6, LP11 and LP28. The ones whose RCC content decreased the most were LP4, PL12, LP26, LP20, LP24 and LP14.

Regarding the MS varieties, the behavior in the control plants was more heterogeneous than in the LP varieties, with some varieties increasing their RCC content and some others lowering it. The percentages of the MS salinity-treated varieties were all negative, with a

great variation between them. Varieties like MS17 and MS25 presented a 100% of RCC decrease, meaning all the plants of those varieties were dead at the 7th day of measurement. Varieties like MS12, MS3 and IR64-*Saltol* were heavily damaged, presenting the highest RCC loss. On the other hand, varieties like MS23, MS2 and MS4 were the ones that lost the less chlorophyll content.

4.2.3 SES score on hydroponic assays

SES score was determined visually according to the plants' phenotype in the days 1, 3, 5 and 7 after salinization of the media (80 mM) (Figures 24 and 25). The higher the SES score, the more phenotypically damaged the plants are by salinity. Control plants were not evaluated since the SES scale determines salinity damage.

Concerning the LP varieties, the 1st day after salinization almost all varieties had not manifested yet any response to the salinization of the media. The 3rd day after salinization many varieties kept the score of 1 in the SES scale (in the figure, some light blue dots are placed behind the dark blue dots). Nonetheless, varieties like LP3, LP4, LP9 or LP24 began to show salinity damage symptoms. The 5th day after salinization a differential behavior of the parentals started to show, with the sensible parental (PL12) showing a higher value than the resistant parental (FL478). Varieties like LP7, LP18 and LP29 showed even a lower score than FL478, indicating less salinity damage. However, some varieties like LP4, LP15, LP21 or LP26 showed a higher score than the sensitive parental, indicating more salinity damage. On the 7th day after salinization, the different salinity response was evident in the parentals. The tolerant parental showed a lower SES score, as expected, meaning a better phenotypical response to salinity than PM37. Varieties like LP4, LP21, LP24 or LP35 showed SES scores similar to PM37, pointing out to a severe salinity damage. On the contrary, there were varieties like LP3, LP17, LP18, LP20 or LP23 that showed SES scores closer to FL478. Furthermore, LP2 and LP9 improved the score of FL478 with lower SES values, being the most phenotypically resistant.

Regarding the MS observations, the 1st day after salinization, almost all varieties showed no salinity damage with a SES score = 1, except for MS17 and MS25. The 3rd day after salinization, the resistant parental, IR64-*Saltol*, unexpectedly presented a higher SES score than the sensitive one, PM37. Varieties like MS2, MS3, MS9, MS20, MS21, MS23 and MS23 showed lower SES scores than PM37 in this measurement, meaning almost no phenotypical damage by salinity. Nonetheless, MS17 and MS25 varieties showed great damage in this 3rd day. On the 5th day after salinization, IR64-*Saltol* kept increasing its

SES score. Varieties like MS17 and MS25 surpassed greatly the PM37 score, some others improved it, like MS2, MS23 and MS26, by lowering it. The rest of the varieties presented an intermediate score. The 7th day, the most affected varieties by salinity were MS3, MS9, MS11, MS17 and M25, surpassing the PM37 score. The ones with the lowest SES score, and therefore a greater phenotypical performance, were MS2 and MS6.

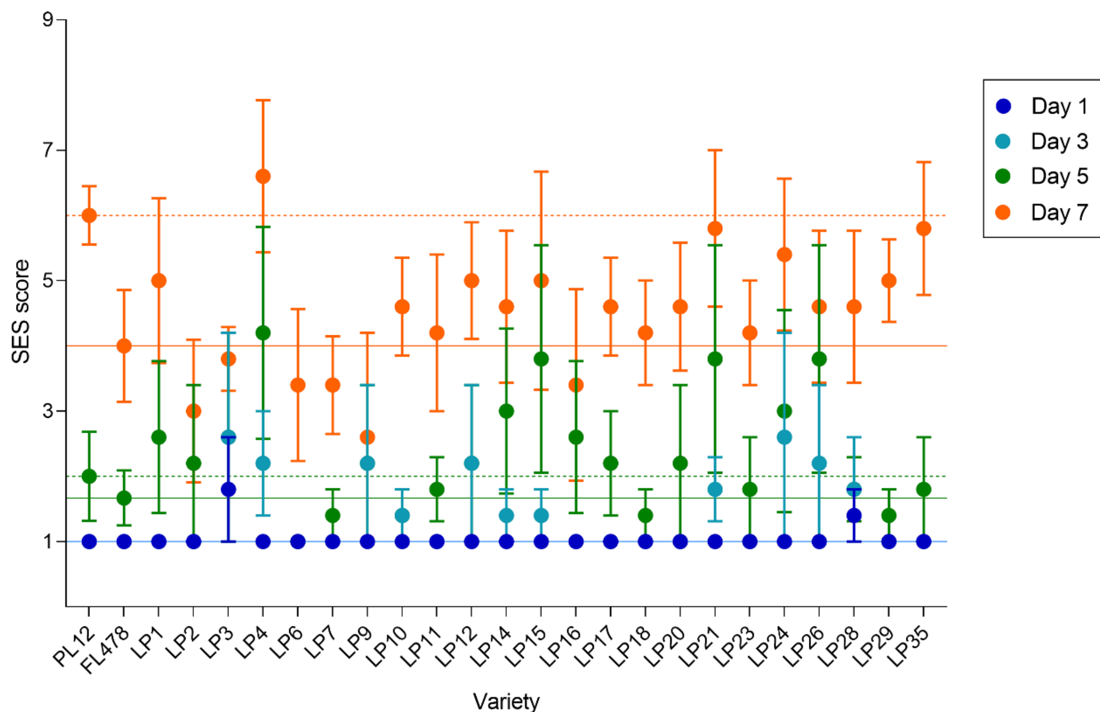


Figure 25. SES score determined for LP varieties in the day 1 (dark blue dots), day 3 (light blue dots), day 5 (green dots) and day 7 (orange dots) after salinization (80 mM). Dotted lines mark PL12 values and continuous lines indicate the FL478 values for each measurement day. Each dot is represented along with the SEM.

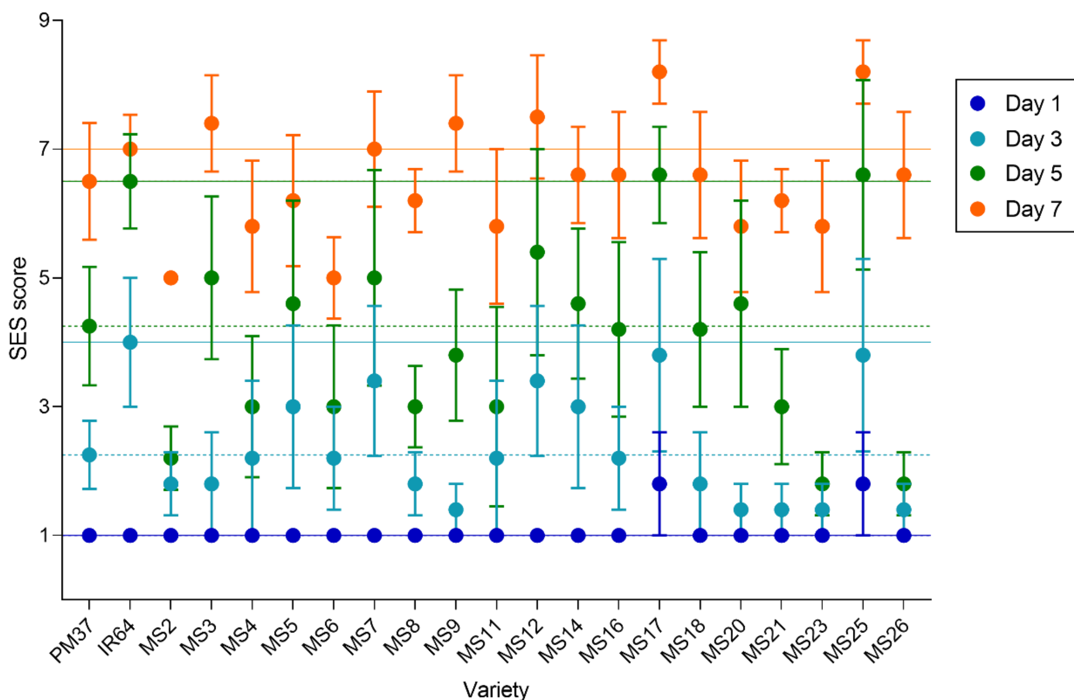


Figure 24. SES score determined for MS varieties in the day 1 (dark blue dots), day 3 (light blue dots), day 5 (green dots) and day 7 (orange dots) after salinization (80 mM). Dotted lines mark PM37 values and continuous lines indicate the IR64-*Saltol* values for each measurement day. Each dot is represented along with the SEM.

4.2.4 Na^+/K^+ ratio measurements on hydroponic assays

The Na^+/K^+ ratio was measured with samples collected for both the LP and MS varieties along with their parentals the 7th day after salinization, as determined in the previous timepoint assay (Figures 26 and 27). Statistics are presented in Table SM 10 and Table SM 11.

The Na^+/K^+ ratio is a measure of the salinity tolerance. The lower the ratio, the better the salt tolerance.

Regarding the LP varieties, it is noticeable that the ratio of the resistant parental, FL478, was lower than the sensitive parental', PL12, as expected. Most of the cross Saltol-introgressed presented intermediate Na^+/K^+ ratio values of the parentals. LP7 and LP9 had the most similar ratios to the resistant parental FL478, followed by other varieties like LP16, LP17, LP18 and LP28. LP3 and LP26 varieties presented the closest values to the sensitive parental, pointing out to similar salt tolerance. LP4 even presented a higher ratio than the sensitive parental, indicating the poorest salt tolerance according to the Na^+/K^+ ratio.

Regarding the MS varieties, the resistant parental, IR64-*Saltol*, had a higher Na^+/K^+ ratio than the sensitive one, PM37, contradicting the expected results. Taking PM37 as reference, it was noticeable that varieties like MS11, MS14, MS20, MS21, and especially MS2, lowered their ratio showing better salt tolerance according to this parameter. Nonetheless, MS12, MS17 and MS25 varieties presented the highest ratios, indicating poor salt tolerance performance.

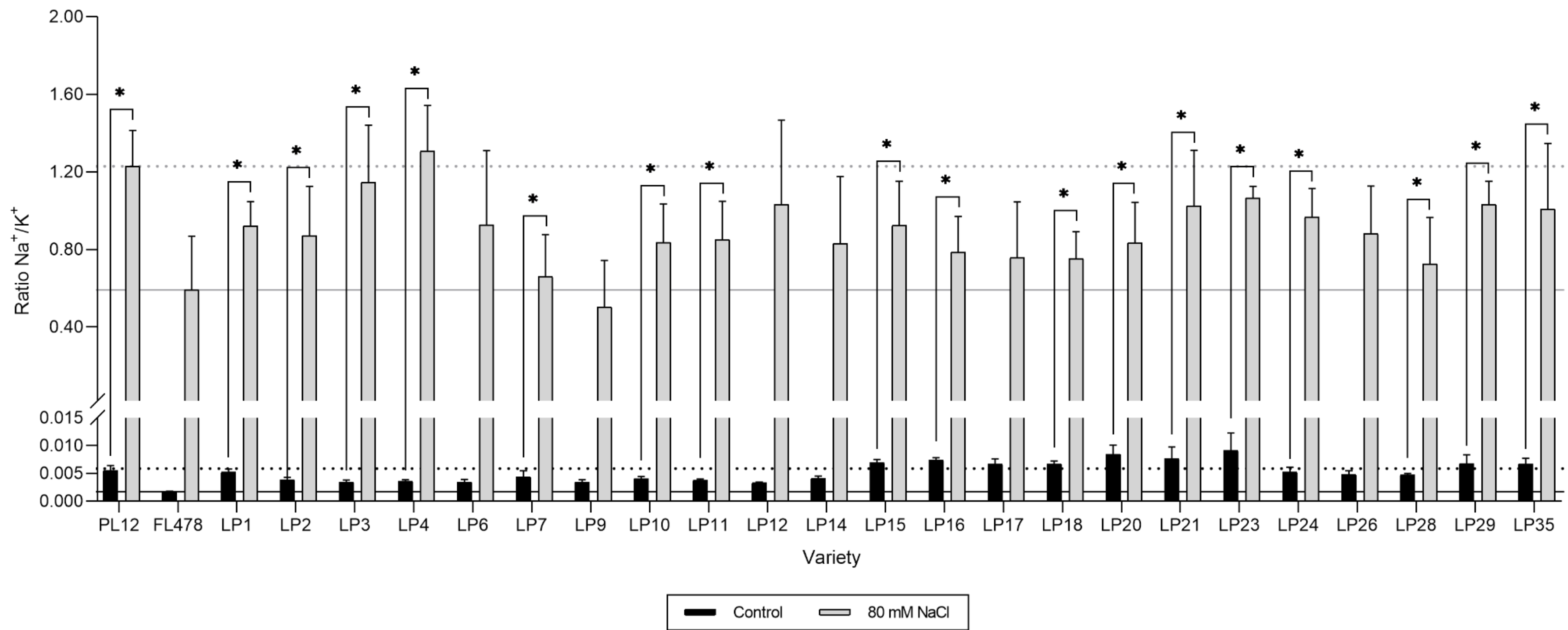


Figure 26. Na^+/K^+ ratio determined for control samples (black bars) grown in a non-salinized media, and for samples grown in a salinized media of 80 mM (grey bars) collected in the 7th day after salinization of the media. Dotted lines represent PL12 control (black) and salinity (grey) values and straight lines represent FL478 control (black) and salinity (grey) values. Each bar is represented along with the SEM. Significant differences between varieties per treatment are shown ($p < 0.05$).

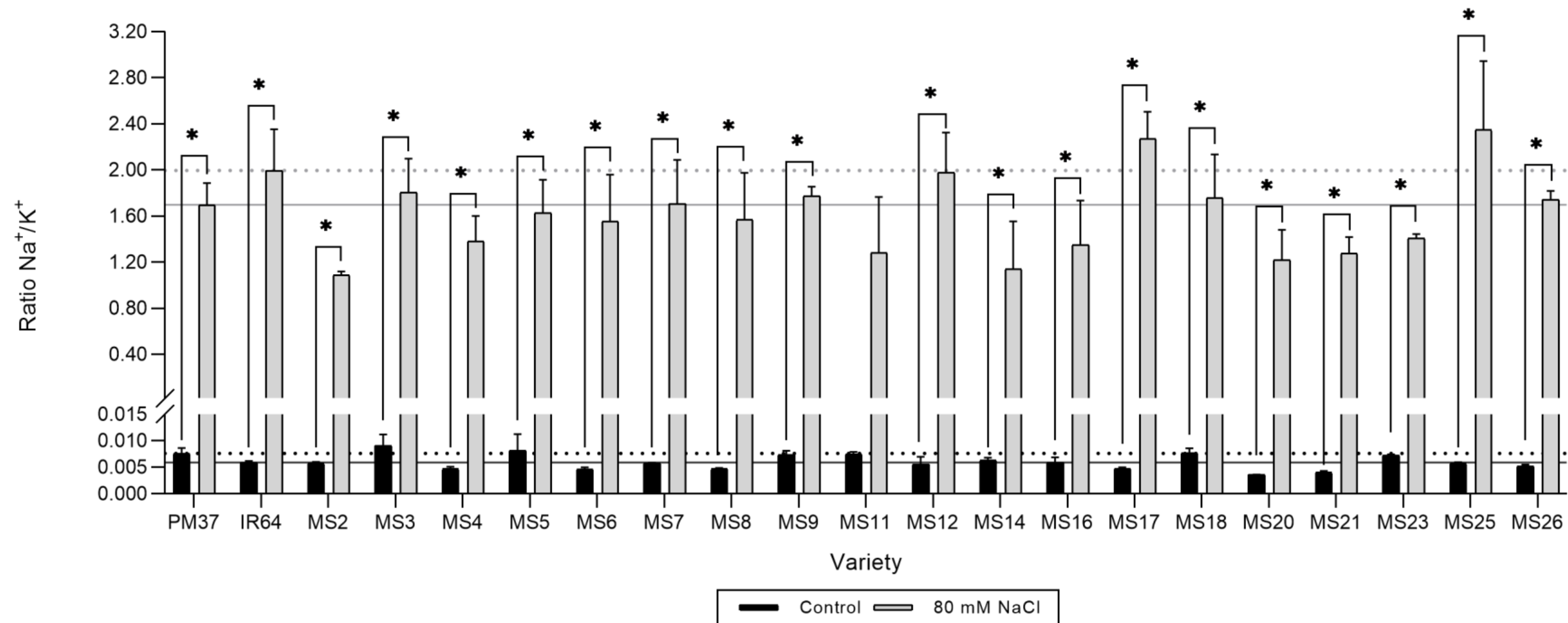


Figure 27. Na^+/K^+ ratio determined for control samples (black bars) grown in a non-salinized media, and for samples grown in a salinized media of 80 mM (grey bars) collected in the 7th day after salinization of the media. Dotted lines represent PM37 control (black) and salinity (grey) values and straight lines represent IR64-*Saltol* control (black) and salinity (grey) values. Each bar is represented along with the SEM. Significant differences between varieties per treatment are shown ($p < 0.05$).

4.2.5 Correlation analysis of the measured parameters on hydroponic assays under salinity conditions

The Spearman test was applied to analyze the correlation between the parameters measured under salinity conditions in the different days after salinization of the media for both LP and MS varieties (Tables 11 and 12).

Table 11. Spearman correlation coefficients for the LP measurements under salinity conditions. Significance at $p < 0.05$ is marked with an asterisk (*). SES = SES analysis. SPAD = SPAD measurements. NaK= Na^+/K^+ ratio measurements. The number indicates the day after salinization in which the measurement was performed (1, 3, 5 or 7).

	SPAD1	SPAD3	SPAD5	SPAD7	SES1	SES3	SES5	SES7
SPAD3	0,215*							
SPAD5	0,296*	0,344*						
SPAD7	0,240*	0,494*	0,475*					
SES1	0,020	0,013	0,011	0,055				
SES3	-0,191*	-0,304*	-0,413*	-0,300*	-0,043			
SES5	-0,256*	-0,440*	-0,519*	-0,495*	-0,081	0,493*		
SES7	-0,290*	-0,415*	-0,502*	-0,594*	-0,024	0,384*	0,600*	
NaK7	0,042	-0,061	-0,122	-0,163	0,166	0,055	0,067	0,245*

Table 12. Spearman correlation coefficients for the MS measurements under salinity conditions. Significance at $p < 0.05$ is marked with an asterisk (*). SES = SES analysis. SPAD = SPAD measurements. NaK= Na^+/K^+ ratio measurements. The number indicates the day after salinization in which the measurement was performed (1, 3, 5 or 7).

	SPAD1	SPAD3	SPAD5	SPAD7	SES1	SES3	SES5	SES7
SPAD3	0,213*							
SPAD5	0,325*	0,383*						
SPAD7	0,113	0,349*	0,680*					
SES1	-0,032	-0,018	-0,114	-0,137				
SES3	-0,268*	-0,449*	-0,585*	-0,517*	0,111			
SES5	-0,279*	-0,421*	-0,709*	-0,682*	0,095	0,649*		
SES7	-0,145	-0,323*	-0,667*	-0,781*	0,107	0,546*	0,751*	
NaK7	-0,303*	-0,172	-0,147	-0,112	0,159	0,173	-0,009	0,023

For the LP varieties, a significant positive moderate correlation was observed for all the SPAD measurements. No correlation was observed between the SES measurements on day 1 after salinization with any of the SPAD measurements. A weak/moderate significant negative correlation was observed between the SPAD measurements and the SES measurements on day 3 after salinization. SES measurements on day 5 and day 7 after salinization presented a moderate/strong significant positive correlation with the SPAD measurements performed on the same days. Also, SES measurements correlated significantly and increasingly from day 3 after salinization onwards. The Na^+/K^+ ratio only correlated positively with the SES measurement on day 7 with significance.
Regarding the MS varieties, a significant moderate to strong positive correlation was observed for all the SPAD measurements, being the correlation between day 5 and day 7 after salinization the strongest. Between the SES and SPAD measurements a negative correlation was observed, increasing along with the day of measurement, showing strong to very strong and significant correlation on days 5 and 7 after salinization. The SES measurements correlated strongly and positively between them in the days 5 and 7. Regarding the Na^+/K^+ ratio, only a weak negative significant correlation was observed with the SPAD measurement on day 1, being the rest of the correlation non-significant and very weak.

4.2.6 Na^+/K^+ ratio measurements on field assays

The Na^+/K^+ ratio was measured with samples collected for both LP and MS varieties 15 days after transplantation into non-salinized and salinized fields (Figures 28 and 29). Statistics are presented in Table SM 12 and Table SM 13.
Regarding the LP varieties, it is noticeable the difference in the salinity ratios between the sensitive parental, PL12, and the resistant parental, FL478, as expected. PL12 is less tolerant to salinity and therefore its Na^+/K^+ ratio was higher (almost 3-fold) than FL478. The LP varieties presented intermediate Na^+/K^+ ratios between both parentals, being LP5, LP13, LP22, LP24, LP25 and LP29 the most similar to the FL478 ratio, while varieties like LP21, LP25 and LP27 showed closer ratios or even higher than PL12.
Regarding the MS varieties, the ratio of the sensitive parental, PM37 was higher than the ratio of the resistant parental, IR64-*Saltol*. Most of the varieties equaled or surpassed the PM37 ratio. Nonetheless, MS14 presented the same ratio as IR64-*Saltol*, and MS28 had an even lower ratio than IR64-*Saltol*, indicating very good salt tolerance.

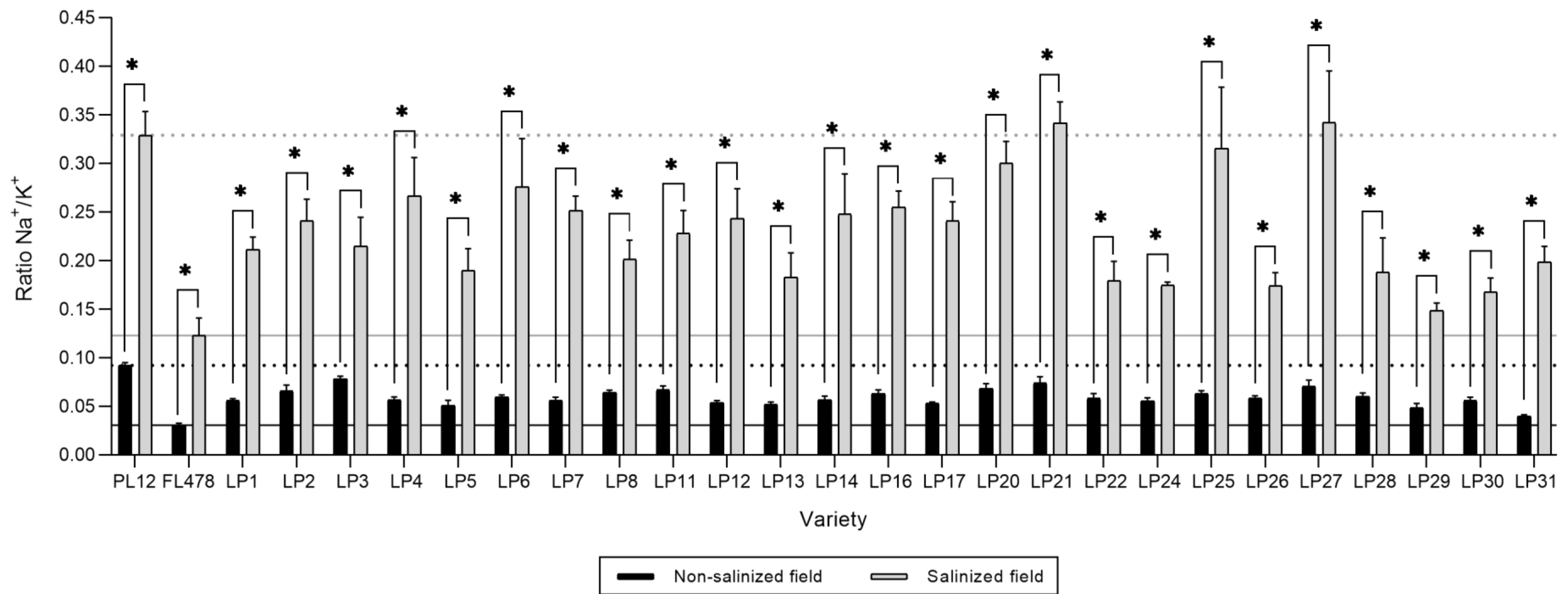


Figure 28. Na^+/K^+ ratio determined for control samples (black bars) grown in a non-salinized field, and for samples grown in a salinized field (grey bars) collected in the 15th day after transplantation into the field. Dotted lines represent PL12 control (black) and salinity (grey) values and straight lines represent FL478 control (black) and salinity (grey) value. Each bar is represented along with the SEM. Significant differences between varieties per treatment are shown ($p < 0.05$).

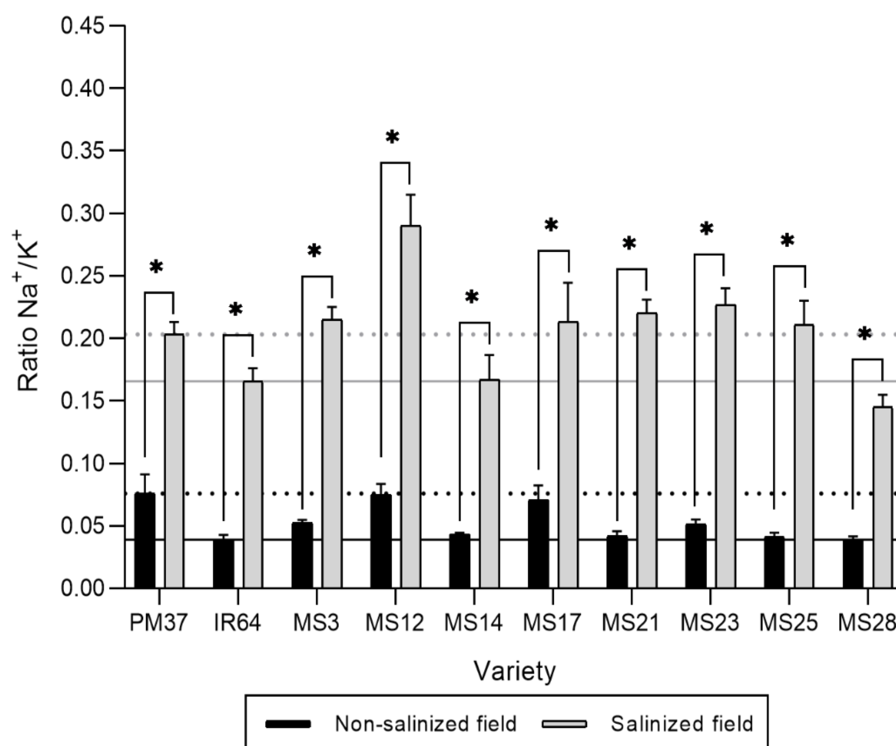


Figure 29. Na^+/K^+ ratio determined for control samples (black bars) grown in a non-salinized field, and for samples grown in a salinized field (grey bars) collected in the 15th day after transplantation into the field. Dotted lines represent PM37 control (black) and salinity (grey) values and straight lines represent IR64-*Saltol* control (black) and salinity (grey) value. Each bar is represented along with the SEM. Significant differences between varieties per treatment are shown ($p < 0.05$).

4.2.7 Yield and correlation with Na^+/K^+ ratio

Yield data for some LP varieties in both non-salinized and salinized fields was obtained at the end of the season (Figure 30).

The yield of the non-salinized fields was higher than the yield of the salinized fields for all varieties except for LP31, whose yield was similar in both conditions.

For the yield and Na^+/K^+ ratio under non-salinity conditions of the LP varieties presented, the Pearson correlation coefficient was 0.422 with a p-value of 0.225, which denoted a positive moderate non-significant relation between both parameters. For the yield and Na^+/K^+ ratio under salinity conditions, the Pearson correlation coefficient was 0.170 with a p-value of 0.661, which denoted a positive weak non-significant relation between both parameters.

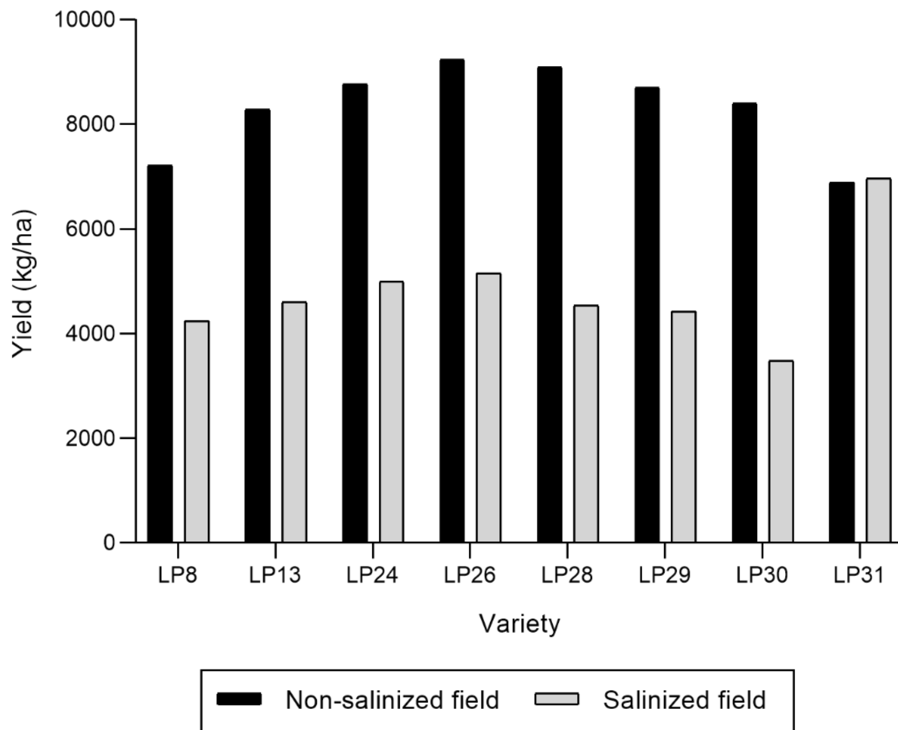


Figure 30. Yield data expressed as kg/ha for some varieties in both non-salinized and salinized fields at Ebro Delta.

4.3 Experiment 3: Studying the rice pearl in Mediterranean varieties by different approaches

4.3.1 Physical measurements

Different physical measurements were determined from pictures taken to 100 grains of each variety and later analyzed with an image software. These pictures are collected in Figure SM 2. The classification of each rice variety was established from the length and width parameters. Average weight was calculated by weighing 100 grains in groups of 10. Results are reflected in Table 13. Statistics are reflected in Table SM 14.

Table 13. Physical parameters of the varieties studied presented as mean ± SEM. Different letters within a measurement express significant differences ($p < 0.05$).

	Montsianell	Bomba	Carnaroli	Guadiamar	PL12
Area (mm x mm)	14.10 ± 0.12^{b}	11.28 ± 0.09^{d}	14.75 ± 0.12^{a}	11.79 ± 0.08^{c}	11.61 ± 0.09^{cd}
Pearl area (%)	31.63 ± 0.75^{b}	19.47 ± 0.81^{c}	44.27 ± 0.85^{a}		
Perimeter (mm)	15.46 ± 0.07^{b}	13.87 ± 0.06^{d}	16.78 ± 0.09^{a}	14.67 ± 0.06^{c}	16.87 ± 0.10^{a}
Length (mm)	5.51 ± 0.02^{b}	4.95 ± 0.02^{c}	6.32 ± 0.03^{a}	5.52 ± 0.02^{b}	6.38 ± 0.04^{a}
Width (mm)	3.17 ± 0.02^{a}	2.92 ± 0.02^{b}	2.79 ± 0.02^{c}	2.66 ± 0.01^{d}	2.07 ± 0.01^{e}
Length/width	1.74 ± 0.01^{d}	1.70 ± 0.01^{e}	2.27 ± 0.02^{b}	2.08 ± 0.01^{c}	3.09 ± 0.02^{a}
Weight (mg)	26.83 ± 0.37^{a}	20.31 ± 0.38^{b}	26.93 ± 0.19^{a}	20.66 ± 0.19^{b}	18.99 ± 0.21^{c}
Grain classification (EU/IRRI)	Medium / Medium bold	Short / Short medium	Long A / Medium medium	Medium / Medium bold	Long B / Long slender

Regarding the varieties' areas, Carnaroli and Montsianell were the ones that presented the highest values. There were significant differences between all varieties, although PL12 shared significance with both Bomba and Guadiamar. The average pear areas values were significantly different, with Carnaroli presenting the highest value, more than two-fold the average of Bomba, which presented the lowest pearl area. This indicates a high variability within the pearl character. Regarding the length, the varieties that showed the same significance were Carnaroli and PL12, Guadiamar and Montsianell, and Bomba as a third group, matching their grain classification, long, medium and short. There was only a small difference in both classifications systems in the case of Guadiamar, which is considered Long A type for the EU classification, since it has a length superior to 6.00 and a length/width ratio inferior to 3. But Guadiamar is considered Medium medium in the IRRI classification due to having a length comprised between 5.51 and 6.60 and a length/width ratio comprised between 2.1 and 3.0. The width was significantly different

for each variety. The heaviest and the lightest varieties were Carnaroli and PL12, both long-grained.

4.3.2 X-ray diffraction patterns

Diffractograms were obtained from chalky grains for all varieties, together with whole grains for crystalline varieties. In the case of pearled varieties, the pearled grains were classified in two groups according to the grain pearl area to check if the size of the pearl affected the diffractogram. Specific grains of each variety, those which presented especially low or high pearl areas, were selected considering their average pearl size. The small pearl grain was defined as having between a 10-25% of pearl area and a large pearl grain having a 30-50% of pearl area, all depending on the variety studied. All diffractograms are grouped in Figure 31.

All varieties presented very similar diffractograms for each kind of grain, with variations in their intensity. Their peaks were placed close to 15º, 17º, 18º and 23º. The patterns shape was almost the same for all varieties. Regarding the pearled varieties, for Montsianell, the chalky grain diffractogram presented a slightly higher intensity, with a maximum value of 2600, than the ones from both pearl measurements, which almost matched completely. In the case of Bomba, the three diffractograms practically matched, being the variety that presented the lowest maximum intensity, with a value of 2200. Carnaroli also had the chalky diffractogram as the more intense, with a maximum value of 3000 counts. The small pearl diffractogram matched the chalky diffractogram in the peak zones, and the large pearl diffractogram presented a completely visible diffractogram due to its lowest intensity. Regarding the crystalline varieties, Guadiamar presented a chalky grain diffractogram with a maximum count value of 2350, slightly higher than the normal grain diffractogram. PL12 presented by far the highest intensities and the highest diffractograms differences in terms of intensity. The maximum value of the most intense diffractogram, the whole grain one, was 3800. On the other hand, the chalky diffractogram presented a maximum value of 2950.

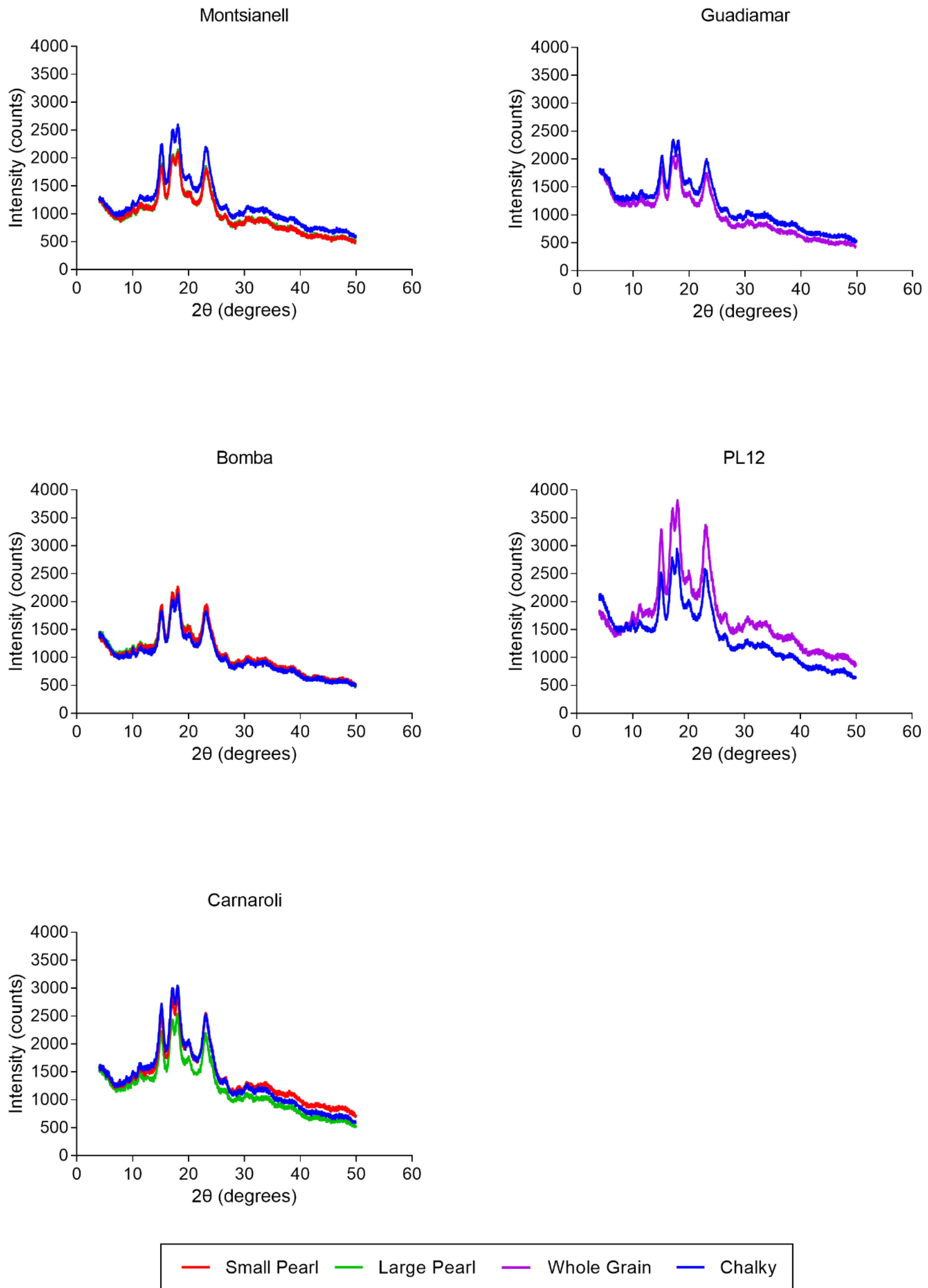


Figure 31. Diffractograms for all the varieties and grain types studied. At the left, the pearled varieties, and at the right, the crystalline varieties. Diffractograms of grains with a small pearl size are represented in red, those of grains with a pearl of big size are represented in green. Whole grain diffractograms for crystalline varieties are represented with a purple line. Chalky grain diffractograms for all varieties are represented in blue.

Crystallinity percentages were calculated from the area contained beneath the peaks of the diffractograms. Values are reflected in Table 14. Statistics are reflected in Table SM 15.

Table 14. Crystallinity percentages for the varieties studied. Values are presented as mean ± SEM. Different letters within a variety express significant differences ($p < 0.05$).

	Montsianell	Bomba	Carnaroli	Guadiamar	PL12
Large pearl (%)	45.12 ± 1.08[a]	45.48 ± 2.31[a]	40.02 ± 0.86[a]		
Small pearl (%)	44.90 ± 1.62[a]	44.63 ± 1.01[a]	40.06 ± 2.17[a]		
Whole grain (%)				54.22 ± 3.97[a]	43.92 ± 1.45[b]
Chalky grain (%)	40.93 ± 1.44[a]	48.57 ± 0.92[a]	38.53 ± 1.19[a]	50.34 ± 3.03[a]	53.21 ± 2.04[a]

As well as in the diffractograms, no significant differences were appreciated in the crystallinity values within the same variety, except for PL12, whose diffractograms varied more clearly in intensity. The pointiness of the diffractogram peaks determine the crystallinity values. For Montsianell and Carnaroli, their peaks were pointier in the chalky diffractograms than in the pearl ones, with the chalky crystallinity percentage being the lowest of the three diffractograms. The same happened with PL12 and the whole grain diffractogram, although having the highest intensity level, it had a lower crystallinity index value than the chalky diffractogram due to this pointier aspect.

4.3.3 Amylose content determination

The amylose content was determined from the different fractions depending on the type of grain. Results are reflected in Figure 32. Statistics are found in Table SM 16.

In the case of the pearled varieties, no significant differences were observed between the whole grain and its fractions, but they appeared between them and the chalky grain for Montsianell and Bomba. Regarding Carnaroli, no significant differences were observed between any of the groups, although the chalky value was lower than those of the whole grain and their fractions. Considering the crystalline varieties, significant differences in the percentage of amylose content was observed between the whole grain and the chalky grain for both Guadiamar and PL12 varieties.

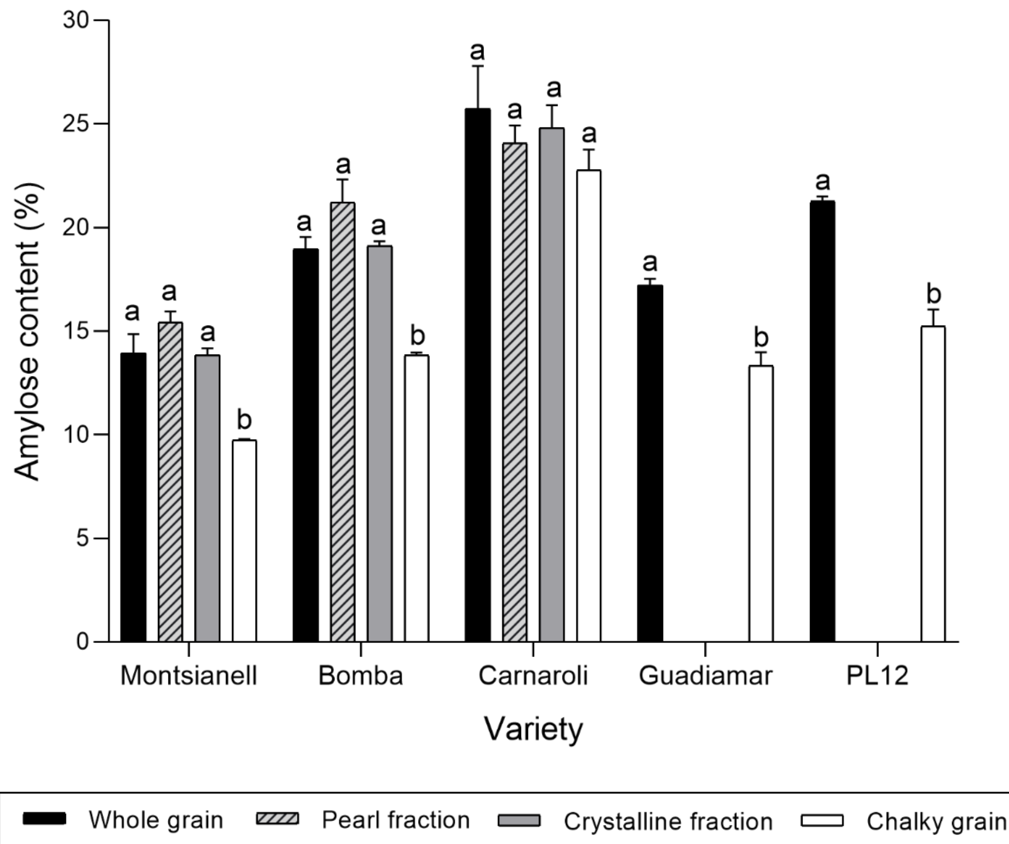


Figure 32. Percentage of amylose content for each variety and fraction. Black bars represent the amylose content in normal whole grains, listed grey bars represent the content of the pearl fraction in the pearled varieties, grey bars represent the content of the crystalline fraction in the pearled varieties, and white bars represent the content for whole chalky grains in all varieties. Values are presented as mean ± SEM. Different letters within a variety indicate significative differences between groups ($p < 0.05$).

4.3.4 Relative storage proteins content determination

SDS-PAGE was performed with 15% acrylamide gels in order to properly separate and quantify the different storage proteins fractions. The position of each group of proteins within the gel is observed in Figure 33. All gel pictures can be observed in Figure SM 3.

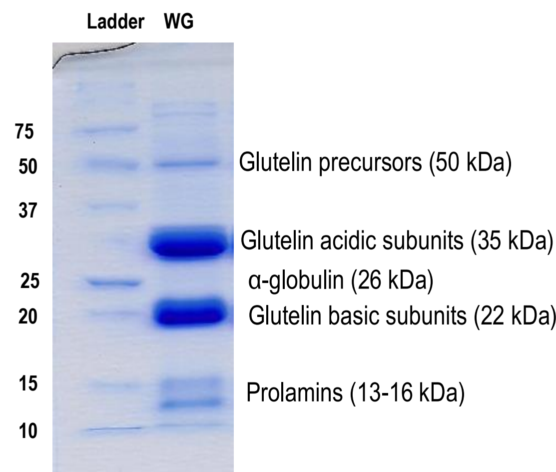


Figure 33. Position of the main protein groups of the rice grain in a 15% acrylamide gel. The values of the ladder bands (kDa) are indicated at the left.

A densitometry analysis was carried on to quantify the relative percentages of each protein fraction regarding the total storage proteins content, which are reflected in Table 15. Statistics are reflected in Table SM 17. The globulin fraction was not considered since its band was almost inappreciable in all gels.

Table 15. Relative percentages of various storage protein fractions of the rice grain for each variety. Values are presented as mean ± SEM. Different letters between groups within a variety indicate significant differences for that specific fraction ($p < 0.05$). WG = whole grain. PF = pearl fraction. CF= crystalline fraction. CG= chalky grain.

		Glutelin precursor (%)	Glutelin acidic subunit (%)	Glutelin basic subunit (%)	Prolamins (%)
Montsianell	WG	3.24 ± 0.09[b]	21.83 ± 0.88[a]	16.09 ± 0.66[a]	11.58 ± 0.32[a]
	PF	3.43 ± 0.02[b]	21.50 ± 0.72[a]	16.91 ± 0.52[a]	12.09 ± 0.25[a]
	CF	3.28 ± 0.05[b]	21.70 ± 0.61[a]	16.83 ± 0.60[a]	11.69 ± 0.34[a]
	CG	4.13 ± 0.11[a]	20.31 ± 0.30[a]	15.17 ± 0.36[a]	9.51 ± 0.07[b]
Bomba	WG	3.00 ± 0.34[a]	15.70 ± 0.38[a]	13.42 ± 0.29[a]	11.67 ± 0.53[a]
	PF	2.85 ± 0.25[a]	16.59 ± 0.46[a]	14.55 ± 0.15[a]	11.47 ± 0.89[a]
	CF	3.26 ± 0.3[a]	17.69 ± 1.14[a]	15.81 ± 0.98[a]	13.92 ± 1.70[a]
	CG	3.77 ± 0.18[a]	17.56 ± 1.08[a]	15.68 ± 0.86[a]	12.18 ± 1.43[a]
Carnaroli	WG	1.71 ± 0.36[a]	16.00 ± 0.23[a]	10.64 ± 0.73[a]	11.80 ± 0.48[a]
	PF	1.67 ± 0.34[a]	16.19 ± 0.23[a]	10.90 ± 0.56[a]	11.86 ± 0.35[a]
	CF	1.59 ± 0.29[a]	15.72 ± 0.55[a]	10.40 ± 0.05[a]	12.32 ± 0.69[a]
	CG	1.56 ± 0.21[a]	16.27 ± 0.70[a]	10.51 ± 0.22[a]	11.46 ± 0.90[a]
Guadiamar	WG	3.46 ± 0.10[b]	19.91 ± 0.32[a]	18.15 ± 0.39[a]	14.12 ± 0.25[a]
	CG	3.88 ± 0.03[a]	19.03 ± 0.24[a]	17.56 ± 0.06[a]	11.87 ± 0.24[b]
PL12	WG	3.36 ± 0.12[a]	21.53 ± 0.58[a]	16.17 ± 1.02[a]	14.13 ± 0.64[a]
	CG	3.61 ± 0.03[a]	19.92 ± 0.41[a]	15.64 ± 0.49[a]	12.11 ± 0.30[b]

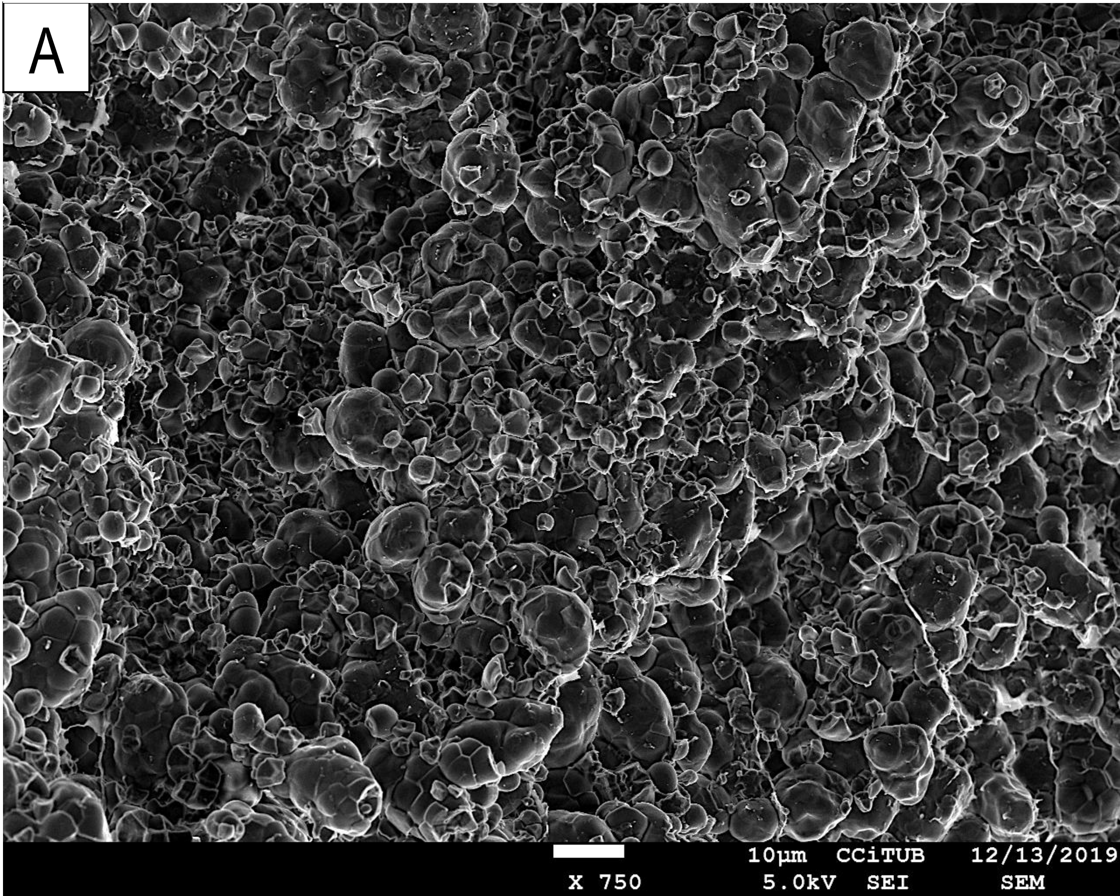
Regarding the pearled varieties, a pattern cannot be established in all three varieties regarding the quantities of the different protein groups for each fraction. For Montsianell, significant differences appeared between the measurements of the normal grain and chalky grain in the quantity of glutelin precursor and prolamins. Besides, although there was no statistical significance, in both glutelin subunit measurements the chalky grain presented the lowest values. In the case of Bomba, no significant differences were statistically observed, but the measurements followed some tendencies. The glutelin precursor presented the highest value for the chalky grain, same as Montsianell. In the

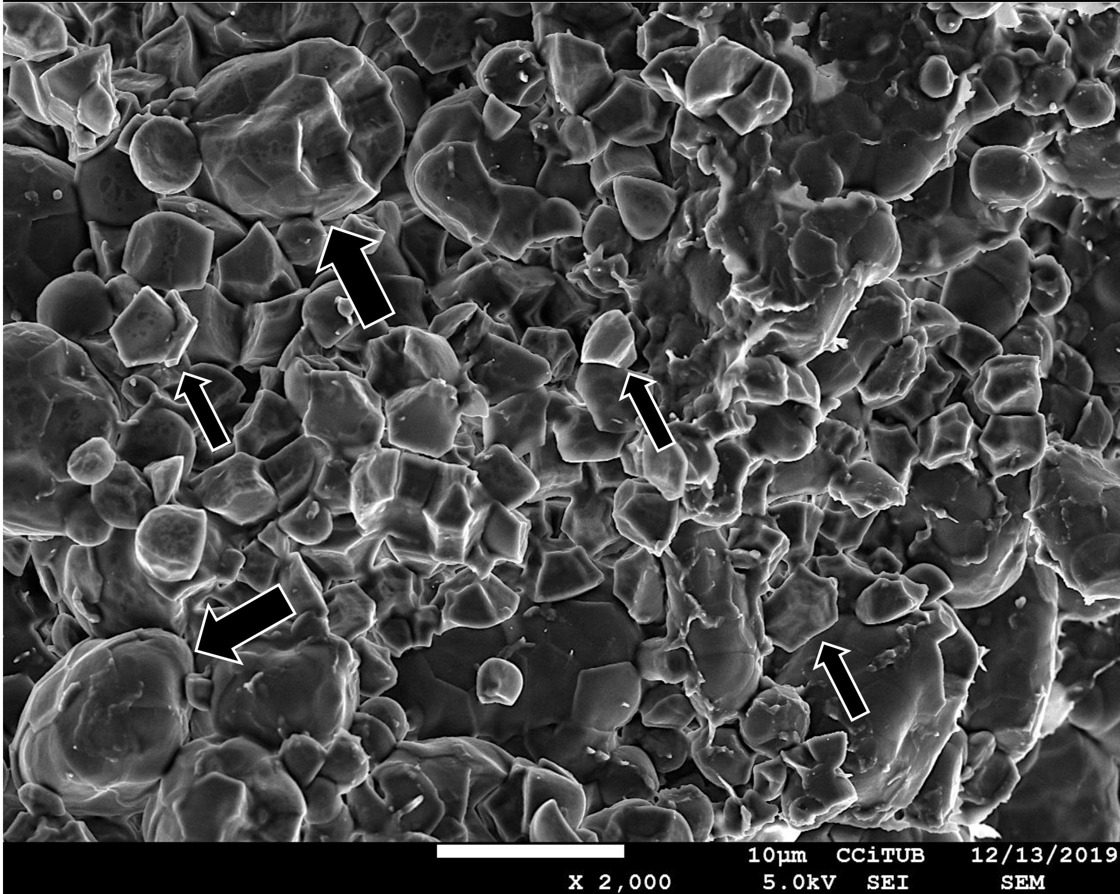
case of both glutelin subunits, the highest value appeared in the crystallin part and the lowest, for the whole grain. The lowest value of the prolamins appeared in the pearl fraction, while the highest corresponded to the crystalline fraction. In the case of Carnaroli, different distribution patterns of the protein groups were followed, also with no significant differences. Contrary to Montsianell and Bomba, the glutelin precursor presented the lowest value in the chalky grain, and the highest in the whole grain. For the glutelin acidic subunit fraction, the chalky grain had the highest value, and the crystalline fraction of the pearled grain, the lowest. In the case of the glutelin basic subunit, the highest and the lowest value corresponded to the pearled and crystalline fraction respectively. For the prolamins, the lowest value belonged to the chalky grain, as it happened with Montsianell.

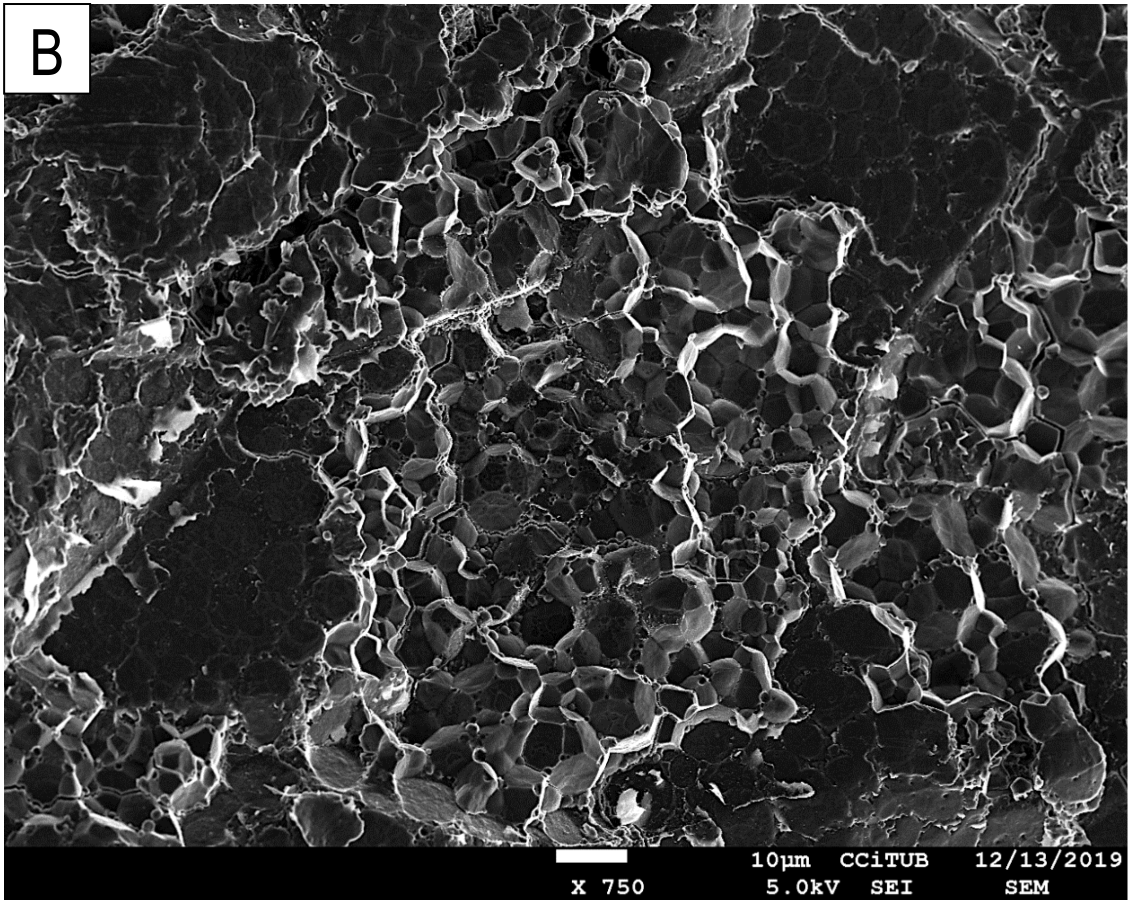
Regarding the crystalline varieties, Guadiamar and PL12, they followed the same pattern in the distribution of the different protein fractions. For the glutelin precursor, the lowest value appeared in the whole grain, and the highest, in the chalky grain, with significant differences for Guadiamar. For all three glutelin subunits and prolamins, the highest value belonged to the whole grain and the lowest to the chalky grain, with significant differences for the prolamins in both varieties.

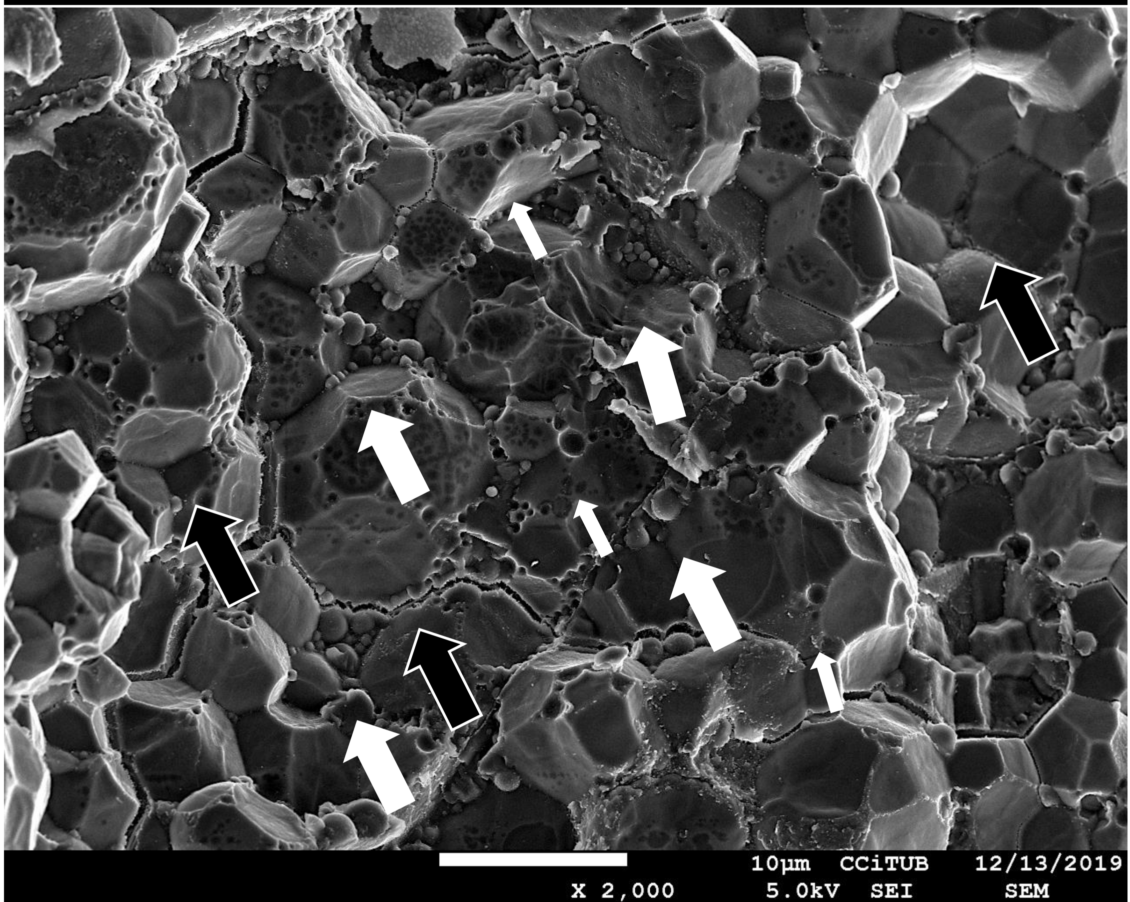
4.3.5 Scanning electron microscopy (SEM)

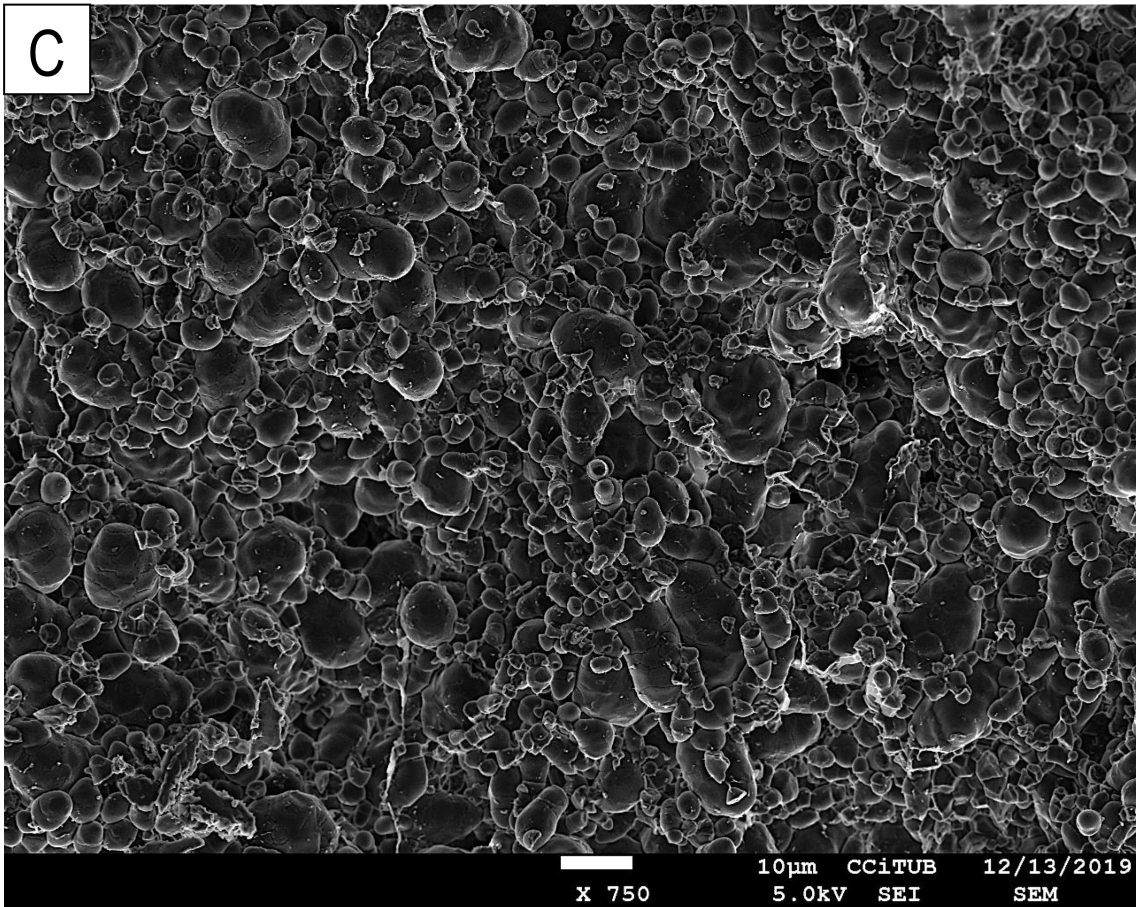
Transversal sections of both normal and chalky grains of each variety were observed with scanning electron microscopy. For the pearled varieties, pictures were taken considering the two distinguishable parts of the grain, the pearl part and crystalline part. In the case of crystalline varieties, the pictures taken were representative for the whole grain area. Chalky grains were also photographed for each variety. Many pictures were taken, and the most representative for each type variety and area were selected. The pictures are compiled in Figure 34.

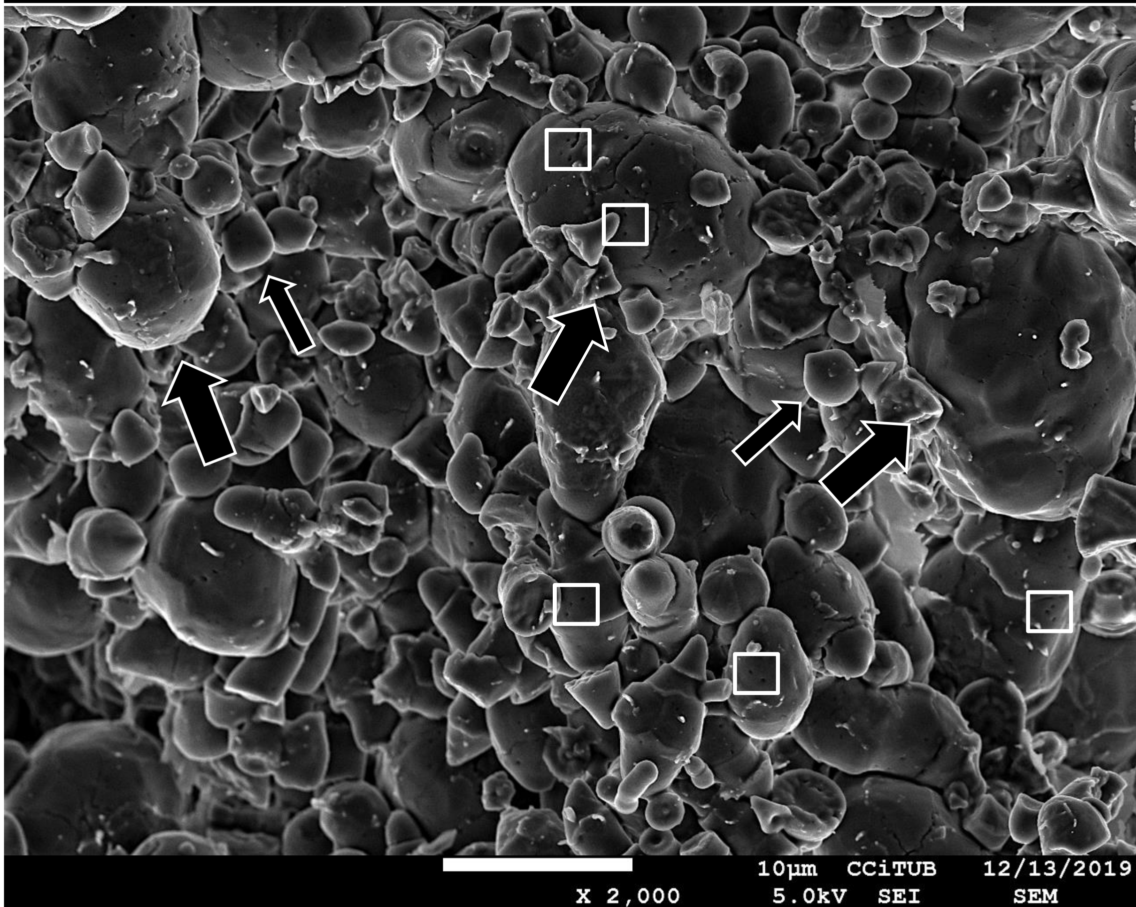

A
10μm CCiTUB 12/13/2019
X 750 5.0kV SEI SEM

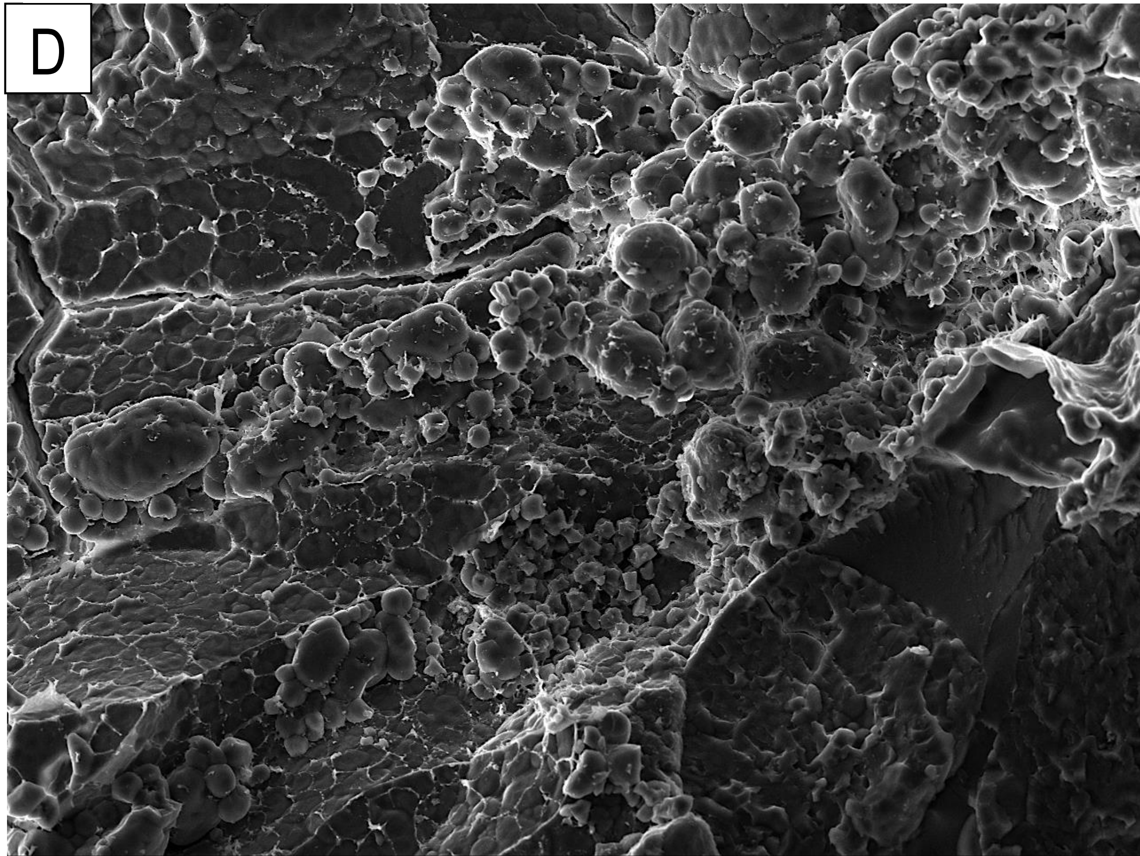

10μm CCiTUB 12/13/2019
X 2,000 5.0kV SEI SEM

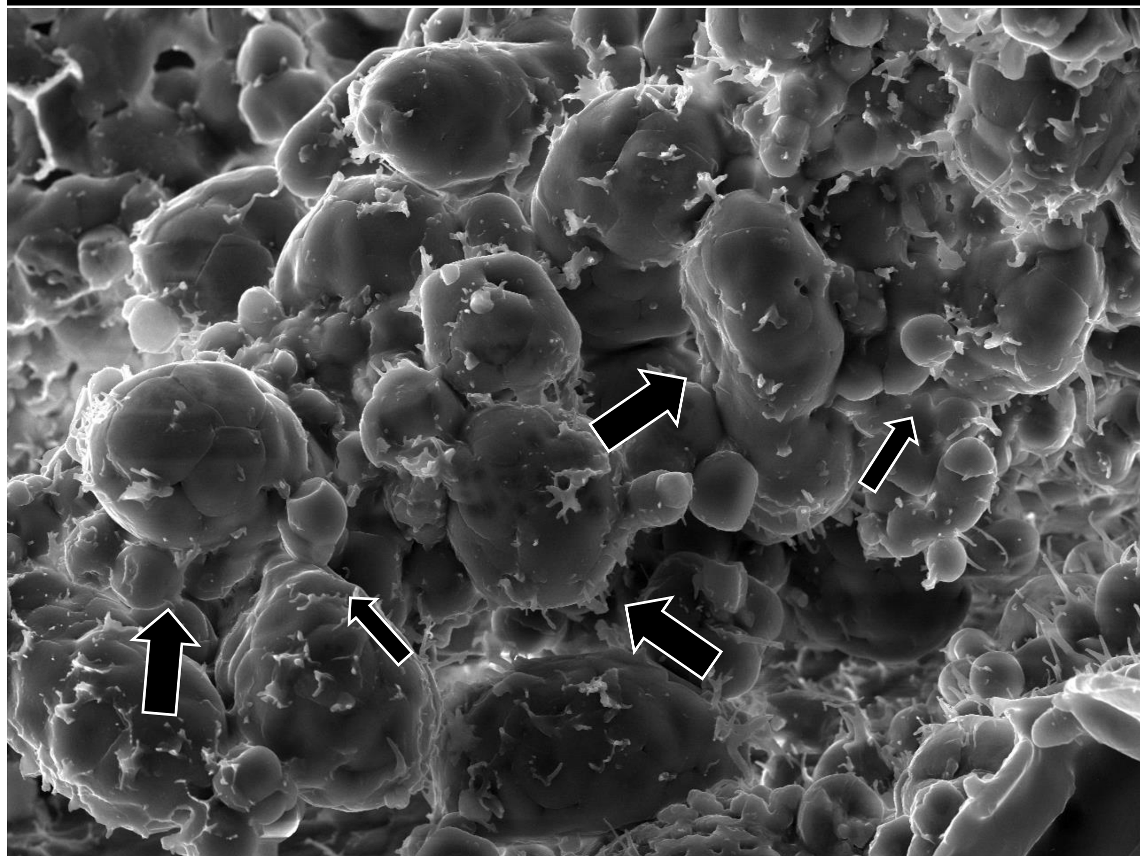

B
10μm CCiTUB 12/13/2019
X 750 5.0kV SEI SEM


10μm CCiTUB 12/13/2019
X 2,000 5.0kV SEI SEM


C
10µm CCiTUB 12/13/2019
X 750 5.0kV SEI SEM


10µm CCiTUB 12/13/2019
X 2,000 5.0kV SEI SEM


D
10µm CCiTUB 2/6/2020
X 750 10.0kV SEI SEM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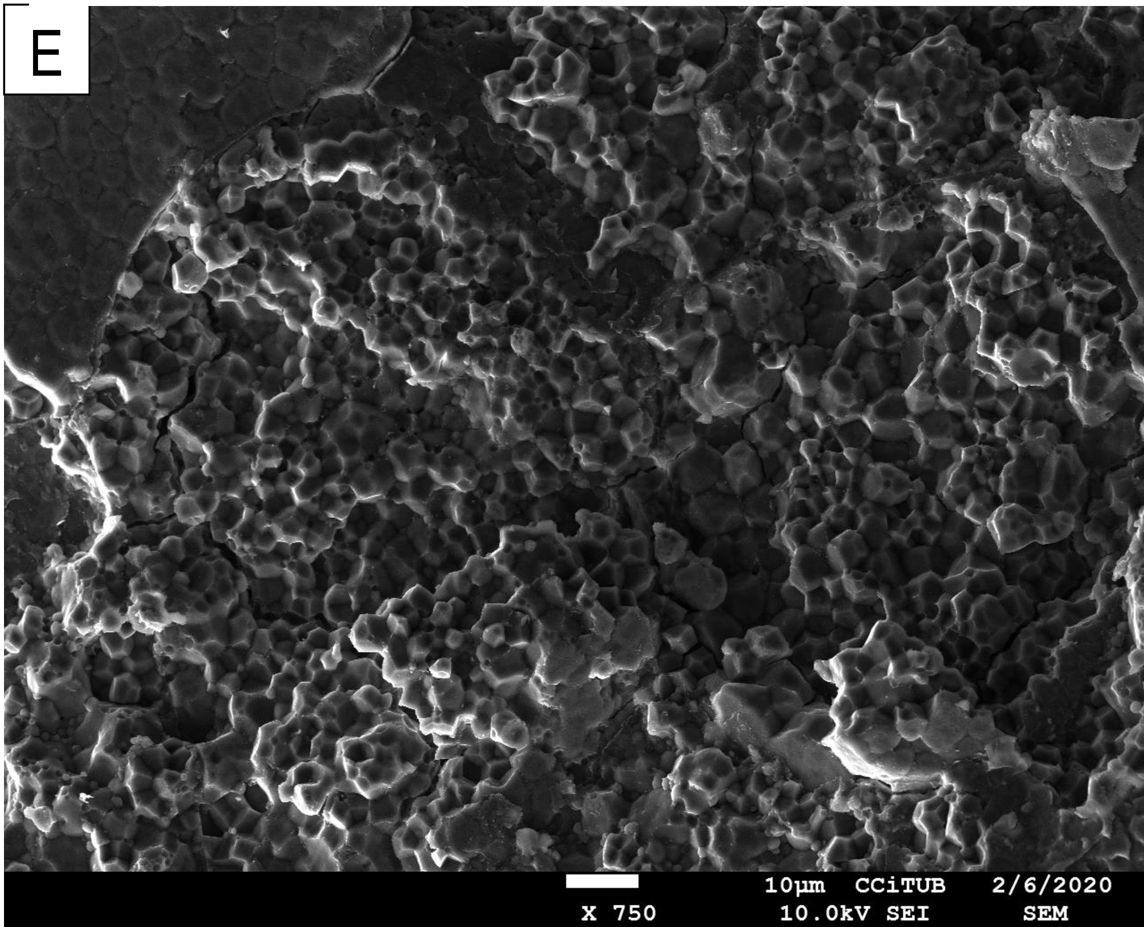
X 2,000 10.0kV SEI SEM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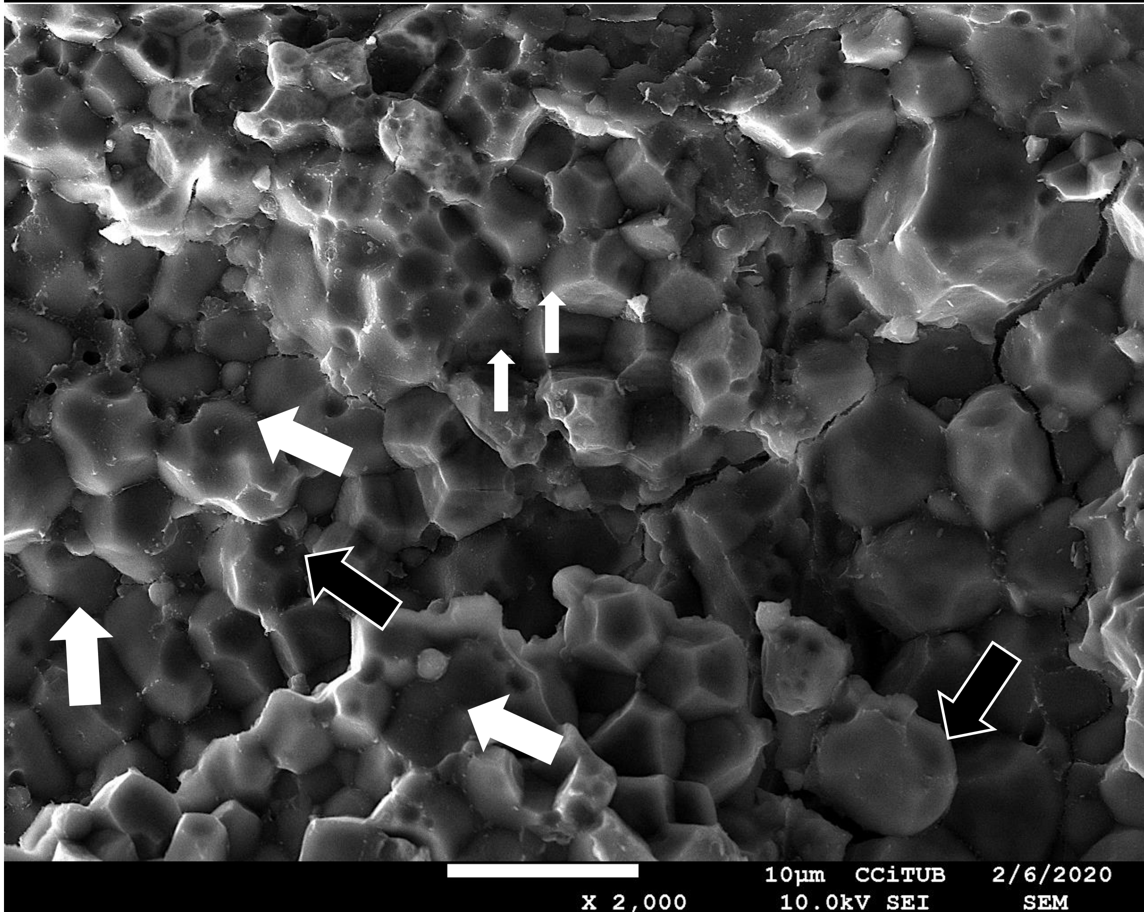
X 750 10.0kV SEI SEM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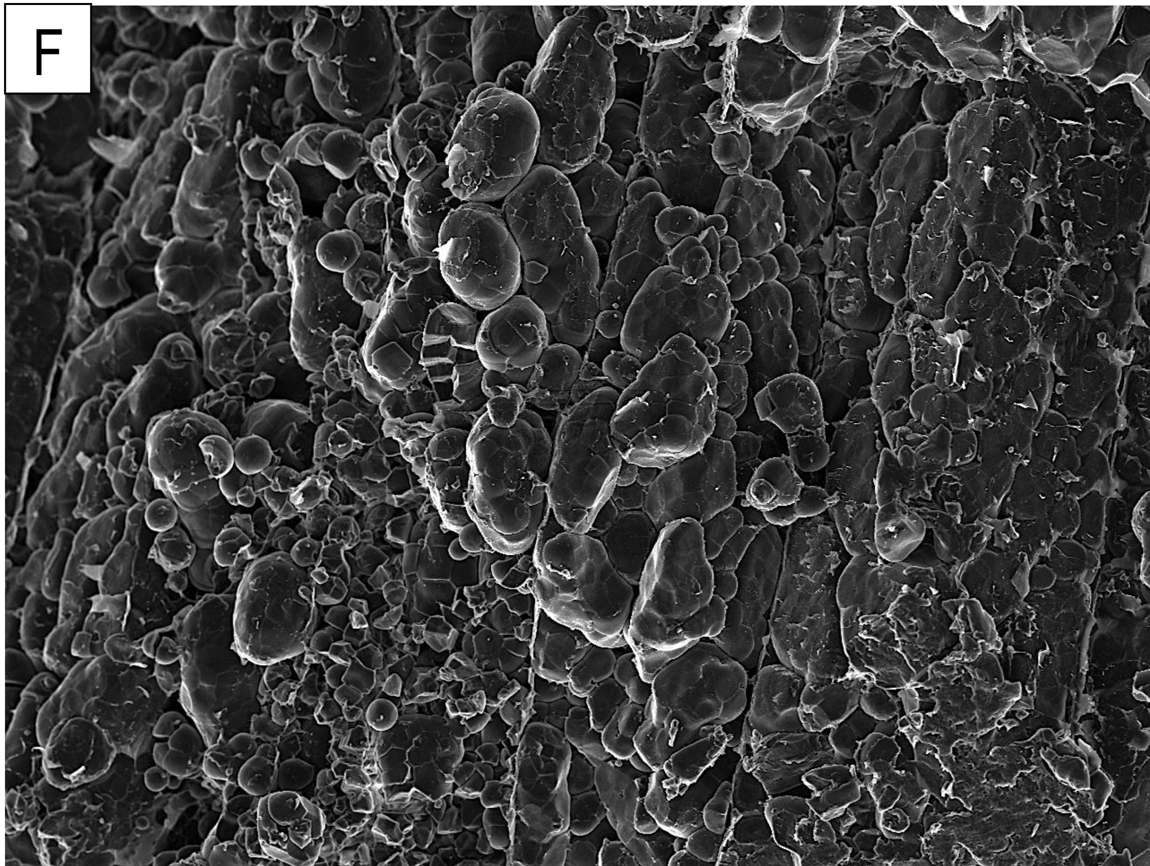
X 2,000 10.0kV SEI SEM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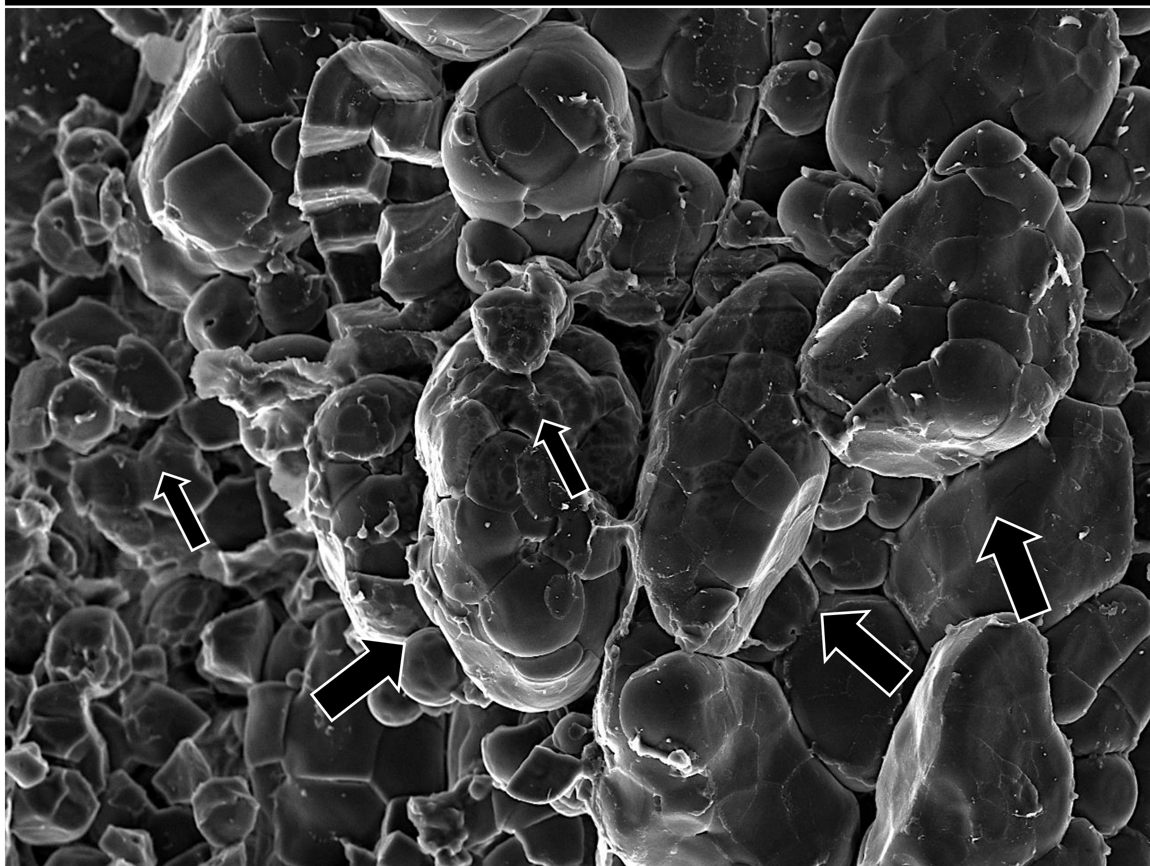
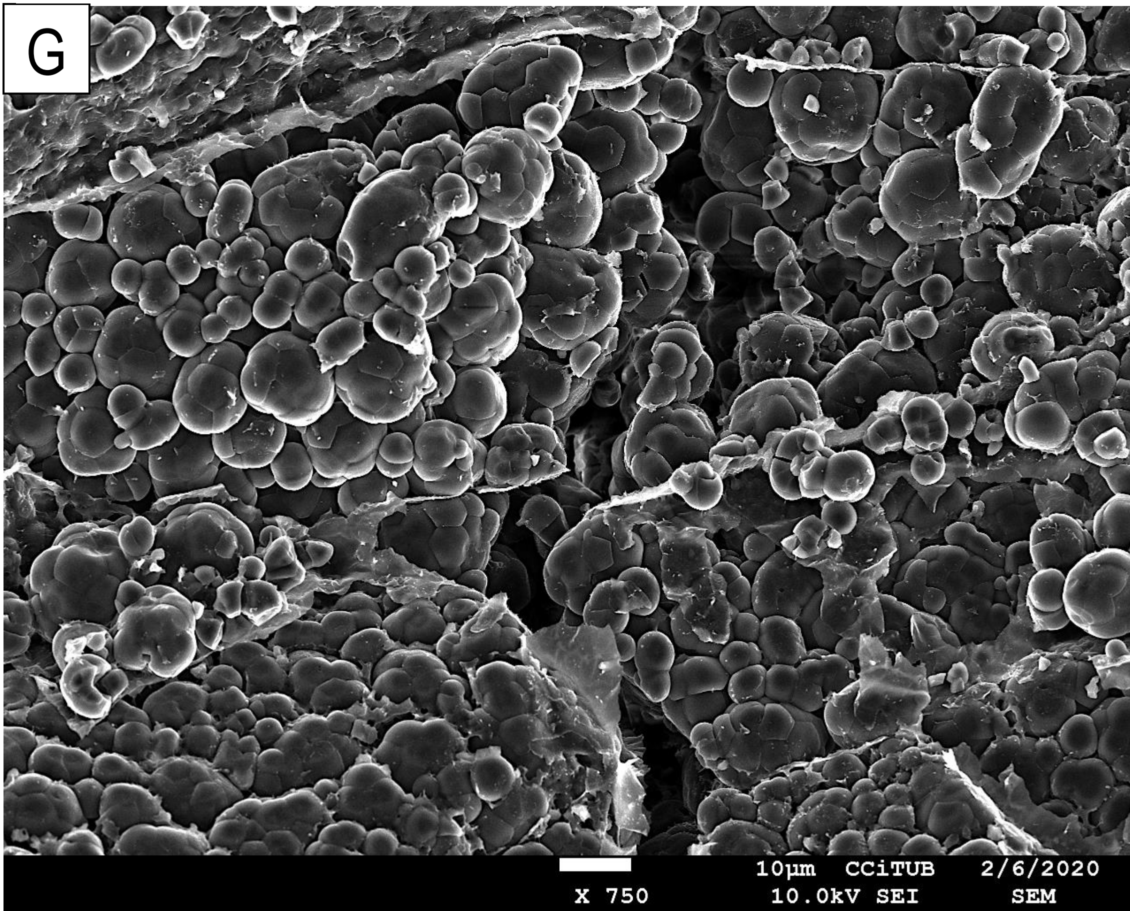
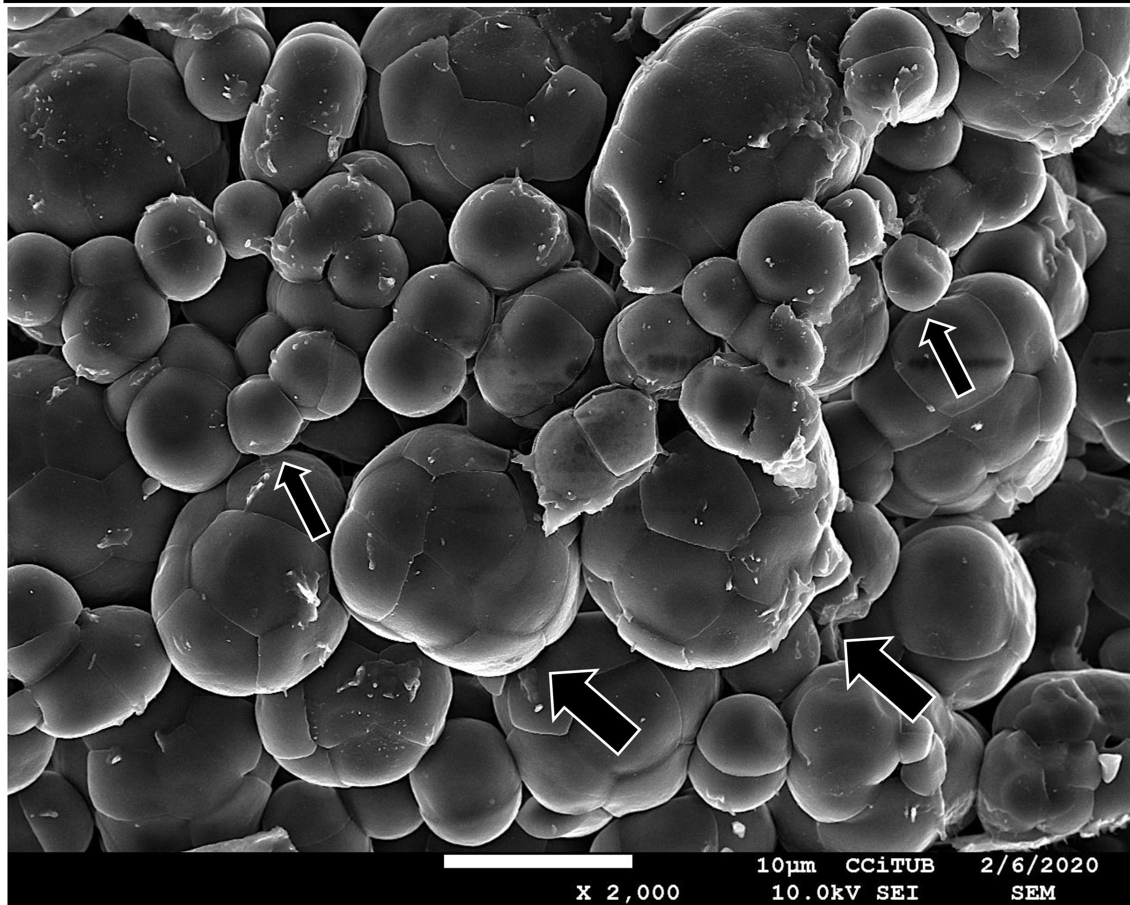
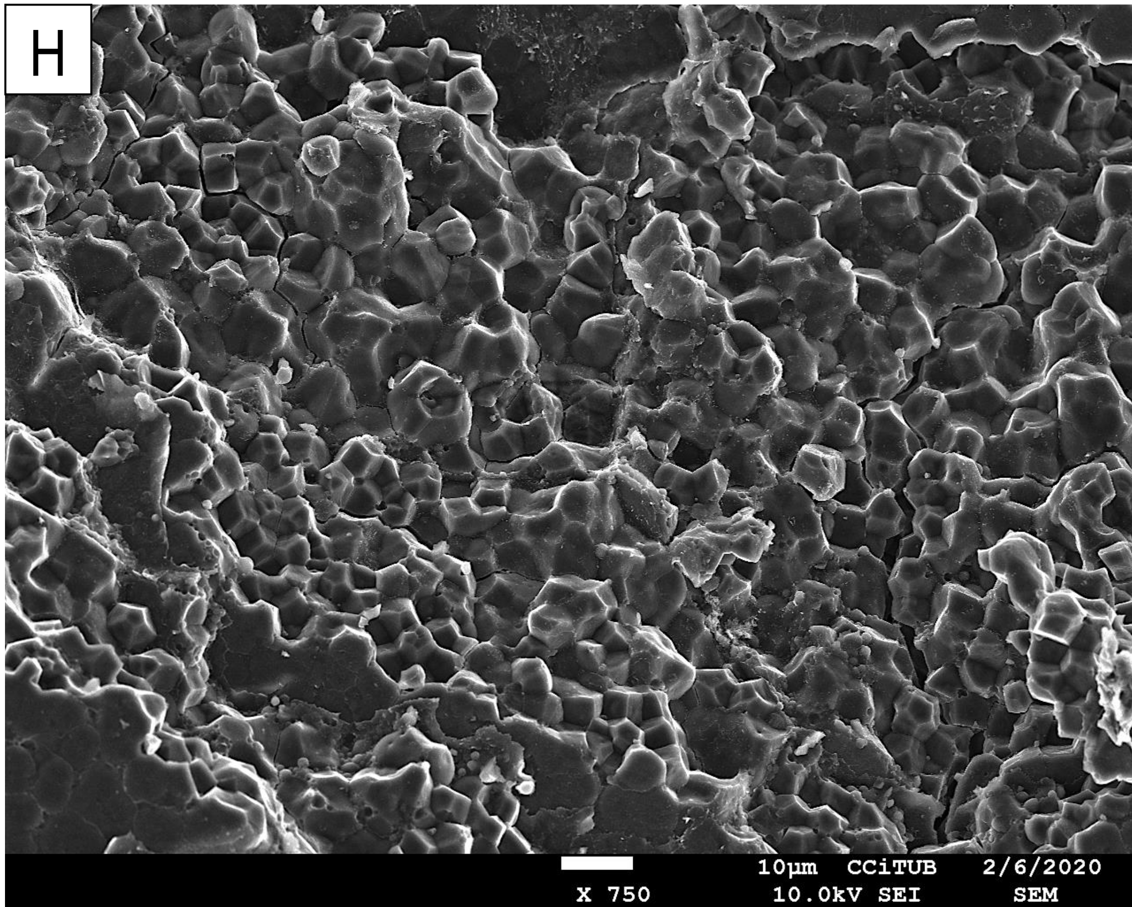
X 750 5.0kV SEI SEM


10µm CCiTUB 1/23/2020
X 2,000 5.0kV SEI SEM


G
10µm CCiTUB 2/6/2020
X 750 10.0kV SEI SEM


10µm CCiTUB 2/6/2020
X 2,000 10.0kV SEI SEM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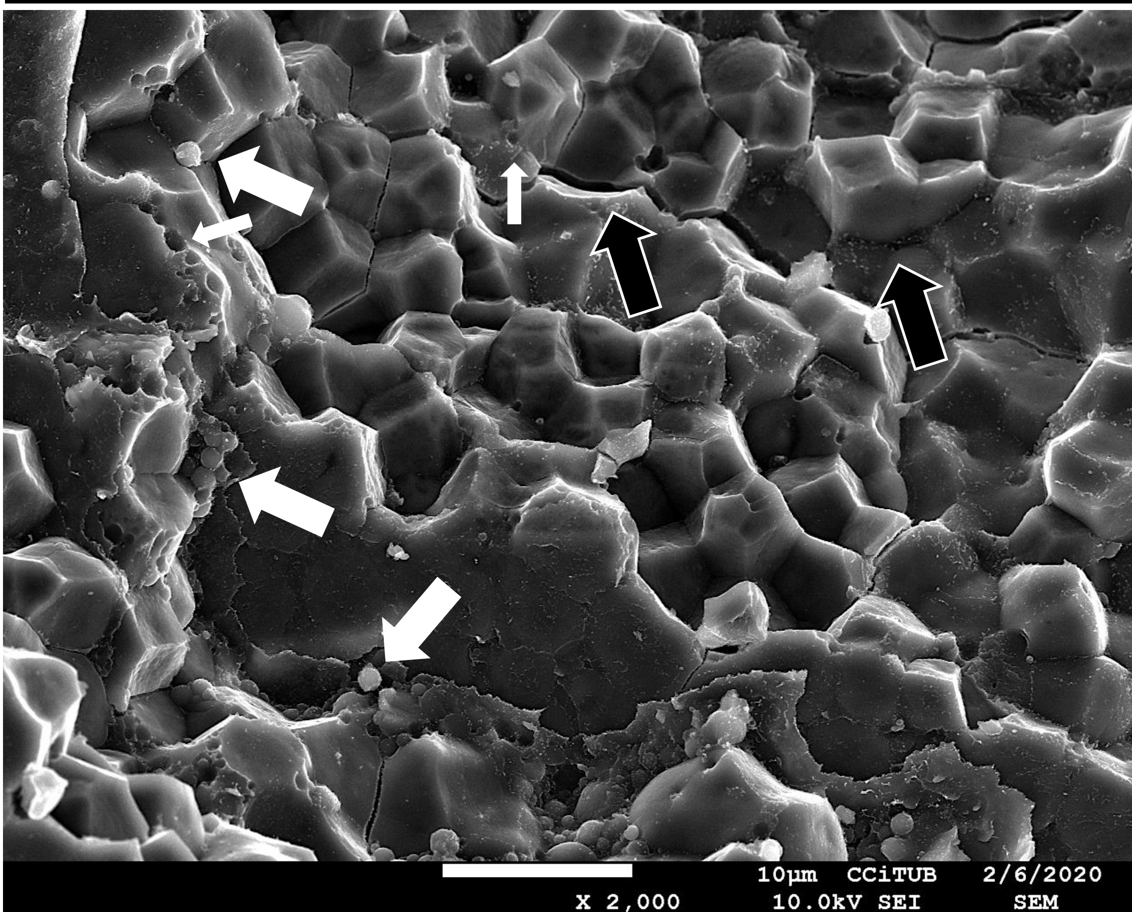
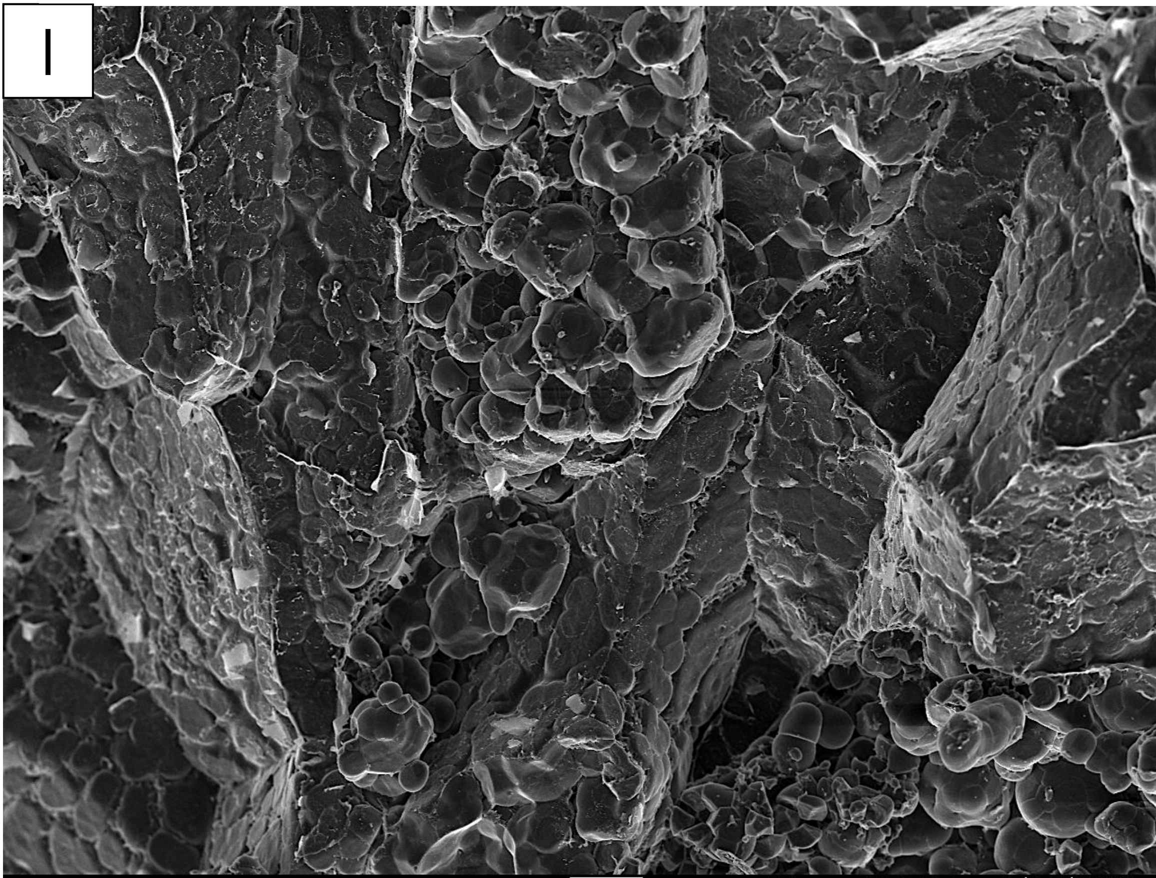
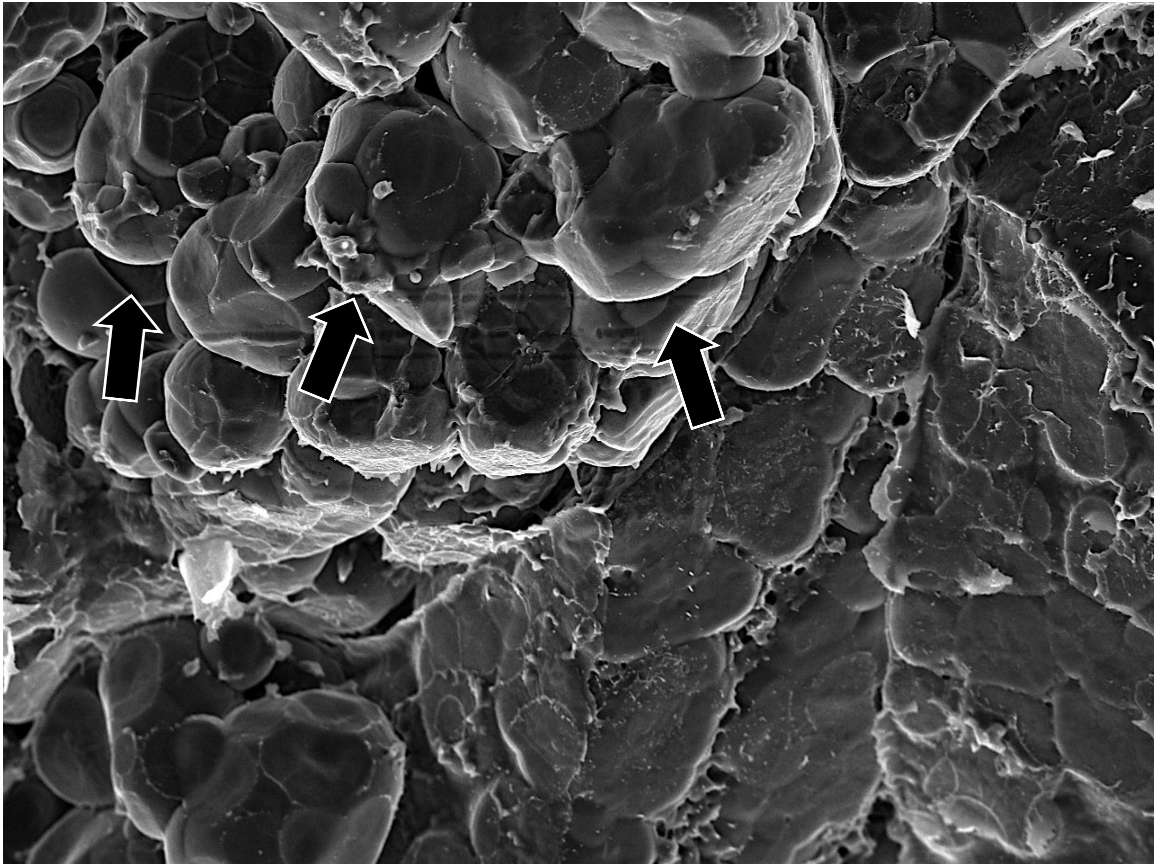
10µm CCiTUB 2/6/2020
X 750 10.0kV SEI SEM


10µm CCiTUB 2/6/2020
X 2,000 10.0kV SEI SEM


I
10µm CCiTUB 1/23/2020
X 750 5.0kV SEI SEM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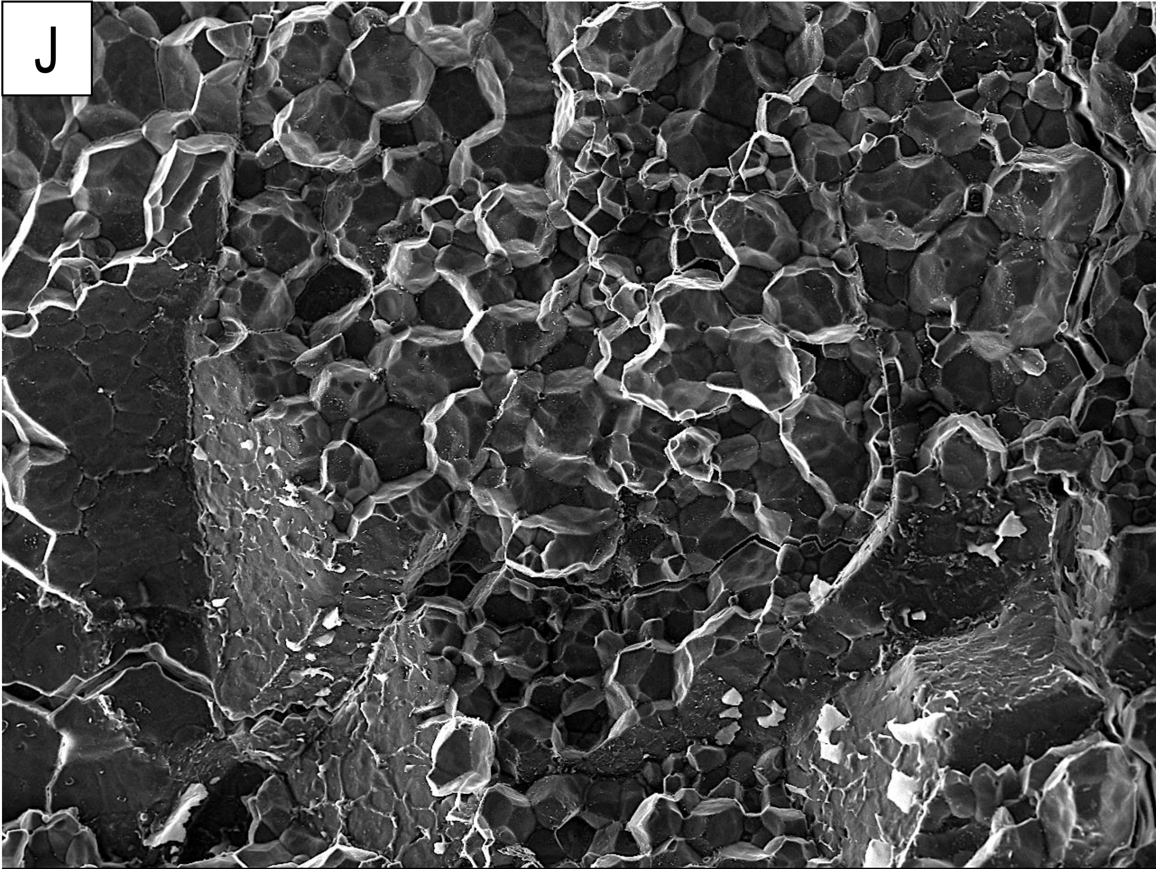
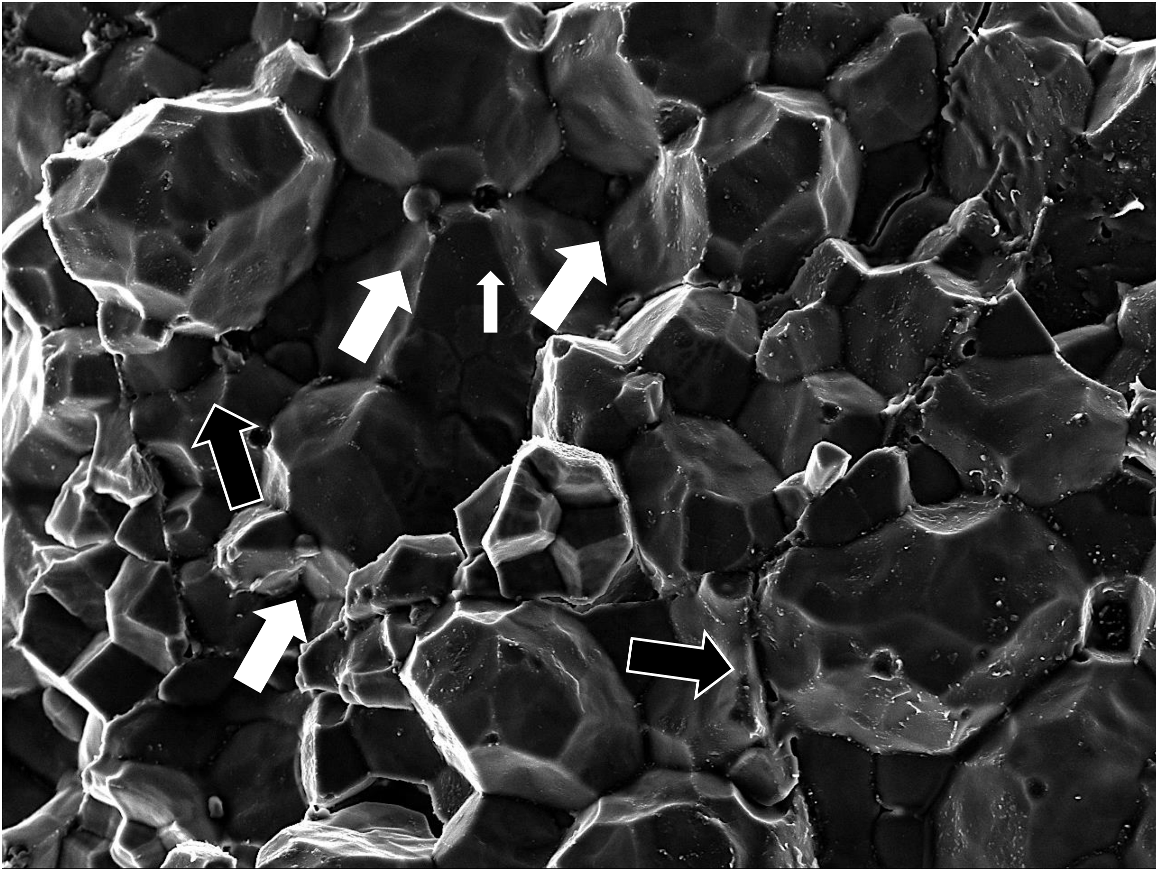
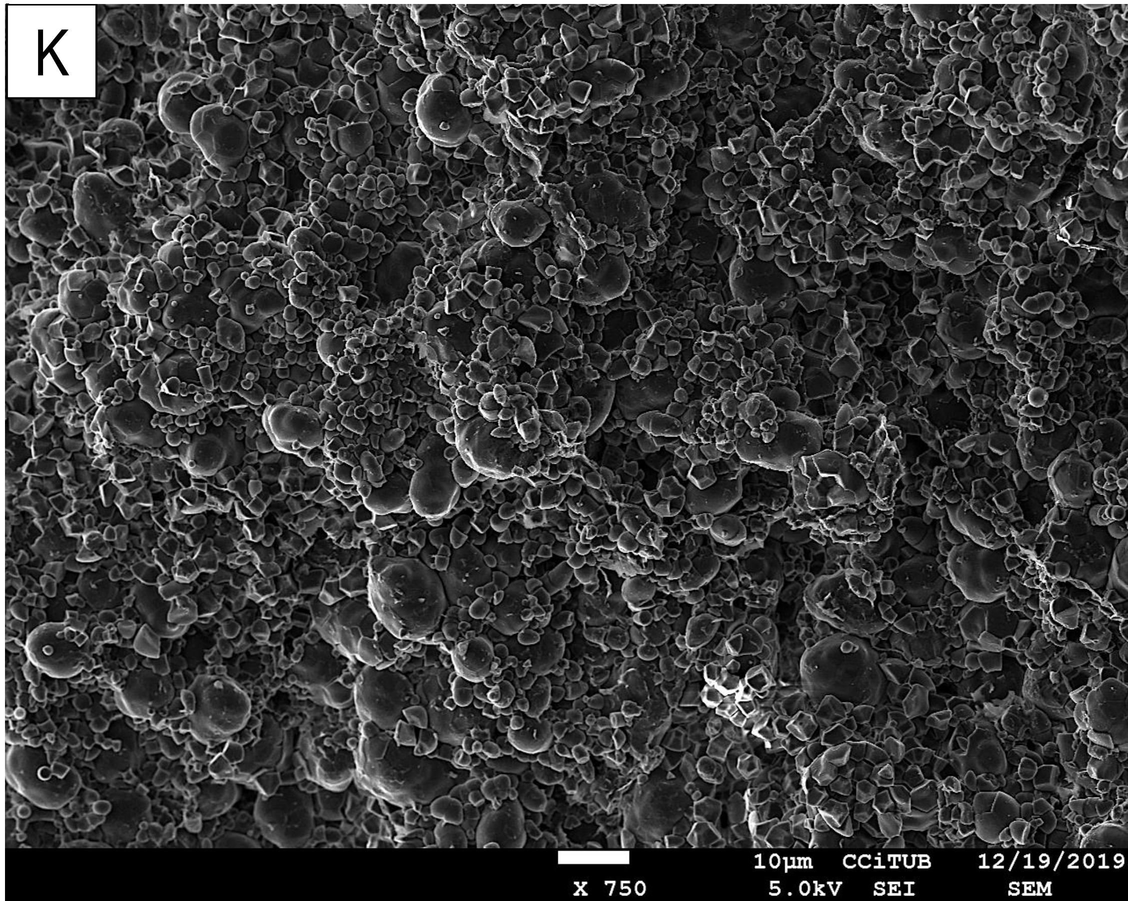
X 2,000 5.0kV SEI SEM


J
10μm CCiTUB 12/19/2019
X 750 5.0kV SEI SEM


10μm CCiTUB 12/19/2019
X 2,000 5.0kV SEI SEM


K
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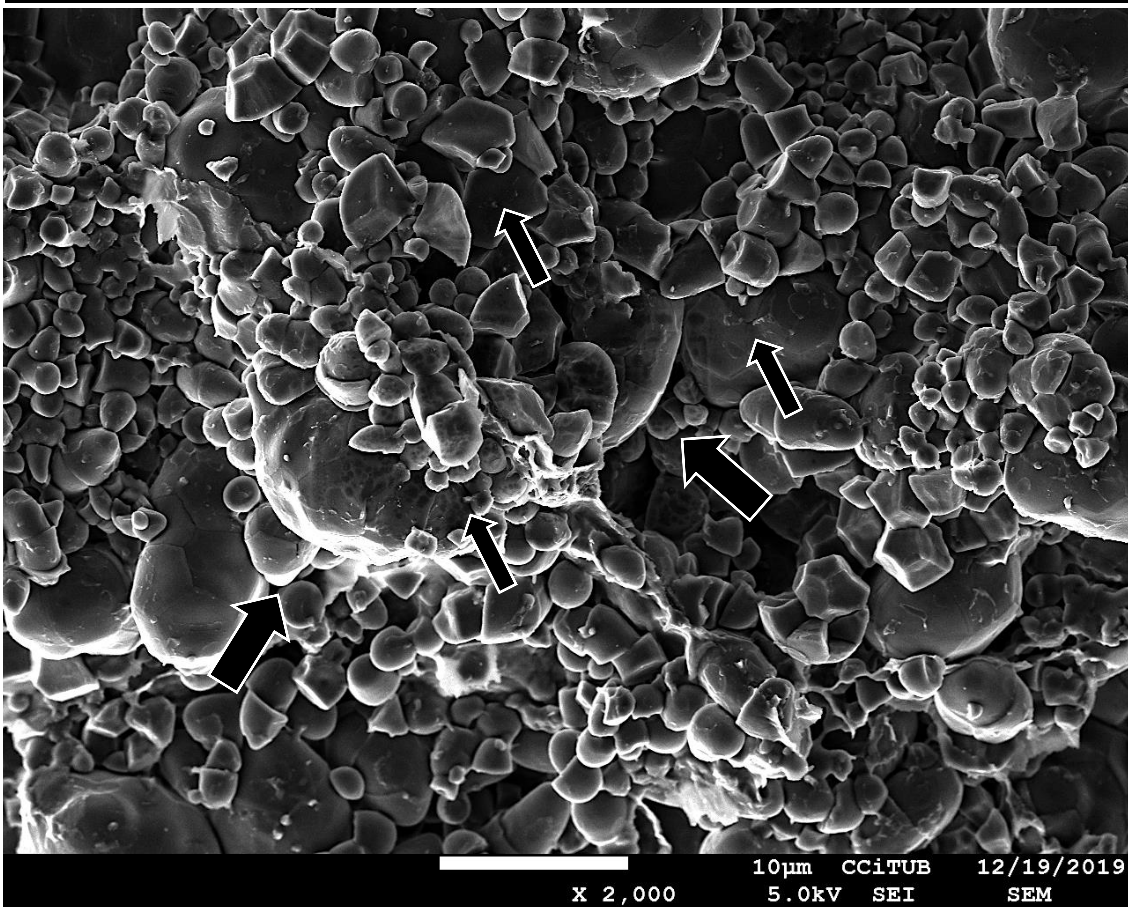
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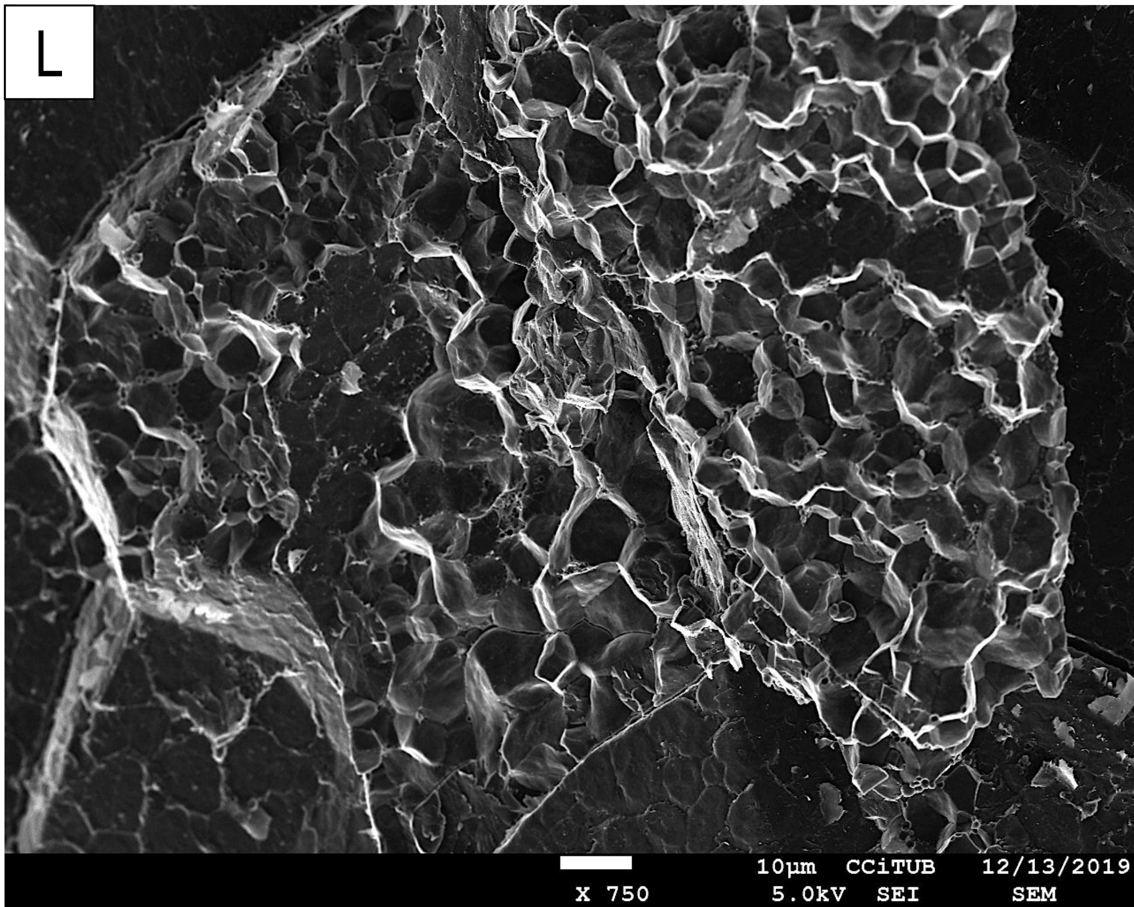
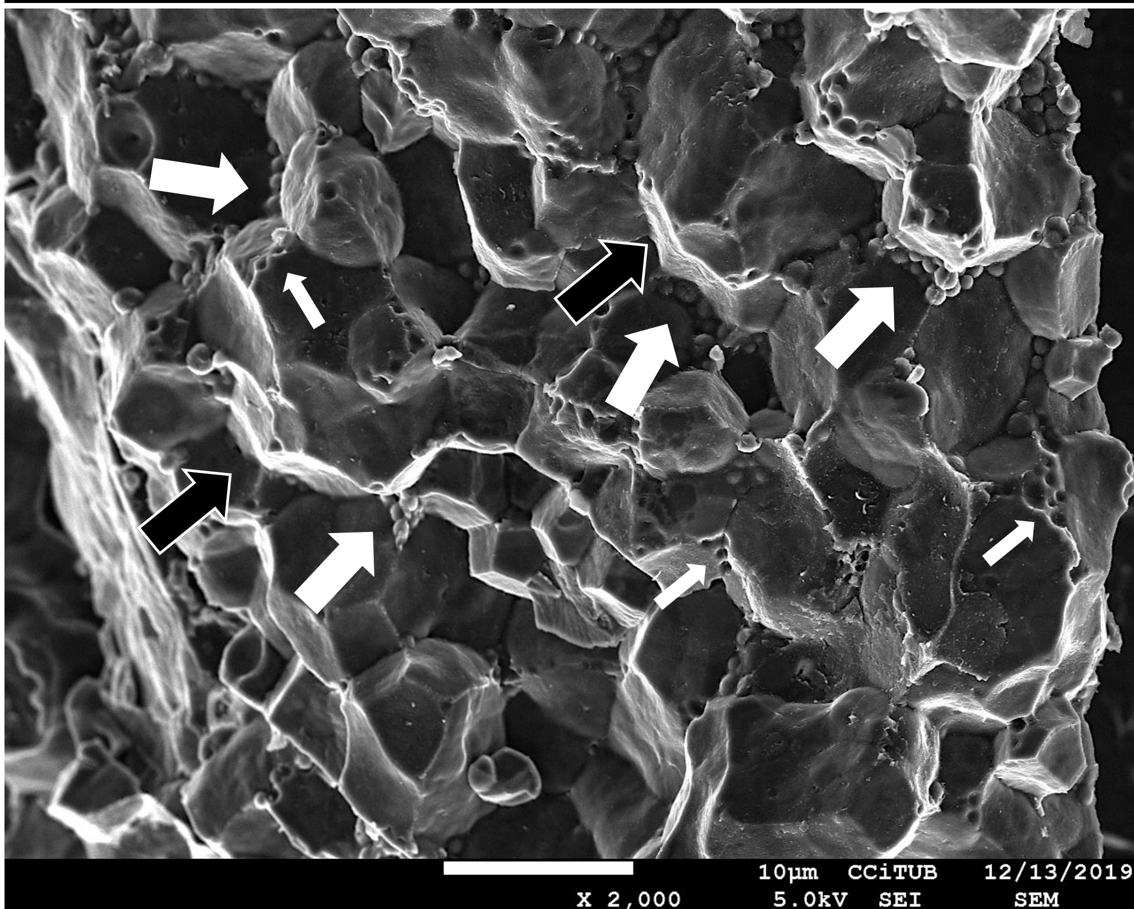
X 2,000 5.0kV SEI SEM


L
10µm CCiTUB 12/13/2019
X 750 5.0kV SEI SEM


10µm CCiTUB 12/13/2019
X 2,000 5.0kV SEI SEM

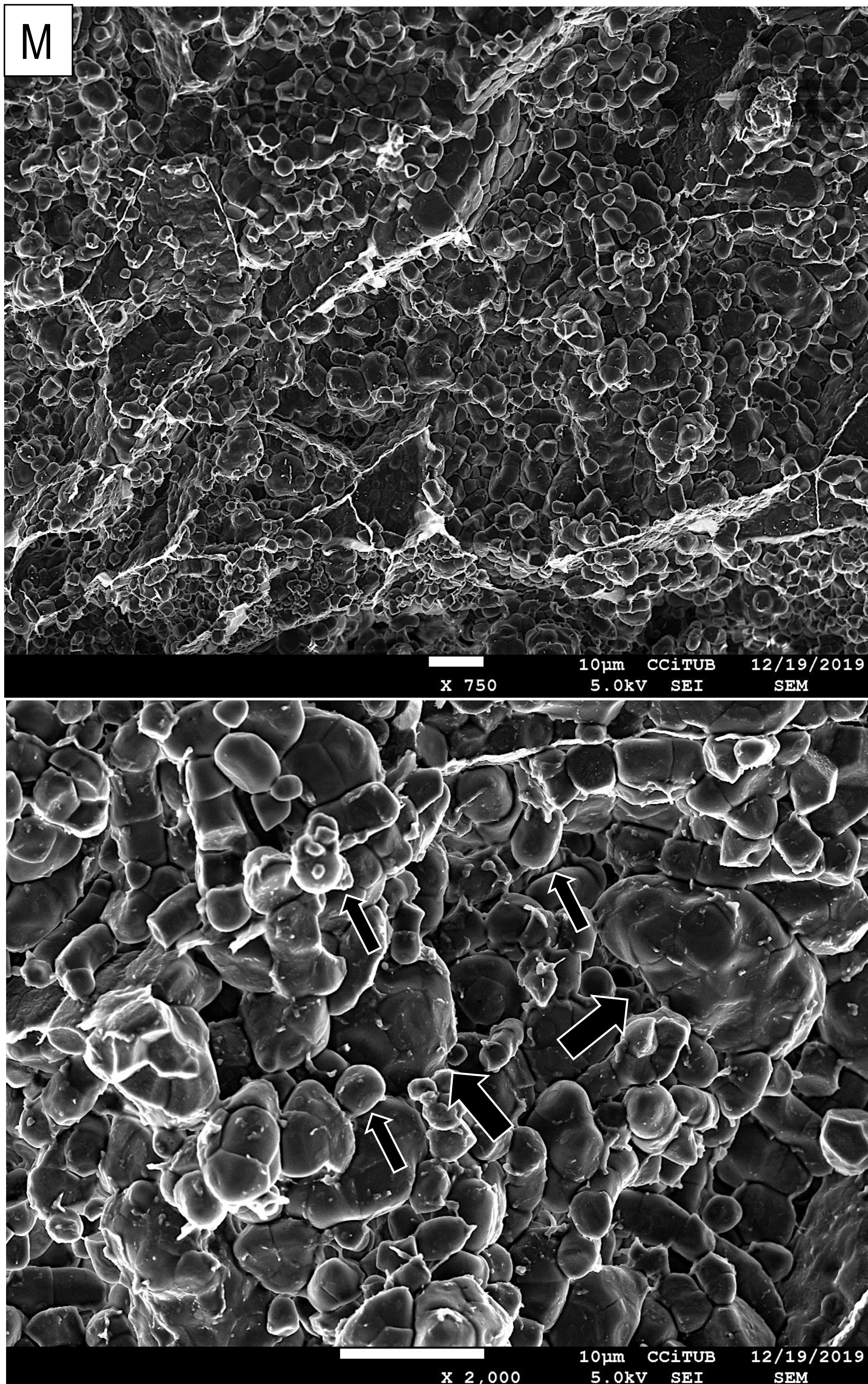


Figure 34. SEM images of diverse types of grains transversally cut. A= Montsianell pearl part. B= Montsianell crystalline part. C=Montsianell chalky grain. D= Bomba pearl part. E= Bomba crystalline part. F= Bomba chalky grain. G= Carnaroli pearl part. H= Carnaroli crystalline part. I= Carnaroli chalky grain. J= Guadiamar normal grain. K= Guadiamar chalky grain. L= PL12 normal grain. M= PL12 chalky grain. Large black arrows point to amyloplasts, small black arrows point to free starch granules, large white arrows point to protein bodies and small white arrows point to traces of protein bodies. White squares enclose micropores derived from α-amylase attacks.

Regarding the pearled varieties, Montsianell, Bomba and Carnaroli, the SEM images showed that there is a clear difference between the pearl part and the crystalline part of the grain in all three varieties. The pearl part presented a disorganized distribution of the starch granules and the amyloplasts, that contain a group of starch molecules, with many spaces between them. These amyloplasts were rounded and broke easily into separate starch granules when the grain was cut, as it can be observed in picture 33A. The separate starch granules presented an irregular shape. Despite of the similar arrangement of the pearl parts in all three varieties, Carnaroli seemed to be the variety that preserved the most the integrity of the amyloplasts when cut. Contrarily, the crystalline zones presented the starch granules perfectly arranged in the amyloplasts, with polyhedral shapes. There were almost no loose starch granules, and the protein bodies and their slots could be observed. Regarding the chalky grains of the pearled varieties, their appearance was practically the same as the pearl parts, with the disorganized and irregular amyloplasts and starch granules. As well as in the pearl part, the Carnaroli grains seemed to preserve more the integrity of the amyloplasts. The Montsianell chalky grain also presented a specificity, as many micropores can be observed in the starch granules surface. These were probably caused by α-amylase attacks.
As for the crystalline varieties, Guadiamar and PL12, their appearance was the same than the crystalline parts of the pearled grains, with the starch granules perfectly arranged inside the amyloplasts, with a polyhedral form and surrounded by protein bodies or their slots. The chalky grains of both varieties presented the same appearance as the chalky grains from the pearled varieties.

4.3.6 Chalk5 promoter analysis

In order to check if the *Chalk5* gene was the one causing the pearled phenotype in the japonica varieties studied, a concrete region of its promoter was sequenced with special attention to the positions -721 and -485. Li et al. (2014) proved that in indica varieties, the presence of a cytosine (C) and an adenine (A) in each position respectively, allowed the binding of cis elements that increased the gene expression, causing ultimately a pearled phenotype. The presence of a thymine (T) in both positions inhibited the binding of the regulatory elements, causing a low expression of the *Chalk5* gene and presenting ultimately a crystalline grain phenotype.

At least three individuals were sequenced per variety, and compared against the sequence of Zhensan97, a model pearled indica variety. All the sequencing work is gathered in Figure 35.

A

```
      1        10                  20        30          40
ZS97  CTAACCTTGACACTTGT.............ACTTGTGCGCTCCTTTGAACAG...CTTTT
m1    CTAACCTCGACACTTGN.NCNGCACAAGACACTAGTGCGCTCCCTTGNACAG..CTTTTN
m2    CTAACCTCNACNCTTGN.ACNGCACAAGACACTANTGCGCTCCCTTGNNCATGCNTTTTN
m3    CTAACCTCGACACTTGNNACTGCACAAGACACTAGTGCGCTCCCTTGAACAG..CTTTTT
m4    CTAACCTCGACACTTGTTACTGCACAAGACACTAGTGCGCTCCCTTGAACAG..CTTTTT
```

```
          50        60        70        80        90       100
ZS97  TTATGGCTTTTTCACTAGAAGC.TGTAACTACTTTGGTTTCTTTTGGCCATCTTTCCTCC
m1    TTATGGCTTTTTCACTATAAGC.TGTAACAACTTTGGTTTCTTTTGGCCATCTTTCCTCC
m2    TTATGGNTTNTTCACTATAAGNCTGNAACAACTTTGGNTTCTTTTGGCCATCTTTCCTCC
m3    TTATGGCTTTTTCACTAGAAGC.TGTAACAACTTTGGTTTCTTTTGGCCATCTTTCCTCC
m4    TTATGGCTTTTTCACTAGAAGC.TGTAACAACTTTGGTTTCTTTTGGCCATCTTTCCTCC
```

```
          110       120       130       140       150       160
ZS97  GGTTAGAACAGGATTGCATGCATCTTAACAGCAAAGAGAGATGCAAACTCTTGAAGTTTA
m1    AATTAGAACAGGATTGCATACATCTTAACAGCAAAGAGAGATGCAAACTATTAAAGTTTA
m2    ANTTAGAACAGGATTGCATACATCTTAACAGCAAAGAGAGATGCAAACTATTAAAGTTTA
m3    AATTAGAACAGGATTGCATACATCTTAACAGCAAAGAGAGATGCAAACTATTAAAGTTTA
m4    AATTAGAACAGGATTGCATACATCTTAACAGCAAAGAGAGATGCAAACTATTAAAGTTTA
```

```
          170       180       190       200       210       220
ZS97  TCATCACAGGAAAACTTATGACATCTACAACTTTGAAATTGAAATATAGCAAGACAAATC
m1    TCATCACAGGAAAACTTATGACATTTACAACTTTGAAATTGAAATATAGCAAGATAAATC
m2    TCATCACAGGAAAACTTATGACATTTACAACTTTGAAATTGAAATATAGCAAGATAAATC
m3    TCATCACAGGAAAACTTATGACATTTACAACTTTGAAATTGAAATATAGCAAGATAAATC
m4    TCATCACAGGAAAACTTATGACATTTACAACTTTGAAATTGAAATATAGCAAGATAAATC
```

```
          230       240       250       260       270       280
ZS97  AAGTAGCCTTTCCAAGTTTCTTTTGACCCAAGAAGAGAGAAGTGCCAAGGATCTGCATGA
m1    AAGTAGCCTTTCCAAGTTTCTTTTGACCAAAGAAGAGAAAAGTGCCAAAGATCTACATGA
m2    ANGTAGCCTTTCCAAGTTTCTTTTGACCAAAGAAGAGAAAAGTGCCAAAGATCTACATGA
m3    AAGTAGCCTTTCCAAGTTTCTTTTGACCAAAGAAGAGAAAAGTGCCAAAGATCTACATGA
m4    AAGTAGCCTTTCCAAGTTTCTTTTGACCAAAGAAGAGAAAAGTGCCAAAGATCTACATGA
```

```
          290       300       310       320       330       340
ZS97  CCCAGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGA
m1    CCCAGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGA
m2    CCCAGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGA
m3    CCCAGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGA
m4    CCCAGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGA
```

```
          350       360       370       380       390       400
ZS97  GGGAAATTTTGTTGCATGTGGGTGAACCCCAAAGCTTACCTAATGGCATTGTCCGTCAGC
m1    GGGAAATTTTGTTGCATATGGGTGAACCCCAAAGCTTACCTAANGGCATTGTCCGTCAGC
m2    GGGAAATTTTGTTGCATATGGGTGAACCCCAAAGCTTACCTAATGGCATTGTCCGTCAGC
m3    GGGAAATTTTGTTGCATATGGGTGAACCCCAAAGCTTACCTAANGGCATTGTCCGTCAGC
m4    GGGAAATTTTGTTGCATATGGGTGAACCCCAAAGCTTACCTAANGGCATTGTCCGTCAGC
```

```
          410       420       430       440       450       460
ZS97  ATTTTCAGTGCGTCCAAACCTCAATGAGACGCACCGTATATAACTTATGCTCATGCGTCA
m1    ATTTCCAGTGCGTCCAAACCTCAATGAGACGCACCGTAGATAACTTATGCTCATGCGTCA
m2    ATTTCCAGTGCGTCCAAACCTCAATGAGACGCACCGTANATAACTTATGCTCATGCGTCA
m3    ATTTCCAGNGCGTCCAAACCTCAATGAGACGCACCGTANATAACTTATGCTCATGCGTCA
m4    ATTTCCAGTGCGTCCAAACCTCAATGAGACGCACCGTANATAACTTATGCTCATGCGTCA
```

```
          470       480       490       500       510       520
ZS97  GATACACCTAATTTGAACCGACGGTAAAGGATTCCAGACGGTGCCGTTTGTAGTGGCTTT
m1    GATACACCTAATTTGAACCGACGGTAAAGGATTCTAGACGGTGCCGTTTGTAGCGGCTTT
m2    NATACACCTAATTTGAACCGACGGTAAAGGATTCTAGACGGTGCCGTTTGTAGCGGCTTT
m3    GATACACCTAATTTGAACCGACGGTAAAGGATTCTAGACGGTGCCGTTTGTAGCGGCTTT
m4    GATACACCTAATTTGAACCGACGGTAAAGGATTCTAGACGGTGCCGTTTGTAGCGGCTTT
```

```
             530       540       550       560       570       580
ZS97  TTCGCTACTTTTTTTGGTGAGATAGAAGACAGTGACAAAATAAGTTGCAAAAACAACAAA
m1    TTCGCTACTTTTCTTGGTGAGATAGAAGACAGTGACAAAATAAGTTGCAAAAACAACAAA
m2    TTCGCTACTTTTCTTGGTGAGATAGAAGACAGTGACAAAATAAGTTGCAAAAACAACAAA
m3    TTCGCTACTTTTCTTGGTGAGATAGAAGACAGTGACAAAATAAGTTGCAAAAACAACAAA
m4    TTCGCTACTTTTCTTGGTGAGATAGAAGACAGTGACAAAATAAGTTGCAAAAACAACAAA

             590       600       610       620       630       640
ZS97  AATGTTTATACACATGTACAGCATGAGCTCTACGGTGTCCCTGGAGAGCACAAGCTGTCA
m1    AATGTTTATACNCATGTACAGCATGAGCTCTACGGNGTCCCTGGAGAGCACAAGCTGTCN
m2    AATGTTTATACNCATGTACAGCATGAGCTCTACGGNGTCCCTGGAAAGCACAAGCTGTCA
m3    AATGTTTATACACATGTACAGCATGAGCTCTACGGGGTCCCTGGAGAGCACAAGCTGTCA
m4    AATGTTTATACNCATGTACAGCATGAGCTCTACGGTGTCCCTGGANAGCACAAGCTGTCA

             650       660       670       680       690       700
ZS97  CCAAGCCATAACAAACGGTCGAACTGATCT.TGAACACTGGATCCAATGCATGGTTTGTT
m1    CCAAGCCATAACAAACGGTCGAACTGATCNTTGAACACTGGATCCAATGCATGGTTTGTC
m2    CCAAGCCATAACAAACGGTCGAACTGATCT.TGAACACTGGATCCAATGCATGGTTTGTC
m3    CCAAGCCATAACAAACGGTCGAACTGATCT.TGAACACTGGATCCAATGCATGGTTTGTC
m4    CCAAGCCATAACAAACGGTCGAACTGATCT.TGAACACTGGATCCAATGCATGGTTTGTC

               710       720
ZS97  CAGTGCTCAAAGAGAACACCAGC
m1    .......................
m2    NA....................G
m3    ANGN..................G
m4    N.....................A
```

B

```
      1         10        20        30        40
ZS97  CTAACCTTG.........AC...ACTTGT.ACTTGTGCGCTCCTTTGAACAGCT.TTTTT
b1    CTAACCTCG.CACTTG.TACTGCACAAGACACTAGTGCGCTCCCTTGAACAGCTTTTTTT
b2    CTAACCTCGACACTTGTTACTGCACAAGACACTAGTGCGCTCCCTTGAACAGCTTTTTTT
b3    CTAACCTCGACACTTG.TACTGCACAAGACACTAGTGCGCTCCCTTGAACAGCTTTTTTT
b4    CTAACCTCGACACTTG.TACTGCACAAGACACTAGTGCGCTCCCTTGAACAGCTTTTTTT

      50        60        70        80        90        100
ZS97  ATGGCTTTTTCACTAGAAGCTGTAACTACTTTGGTTTCTTTTGGCCATCTTTCCTCCGGT
b1    ATGGCTTTTTCACTAGAAGCTGTAACAACTTTGGTTTCTTTTGGCCATCTTTCCTCTAAT
b2    ATGGCTTTTTCACTAGAAGCTGTAACAACTTTGGTTTCTTTTGGCCATCTTTCCTCTAAT
b3    ATGGCTTTTTCACTAGAAGCTGTAACAACTTTGGTTTCTTTTGGCCATCTTTCCTCTAAT
b4    ATGGCTTTTTCACTAGAAGCTGTAACAACTTTGGTTTCTTTTGGCCATCTTTCCTCTAAT

      110       120       130       140       150       160
ZS97  TAGAACAGGATTGCATGCATCTTAACAGCAAAGAGAGATGCAAACTCTTGAAGTTTATCA
b1    TAGAACAGGATTGCATACATCTTAACAGCAAAGAGAGATGCAAACTATTAAAGTTTATCA
b2    TAGAACAGGATTGCATACATCTTAACAGCAAAGAGAGATGCAAACTATTAAAGTTTATCA
b3    TAGAACAGGATTGCATACATCTTAACAGCAAAGAGAGATGCAAACTATTAAAGTTTATCA
b4    TAGAACAGGATTGCATACATCTTAACAGCAAAGAGAGATGCAAACTATTAAAGTTTATCA

      170       180       190       200       210       220
ZS97  TCACAGGAAAACTTATGACATCTACAACTTTGAAATTGAAATATAGCAAGACAAATCAAG
b1    TCACAGGAAAACTTATGACATTTACAACTTTGAAATTGAAATATAGCAAGATAAATCAAG
b2    TCACAGGAAAACTTATGACATTTACAACTTTGAAATTGAAATATAGCAAGATAAATCAAG
b3    TCACAGGAAAACTTATGACATTTACAACTTTGAAATTGAAATATAGCAAGATAAATCAAG
b4    TCACAGGAAAACTTATGACATTTACAACTTTGAAATTGAAATATAGCAAGATAAATCAAG

      230       240       250       260       270       280
ZS97  TAGCCTTTCCAAGTTTCTTTTGACCCAAGAAGAGAGAAGTGCCAAGGATCTGCATGACCC
b1    TAGCCTTTCCAAGTTTCTTTTGACCAAAGAAGAGAAAAGTGCCAAAGATCTACATGACCC
b2    TAGCCTTTCCAAGTTTCTTTTGACCAAAGAAGAGAAAAGTGCCAAAGATCTACATGACCC
b3    TAGCCTTTCCAAGTTTCTTTTGACCAAAGAAGAGAAAAGTGCCAAAGATCTACATGACCC
b4    TAGCCTTTCCAAGTTTCTTTTGACCAAAGAAGAGAAAAGTGCCAAAGATCTACATGACCC
                                                         ▲

      290       300       310       320       330       340
ZS97  AGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGAGGG
b1    AGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGAGGG
b2    AGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGAGGG
b3    AGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGAGGG
b4    AGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGAGGG

      350       360       370       380       390       400
ZS97  AAATTTGTTGCATGTGGGTGAACCCCAAAGCTTACCTAATGGCATTGTCCGTCAGCATT
b1    AAATTTGTTGCATATGGGTGAACCCCAAAGCTTACCTAANGGCATTGTCCGTCAGCATT
b2    AAATTTGTTGCATATGGGTGAACCCCAAAGCTTACCTAANGGCATTGTCCGTCAGCATT
b3    AAATTTGTTGCATATGGGTGAACCCCAAAGCTTACCTAANGGCATTGTCCGTCAGCATT
b4    AAATTTGTTGCATATGGGTGAACCCCAAAGCTTACCTAANGGCATTGTCCGTCAGCATT

      410       420       430       440       450       460
ZS97  TTCAGTGCGTCCAAACCTCAATGAGACGCACCGTATATAACTTATGCTCATGCGTCAGAT
b1    TCCAGTGCGTCCAAACCTCAATGAGACGCACCGTANATAACTTATGCTCATGCGTCANAT
b2    TCCAGNGCGTCCAAACCTCAATGAGACGCACCGTANATAACTTATGCTCATGCGTCANAT
b3    TCCAGNGCGTCCAAACCTCAATGAGACGCACCGTAGATAACTTATGCTCATGCGTCAGAT
b4    TCCAGTGCGTCCAAACCTCAATGAGACGCACCGTAGATAACTTATGCTCATGCGTCAGAT

      470       480       490       500       510       520
ZS97  ACACCTAATTTGAACCGACGGTAAAGGATTCCAGACGGTGCCGTTTGTAGTGGCTTTTTC
b1    ACACCTAATTTGAACCGACGGTAAAGGATTCTAGACGGTGCCGTTTGTAGCGGCTTTTTC
b2    ACACCTAATTTGAACCGACGGTAAAGGATTCTAGACGGTGCCGTTTGTAGCGGCTTTTTC
b3    ACACCTAATTTGAACCGACGGTAAAGGATTCTAGACGGTGCCGTTTGTAGCGGCTTTTTC
b4    ACACCTAATTTGAACCGACGGTAAAGGATTCTAGACGGTGCCGTTTGTAGCGGCTTTTTC
                                                   ▲
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```
        530       540       550       560       570       580
ZS97    GCTACTTTTTTTGGTGAGATAGAAGACAGTGACAAAATAAGTTGCAAAAACAACAAAAAT
b1      GCTACTTTTCTTGGTGAGATAGAANACAGTGACAAAATAAGTTGCAAAAACAACAAAAAT
b2      GCTACTTTTCTTGGTGAGATAGAAGACAGTGACAAAATAAGTTGCAAAAACAACAAAAAT
b3      GCTACTTTTCTNGGTGAGATAGAAGACAGNGACAAAATAAGTTGCAAAAACAACAAAAAT
b4      GCTACTTTTCTTGGTGAGATAGAAGACAGTGACAAAATAAGTTGCAAAAACAACAAAAAT

        590       600       610       620       630       640
ZS97    GTTTATACACATGTACAGCATGAGCTCTACGGTGTCCCTGGAGAGCACAAGCTGTCACCA
b1      GTTTATACNCATGTACAGCATGAGCTCTACGGNGTCCCTGGAGAGCACAAGCTGTCNCCA
b2      GTTTATACNCATGTACAGCATGAGCTCTACGGTGTCCCTGGAGAGCACAAGCTGTCNCCA
b3      GTTTATACCCATGTACAGCATGAGCTCTACGGGGTCCCTGGAGAGCACAAGCTGTCACCA
b4      GTTTATACACATGTACAGCATGAGCTCTACGGTGTCCCTGGAGAGCACAAGCTGTCACCA

        650       660       670       680       690       700
ZS97    AGCCATAACAAACGGTCGAACTGATCTTGAACACTGGATCCAATGCATGGTTTGTTCAGT
b1      AGCCATAACAAACGGTCGAACTGATCTTGAACACTGGATCCNATGCATGGTTTGTCCNGG
b2      AGCCATAACAAACGGTCGAACTGATCTTGAACACTGGATCCAATGCATGGTTTGTCCNNG
b3      AGCCATAACAAACGGTCGAACTGATCTTGAACACTGGACCCAATGCANGGTTTGTCNNGN
b4      AGCCATAACAAACGGTCGAACTGATCTTGAACACTGGATCCAATGCATGGTTGCCNNNGG

        710       720
ZS97    GCTCAAAGAGAACAC.CAGC
b1      GGCCC.........AAAAAA
b2      GGGCC.........CAAAAA
b3      G.CCC.........AAAA.A
b4      GGCCC.........NAA..A
```

C

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        1        10        20        30        40
ZS97  CTAACCTTG.........AC...ACTTGT.ACTTGTGCGCTCCTTTGAACAGCT.TTTTT
c1    CTAACCTCNACACTTGN.ACNGCACAAGACACTAGTGCGCTCCCTTGNNCAGCTTTTTTT
c2    CTAACCTCGACACTTGT.ACTGCACAAGACACTAGTGCGCTCCCTTGAACAGCTTTTTTT
c3    CTAACCTCGACACTTGN.ACTGCACAAGACACTAGTGCGCTCCCTTGAACAGCTTTTTTT
c4    CTAACCTCGACACTTGTTACTGCACAAGACACTAGTGCGCTCCCTTGAACAGCTTTTTTT

        50        60        70        80        90        100
ZS97  ATGGCTTTTTCACTAGAAGCTGTAACTACTTTGGTTTCTTTTGGCCATCTTTCCTCCGGT
c1    ATGGCTTTTTCACTAGAAGCTGTAACAACTTTGGTTTCTTTTGGCCATCTTTCCTCTAAT
c2    ATGGCTTTTTCACTAGAAGCTGTAACAACTTTGGTTTCTTTTGGCCATCTTTCCTCTAAT
c3    ATGGCTTTTTCACTAGAAGCTGTAACAACTTTGGTTTCTTTTGGCCATCTTTCCTCTAAT
c4    ATGGCTTTTTCACTAGAAGCTGTAACAACTTTGGTTTCTTTTGGCCATCTTTCCTCTAAT

        110       120       130       140       150       160
ZS97  TAGAACAGGATTGCATGCATCTTAACAGCAAAGAGAGATGCAAACTCTTGAAGTTTATCA
c1    TAGAACAGGATTGCATACATCTTAACAGCAAAGAGAGATGCAAACTATTAAAGTTTATCA
c2    TAGAACAGGATTGCATACATCTTAACAGCAAAGAGAGATGCAAACTATTAAAGTTTATCA
c3    TAGAACAGGATTGCATACATCTTAACAGCAAAGAGAGATGCAAACTATTAAAGTTTATCA
c4    TAGAACAGGATTGCATACATCTTAACAGCAAAGAGAGATGCAAACTATTAAAGTTTATCA

        170       180       190       200       210       220
ZS97  TCACAGGAAAACTTATGACATCTACAACTTTGAAATTGAAATATAGCAAGACAAATCAAG
c1    TCACAGGAAAACTTATGACATTTACAACTTTGAAATTGAAATATAGCAAGATAAATCAAG
c2    TCACAGGAAAACTTATGACATTTACAACTTTGAAATTGAAATATAGCAAGATAAATCAAG
c3    TCACAGGAAAACTTATGACATTTACAACTTTGAAATTGAAATATAGCAAGATAAATCAAG
c4    TCACAGGAAAACTTATGACATTTACAACTTTGAAATTGAAATATAGCAAGATAAATCAAG

        230       240       250       260       270       280
ZS97  TAGCCTTTCCAAGTTTCTTTTGACCCAAGAAGAGAGAAGTGCCAAGGATCTGCATGACCC
c1    TAGCCTTTCCAAGTTTCTTTTGACCAAAGAAGAGAAAAGTGCCAAAGATCTACATGACCC
c2    TAGCCTTTCCAAGTTTCTTTTGACCAAAGAAGAGAAAAGTGCCAAAGATCTACATGACCC
c3    TAGCCTTTCCAAGTTTCTTTTGACCAAAGAAGAGAAAAGTGCCAAAGATCTACATGACCC
c4    TAGCCTTTCCAAGTTTCTTTTGACCAAAGAAGAGAAAAGTGCCAAAGATCTACATGACCC

        290       300       310       320       330       340
ZS97  AGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGAGGG
c1    AGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGAGGG
c2    AGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGAGGG
c3    AGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGAGGG
c4    AGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGAGGG

        350       360       370       380       390       400
ZS97  AAATTTTGTTGCATGTGGGTGAACCCCAAAGCTTACCTAATGGCATTGTCCGTCAGCATT
c1    AAATTTTGTTGCATATGGGTGAACCCCAAAGCTTACCTAATGGCATTGTCCGTCAGCATT
c2    AAATTTTGTTGCATATGGGTGAACCCCAAAGCTTACCTAANGGCATTGTCCGTCAGCATT
c3    AAATTTTGTTGCATATGGGTGAACCCCAAAGCTTACCTAANGGCATTGTCCGTCAGCATT
c4    AAATTTTGTTGCATATGGGTGAACCCCAAAGCTTACCTAANGGCATTGTCCGTCAGCATT

        410       420       430       440       450       460
ZS97  TTCAGTGCGTCCAAACCTCAATGAGACGCACCGTATATAACTTATGCTCATGCGTCAGAT
c1    TCCAGNGCGTCCAAACCTCAATGAGACGCACCGTANATAACTTATGCTCATGCNTCAGAT
c2    TCCAGTGCGTCCAAACCTCAATGAGACGCACCGTANATAACTTATGCTCATGCGTCAGAT
c3    TCCAGTGCGTCCAAACCTCAATGAGACGCACCGTAGATAACTTATGCTCATGCGTCANAT
c4    TCCAGTGCGTCCAAACCTCAATGAGACGCACCGTANATAACTTATGCTCATGCGTCAGAT

        470       480        490       500       510       520
ZS97  ACACCTAATTTGAACCGACG.GTAAAGGATTCCAGACGGTGCCGTTTGTAGTGGCTTTTT
c1    ACNCCTAATTTGAACCGACGGGTAAAGGATTCTAGACGGNGCCGTTTGNAGCGGCTTTTT
c2    ACACCTAATTTGAACCGACG.GTAAAGGATTCTAGACGGTGCCGTTTGTAGCGGCTTTTT
c3    ACACCTAATTTGAACCGACG.GTAAAGGATTCTAGACGGTGCCGTTTGTAGCGGCTTTTT
c4    ACACCTAATTTGAACCGACG.GTAAAGGATTCTAGACGGTGCCGTTTGTAGCGGCTTTTT
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```
        530       540       550       560       570       580
ZS97  CGCTACTTTTTTTGGTGAGATAGAAGACAGTGACAAAATAAGTTGCAAAAACAACAAAAA
c1    CNCTACTTTTCTTGGGGAGATAGAA.ANAGNGNCNAAATAAGTTGCCAAAACNACNAAAA
c2    CGCTACTTTTCTTGGTGAGATAGAAGACAGTGACAAAATAAGTTGCAAAAACAACAAAAA
c3    CGCTACTTTTCTTGGTGAGATAGAANACAGTGACAAAATAAGTTGCAAAAACAACAAAAA
c4    CGCTACTTTTCTTGGTGAGATAGAAGACAGTGACAAAATAAGTTGCAAAAACAACAAAAA

        590       600       610       620       630       640
ZS97  TGTTTATACACATGTACAGCATGAGCTCTACGGTGTCCCTGGAGAGCACAAGCTGTCACC
c1    NGTTTNTC.CCCTGNNCCGCNTGNGCNCTNCGGGGTCCCNGGGNAGNNNAAGNNGGNCCC
c2    TGTTTATACACATGTACAGCATGAGCTCTACGGTGTCCCTGGAGAGCACAAGCTGTCNCC
c3    TGTTTATACACATGTACAGCATGAGCTCTACGGTGTCCCTGGAGAGCACAAGCTGTCNCC
c4    TGTTTATACACATGTACAGCATGAGCTCTACGGTGTCCCTGGAGAGCACAAGCTGTCACC

        650       660        670       680       690       700
ZS97  AAGCCATAACAAACGGTCG.AACTGATCTTGAACACTGGATCCAATGCATGGTTTGTTCA
c1    CANNCCTAANAAAAGGGCGNAANNGGNNCTGNACNCNGGACCAA..GGNTGGTTTGTCNN
c2    AAGCCATAACAAACGGTCG.AACTGATCTTGAACACTGGATCCAATGCATGGTTTGTCCN
c3    AAGCCATAACAAACGGTCG.AACTGATCTTGAACACTGGATCCAATGCATGGTTTGTCCN
c4    AAGCCATAACAAACGGTCG.AACTGATCTTGAACACTGGATCCAATGCATGGTTTGTCCN

              710       720
ZS97  GTGC..TCAAAGAGAACACCAGC
c1    NGGC..NCNAANNNNAAAN.NAA
c2    GGGG..CCAAA..A........A
c3    NGNGGCNCAAA..A........A
c4    NGGGGCCCAAA..A........A
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D

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           1          10        20                            30        40
ZS97  CTAACC.TTGACACTTGT.ACT............TGTGCGCTCCTTTGAACAGCTTTT.T
g1    CTAACC.TCGACACTTGT.ACTGCNCAAGACACTAGTGCGCTCCCTTGAACAGCTTTTTT
g2    CTAACC.TCGACACTTGTTACTGCACAAGACACTAGTGCGCTCCCTTGAACAGCTTTTTT
g3    CTAACC.TCGACNCTTGN.ACNGCACAAGACACTAGTGCGCTCCCTTGNACAGCTTTTTT
g4    CTAACCGTCGACACTNGN.ACNGCNCNAGACACTANTGCGCTCCCTTGNACAGCTTTTTT
g5    CTAACC.TCGACACTTGN.ACTGCACAAGACACTAGTGCGCTCCCTTGNNCAGCTTTTTT

         50        60        70        80        90        100
ZS97  TATGGCTTTTTCACTAGAAG.CTGTAACTACTTTGGTTTCTTTTGGCCATCTTTCCTCCG
g1    TATGGCTTTTTCACTAGAAG.CTGTAACAACTTTGGTTTCTTTTGGCCATCTTTCCTCCA
g2    TATGGCTTTTTCACTAGAAG.CTGTAACAACTTTGGTTTCTTTTGGCCATCTTTCCTCCA
g3    TATGGCTTTTTCACTAGAAG.CTGTAACAACTTTGGTTTCTTTTGGCCATCTTTCCTCCA
g4    TATGGNTTNTTCNCTATAAGACTGTAACAACTTTGGNTTCTTTTGGCCATCTTTCCTCCA
g5    TATGGCTTTTTCACTAGAAG.CTGTAACAACTTTGGTTTCTTTTGGCCATCTTTCCTCCA

         110       120       130       140       150       160
ZS97  GTTAGAACAGGATTGCATGCATCTTAACAGCAAAGAGAGATGCAAACTCTTGAAGTTTAT
g1    ATTAGAACAGGATTGCATACATCTTAACAGCAAAGAGAGATGCAAACTATTAAAGTTTAT
g2    ATTAGAACAGGATTGCATACATCTTAACAGCAAAGAGAGATGCAAACTATTAAAGTTTAT
g3    ATTAGAACAGGATTGCATACATCTTAACAGCAAAGAGAGATGCAAACTATTAAAGTTTAT
g4    ATTAGAACAGGATTGCATACNTCNTAACAGCAAAGAGAGATGCAAACTATTAAAGTTTAT
g5    ATTAGAACAGGATTGCATACATCTTAACAGCAAAGAGAGATGCAAACTATTAAAGTTTAT

         170       180       190       200       210       220
ZS97  CATCACAGGAAAACTTATGACATCTACAACTTTGAAATTGAAATATAGCAAGACAAATCA
g1    CATCACAGGAAAACTTATGACATTTACAACTTTGAAATTGAAATATAGCAAGATAAATCA
g2    CATCACAGGAAAACTTATGACATTTACAACTTTGAAATTGAAATATAGCAAGATAAATCA
g3    CATCACAGGAAAACTTATGACATTTACAACTTTGAAATTGAAATATAGCAAGATAAATCA
g4    CATCACAGGAAAACTTATGACATTTACAACTTTGAAATTGAAATATAGCAAGATAAATCA
g5    CATCACAGGAAAACTTATGACATTTACAACTTTGAAATTGAAATATAGCAAGATAAATCA

         230       240       250       260       270       280
ZS97  AGTAGCCTTTCCAAGTTTCTTTTGACCCAAGAAGAGAGAAGTGCCAAGGATCTGCATGAC
g1    AGTAGCCTTTCCAAGTTTCTTTTGACCAAAGAAGAGAAAAGTGCCAAAGATCTACATGAC
g2    AGTAGCCTTTCCAAGTTTCTTTTGACCAAAGAAGAGAAAAGTGCCAAAGATCTACATGAC
g3    AGTAGCCTTTCCAAGTTTCTTTTGACCAAAGAAGAGAAAAGTGCCAAAGATCTACATGAC
g4    AGTAGCCTTTCCAAGTTTCTTTTGACCAAAGAAGAGAAAAGTGCCAAAGATCTACATGAC
g5    AGTAGCCTTTCCAAGTTTCTTTTGACCAAAGAAGAGAAAAGTGCCAAAGATCTACATGAC

         290       300       310       320       330       340
ZS97  CCAGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGAG
g1    CCAGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGAG
g2    CCAGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGAG
g3    CCAGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGAG
g4    CCAGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGAG
g5    CCAGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGAG

         350       360       370       380       390       400
ZS97  GGAAATTTTGTTGCATGTGGGTGAACCCCAAAGCTTACCTAATGGCATTGTCCGTCAGCA
g1    GGAAATTTTGTTGCATATGGGTGAACCCCAAAGCTTACCTAANGGCATTGTCCGTCAGCA
g2    GGAAATTTTGTTGCATATGGGTGAACCCCAAAGCTTACCTAANGGCATTGTCCGTCAGCA
g3    GGAAATTTTGTTGCATATGGGTGAACCCCAAAGCTTACCTAANGGCATTGTCCGTCAGCA
g4    GGAAATTTTGTTGCATATGGGTGAACCCCAAAGCTTACCTAATGGCATTGTCCGTCAGCA
g5    GGAAATTTTGTTGCATATGGGTGAACCCCAAAGCTTACCTAANGGCATTGTCCGTCAGCA

         410       420       430       440       450       460
ZS97  TTTTCAGTGCGTCCAAACCTCAATGAGACGCACCGTATATAACTTATGCTCATGCGTCAG
g1    TTTCCAGTGCGTCCAAACCTCAATGAGACGCACCGTANATAACTTATGCTCATGCGTCAN
g2    TTTCCAGTGCGTCCAAACCTCAATGAGACGCACCGTANATAACTTATGCTCATGCGTCAN
g3    TTTCCAGTGNGTCCAAACCTCAATGAGACGCACCGTANATAACTTATGCTCATGCGTCAG
g4    TTTCCAGTGCGTCCAAACCTCAATGAGACGCACCGTANATAACTTATGCTCATGCGTCAG
g5    TTTCCAGNGCGTCCAAACCTCAATGAGACGCACCGTAGATAACTTATGCTCATGCGTCAG
```

```
        470       480       490       500       510       520
ZS97  ATACACCTAATTTGAACCGACGGTAAAGGATTCCAGACGGTGCCGTTTGTAGTGGCTTTT
g1    ATACACCTAATTTGAACCGACGGTAAAGGATTCTAGACGGTGCCGTTTGTAGCGGCTTTT
g2    ATACACCTAATTTGAACCGACGGTAAAGGATTCTAGACGGTGCCGTTTGTAGCGGCTTTT
g3    ATACNCCTAATTTGAACCGACGGTAAAGGATTCTAGACGGTGCCNTTTGTAGCGGCTTTT
g4    ATACACCTAATTTGAACCGACGGTAAAGGATTCTAGACGGTGCCGTTTGTAGCGGCTTTT
g5    ATACACCTAATTTGAACCGACGGTAAAGGATTCTAGACGGTGCCGTTTGTAGCGGCTTTT
```

```
          530        540       550       560       570       580
ZS97  TCGCTACTTTTTTTG.GTGAGATA.GAAGACAGTGACAAAATAAGTTGCAAAAACAAC.A
g1    TCGCTACTTTTCTTG.GTGAGATA.GAAGACAGTGACAAAATAAGTTGCAAAAACAAC.A
g2    TCGCTACTTTTCTNG.GTGAGATA.GAAGACAGTGACAAAATAAGTTGCAAAAACAAC.A
g3    TCGCTACTTTTCTNGGGNGAGATANNAANACAGNGACAAAATAANTTGCAAAAACAACAA
g4    TCGCTACTTTTCTTG.GNGAGATA.GAAGACAGNGACAAAATAAGTTGCAAAAACANC.A
g5    TCGCTACTTTTCTTG.GTGAGATAG.AAGACAGTGACAAAATAAGTTGCAAAAACAAC.A
```

```
             590       600       610       620       630
ZS97  AAAATGTTTATACACAT.GTACAGCATGAGCTCTACGGTGT.CCCTGGAGAGCACAAGCT
g1    AAAATGTTTATACNCAT.GTACAGCATGAGCTCTACGGNGT.CCCTGGAGAGCACAAGCT
g2    AAAATGTTTATACNCAT.GTACAGCATGAGCTCTACGGTGT.CCCTGGAGAGCACAAGCT
g3    AAAANGTTTATNCNCNT.GTACNGCNTGAGCTCTACGGNGTCCCTGGANAGCNCAAGCT
g4    AAAATGTTTATACNCATGGTACAGCATGAGCTCTACGGNGNNCCCTGGAGAGCANAAGCT
g5    AAAATGTTTATACNCATG.TACAGCATGAGCTCTACGGNGT.CCCTGGAGAGCACAAGCT
```

```
     640       650       660       670       680       690
ZS97  G.TCACCAAGCCATAACAAACGGTC.GAACTGATCTTGAACACTGGATCCAATGCATGGT
g1    G.TCACCAAGCCATAACAAACGGTC.GAACTGATCTTGAACACTGGATCCAATGCATGGT
g2    G.TCACCAAGCCATAACAAACGGTC.GAACTGATCTTGAACACTGGATCCAATGCATGGT
g3    GGTCCCCAAGCCATAANAAAAGGNCNAAACTGATCTNGAACACTGGATCCAANGCNNGGT
g4    .GTCACCAAGCCATAACAAACGGTC.GAACTGATCTTGAACACTGGATCCAATGCATGGT
g5    G.TCACCAAGCCATAACAAACGGNC.GAACTGATCTTGAACACTGGATCCAATGCATGGT
```

```
      700        710       720
ZS97  TTGTTC.AGTGCTCAAAGAGAACACCAGC
g1    TTGTCN.NGNGCCCAAAAA.........A
g2    TTGTCN.AGGGCCCCAAAA.........A
g3    TTGTTCNNGNGCCCCAAA..........A
g4    TTGTCC.AGNGCNCAAAA..........A
g5    TTGTCCANGNGCNCAAA...........A
```

E

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     1      10                 20                    30        40
ZS97 CTAACCTTGACACTTGT.ACT.............TGTGCGCTCCTTTGAACAGCTTTTT.T
o1   CTAACCTCGACACTTGT.ACTGCACAAGACACTAGTGCGCTCCCTTGAACAGCTTTTTTT
o2   CTAACCTCGACACTTGT.ACTGCACAAGACACTAGTGCGCTCCCTTGAACAGCTTTTTTT
o3   CTAACCTCGACACTTGTTACTGCACAAGACACTAGTGCGCTCCCTTGAACAGCTTTTTTT
o4   CTAACCTCGACACTTGT.ACTGCACAAGACACTAGTGCGCTCCCTTGAACAGCTTTTTTT
o5   CTAACCTCGACACTTGT.ACTGCACAAGACACTAGTGCGCTCCCTTGAACAGCTTTTTTT

       50        60        70        80        90        100
ZS97 ATGGCTTTTTCACTAGAAGCTGTAACTACTTTGGTTTCTTTTGGCCATCTTTCCTCCGGT
o1   ATGGCTTTTTCACTAGAAGCTGTAACAACTTTGGTTTCTTTTGGCCATCTTTCCTCCAAT
o2   ATGGCTTTTTCACTAGAAGCTGTAACAACTTTGGTTTCTTTTGGCCATCTTTCCTCCAAT
o3   ATGGCTTTTTCACTAGAAGCTGTAACAACTTTGGTTTCTTTTGGCCATCTTTCCTCCAAT
o4   ATGGCTTTTTCACTAGAAGCTGTAACAACTTTGGTTTCTTTTGGCCATCTTTCCTCCAAT
o5   ATGGCTTTTTCACTAGAAGCTGTAACAACTTTGGTTTCTTTTGGCCATCTTTCCTCCAAT

       110       120       130       140       150       160
ZS97 TAGAACAGGATTGCATGCATCTTAACAGCAAAGAGAGATGCAAACTCTTGAAGTTTATCA
o1   TAGAACAGGATTGCATACATCTTAACAGCAAAGAGAGATGCAAACTATTAAAGTTTATCA
o2   TAGAACAGGATTGCATACATCTTAACAGCAAAGAGAGATGCAAACTATTAAAGTTTATCA
o3   TAGAACAGGATTGCATACATCTTAACAGCAAAGAGAGATGCAAACTATTAAAGTTTATCA
o4   TAGAACAGGATTGCATACATCTTAACAGCAAAGAGAGATGCAAACTATTAAAGTTTATCA
o5   TAGAACAGGATTGCATACATCTTAACAGCAAAGAGAGATGCAAACTATTAAAGTTTATCA

       170       180       190       200       210       220
ZS97 TCACAGGAAAACTTATGACATCTACAACTTTGAAATTGAAATATAGCAAGACAAATCAAG
o1   TCACAGGAAAACTTATGACATTTACAACTTTGAAATTGAAATATAGCAAGATAAATCAAG
o2   TCACAGGAAAACTTATGACATTTACAACTTTGAAATTGAAATATAGCAAGATAAATCAAG
o3   TCACAGGAAAACTTATGACATTTACAACTTTGAAATTGAAATATAGCAAGATAAATCAAG
o4   TCACAGGAAAACTTATGACATTTACAACTTTGAAATTGAAATATAGCAAGATAAATCAAG
o5   TCACAGGAAAACTTATGACATTTACAACTTTGAAATTGAAATATAGCAAGATAAATCAAG

       230       240       250       260       270       280
ZS97 TAGCCTTTCCAAGTTTCTTTTGACCCAAGAAGAGAGAAGTGCCAAGGATCTGCATGACCC
o1   TAGCCTTTCCAAGTTTCTTTTGACCAAAGAAGAGAAAAGTGCCAAAGATCTACATGACCC
o2   TAGCCTTTCCAAGTTTCTTTTGACCAAAGAAGAGAAAAGTGCCAAAGATCTACATGACCC
o3   TAGCCTTTCCAAGTTTCTTTTGACCAAAGAAGAGAAAAGTGCCAAAGATCTACATGACCC
o4   TAGCCTTTCCAAGTTTCTTTTGACCAAAGAAGAGAAAAGTGCCAAAGATCTACATGACCC
o5   TAGCCTTTCCAAGTTTCTTTTGACCAAAGAAGAGAAAAGTGCCAAAGATCTACATGACCC

       290       300       310       320       330       340
ZS97 AGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGAGGG
o1   AGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGAGGG
o2   AGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGAGGG
o3   AGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGAGGG
o4   AGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGAGGG
o5   AGAGTTGTTTTATTCAAAAACCACCATTCGAAATGAAGGTAGCTAGATGCATGATGAGGG

       350       360       370       380       390       400
ZS97 AAATTTTGTTGCATGTGGGTGAACCCCAAAGCTTACCTAATGGCATTGTCCGTCAGCATT
o1   AAATTTTGTTGCATATGGGTGAACCCCAAAGCTTACCTAANGGCATTGTCCGTCAGCATT
o2   AAATTTTGTTGCATATGGGTGAACCCCAAAGCTTACCTAANGGCATTGTCCGTCAGCATT
o3   AAATTTTGTTGCATATGGGTGAACCCCAAAGCTTACCTAANGGCATTGTCCGTCAGCATT
o4   AAATTTTGTTGCATATGGGTGAACCCCAAAGCTTACCTAATGGCATTGTCCGTCAGCATT
o5   AAATTTTGTTGCATATGGGTGAACCCCAAAGCTTACCTAANGGCATTGTCCGTCAGCATT

       410       420       430       440       450       460
ZS97 TTCAGTGCGTCCAAACCTCAATGAGACGCACCGTATATAACTTATGCTCATGCGTCAGAT
o1   TCCAGNGCGTCCAAACCTCAATGAGACGCACCGTAGATAACTTATGCTCATGCGTCANAT
o2   TCCAGTGCGTCCAAACCTCAATGAGACGCACCGTAGATAACTTATGCTCATGCGTCANAT
o3   TCCAGTGCGTCCAAACCTCAATGAGACGCACCGTANATAACTTATGCTCATGCGTCANAT
o4   TCCAGNGCGTCCAAACCTCAATGAGACGCACCGTANATAACTTATGCTCATGCGTCANAT
o5   TCCAGNGCGTCCAAACCTCAATGAGACGCACCGTAGATAACTTATGCTCATGCGTCANAT
```

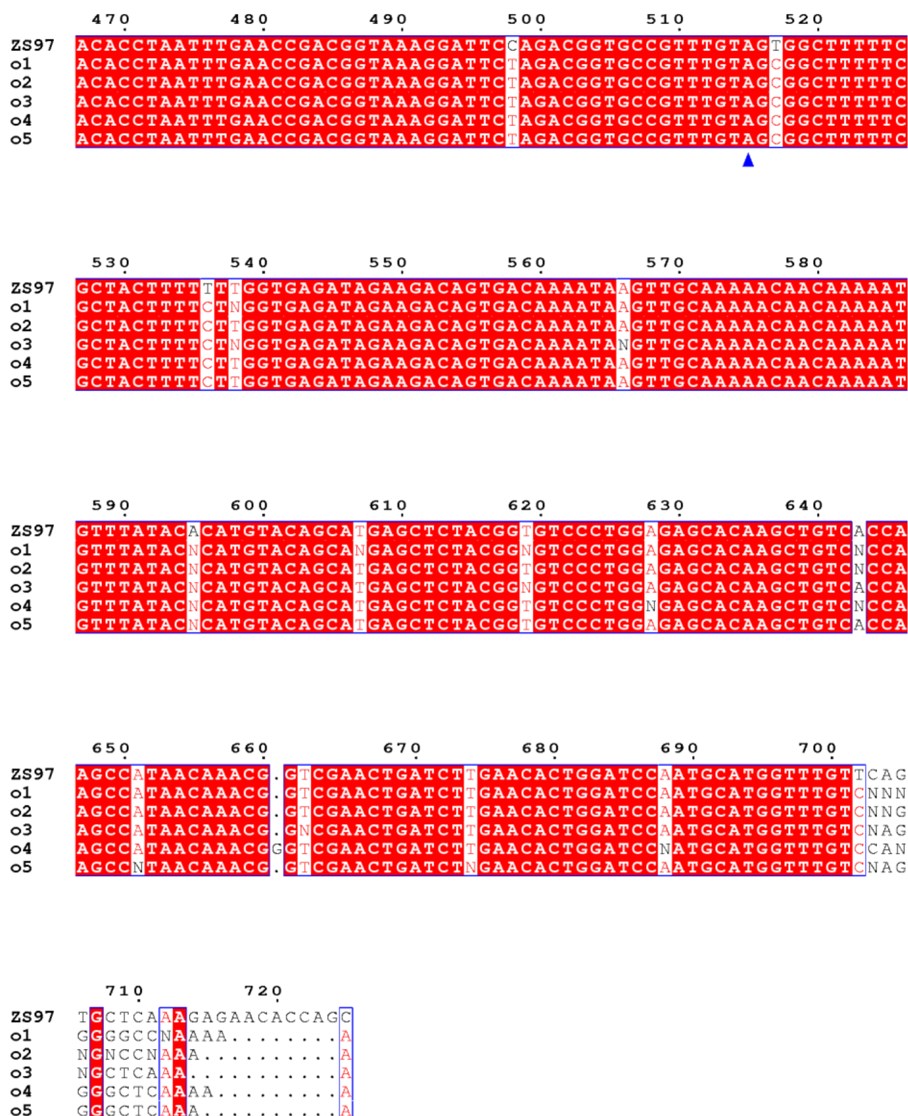

Figure 35. Sequencing of the region of interest of the *Chalk5* gene promoter. The first and second blue triangle marks the -721 and the -485 position respectively. zs97 refers to the genome of reference from Zhensan97. A= Montsianell, B= Bomba, C= Carnaroli, D= Guadiamar, E= PL12.

For all the varieties studied, the nucleotides in the positions -721 and -485 were C and A independent of the presence of pearl. Therefore, in the varieties studied, the expression of the *Chalk5* gene was not regulated in the same way than in indica varieties, and therefore these polymorphisms did not determine the presence or absence of pearl in our Mediterranean japonica varieties studied.

5. Discussion

5.1 Experiment 1: Anther culture of two temperate and two tropical japonica varieties

Anther culture is a very useful technique that allows to obtain doubled haploid plantlets from haploid microspores. However, its efficiency depends on many factors, being the variety used one of the most determinant. Khanna & Raina (1998) studied multiple parameters in the performance of calli induction and plantlet regeneration of three indica varieties (IR-72, IR-54 and Karna Local), and statistically showed that the most influential parameter was variety. Shahnewaz et al. (2004) performed anther culture in six cultivars under the same conditions and obtained varying induction and regeneration percentages depending on the variety. He et al. (2006) obtained plantlet regeneration percentages of some indica hybrids that varied from 1.6% to 82.9% depending on variety. These examples show the need for the optimization of each step in the anther culture protocols considering variety.

This work has adapted a medium grain, temperate japonica Montsianell-optimized anther culture protocol to be used for long grain tropical japonica (303012 and 303013) and long grain temperate japonica (PL12) varieties.

Alemanno et al. (1994) stated that the presence of colchicine in the induction medium stimulated calli induction in the japonica rice variety Miara. However, different results have been obtained in several studies depending on the colchicine concentration, treatment duration and variety in japonica varieties (Alemanno & Guiderdoni, 1994; Chen et al., 2001; Hooghvorst et al., 2018). Therefore, in order to improve anther induction efficiency in this study, two different media were tested, a colchicine-free medium (0D1) and a 150 mg· L^{-1} colchicine-supplemented medium (150D1).

In this study, statistical analysis proved that calli induction was highly related to the presence of colchicine in the medium. The Montsianell cultivar induced calli 20-fold more in 150D1 than in 0D medium. This result is very similar to the one obtained by Hooghvorst et al. (2018), performed under the same conditions and cultivar, with a 7.39% induction rate. In the case of tropical japonica varieties, 303012 and 303013, which were tested for the first time, induction rates were higher in 150D1 medium than in 0D1 medium, although low induction rates were obtained, being less than 1% in all cases. This shows again the high influence of the variety used in the anther culture efficiency. An

explanation for the varying induction percentages could be that temperate japonica and tropical japonica varieties are genetically divergent in spite of belonging to the same japonica sub-species (Kovach et al., 2007). Further studies should be performed to improve the anther culture protocol for the tropical japonica cultivars.

Plantlet regeneration from calli was the next step assayed in this study. Three different media were tested, the staple XACRM medium described by Serrat et al. (2014), and two media derived from XACRM, named SARM and GERM, with different additives in order to increase osmotic stress and therefore, to force regeneration. Media affected plantlet regeneration in this study as proved by the statistics. SARM was the only medium that produced regeneration in all varieties, although the rates were lower for the tropical japonica varieties than for the temperate japonica ones. In general, Montsianell was the variety that presented the highest percentage of plantlet regeneration. Also, it was the variety that better responded to any of the three regeneration media, being GERM the one that regenerated the most plantlets. Nevertheless, SARM medium was the one that provided the highest regeneration percentage in all three long grain varieties. Yoshida et al. (1994) tried different combinations of sorbitol percentage and hormone concentration in the regeneration medium of two japonica rice cultivars. It would be interesting to carry out an essay of SARM medium with different hormone and sorbitol concentrations to improve regeneration percentages in all the varieties studied.

A common problem in anther culture is the regeneration of albino plants (Mishra & Rao, 2016). Albinism makes the regenerated plantlets useless since they end up dying. The albino plantlets lack chlorophyll, due to the inability of protoplasts to transform into chloroplasts (Makowska & Oleszczuk, 2014). Zubko and Day (2002) affirmed that most albino plants lack some parts of the plastid genome. This impairment of DNA is the basic cause of albinism in rice (Kumari et al., 2009). In this study, the ratio of calli regenerating green plantlets / calli regenerating albino plantlets was inferior to 1 in Montsianell, PL12 and 303013 varieties, meaning that fewer calli regenerated green plantlets. Genotype, between other factors, determines the percentage of albino plants in anther culture, which may vary between 5% and 100% (Talebi et al., 2007). Yamagishi et al. (1998) obtained a 36% of albino regeneration for Nipponbare temperate japonica variety. Alemanno et al. (1994) obtained a 15.5% of albino plants when regenerating plantlets from japonica variety Miara. The total percentage of albino plantlets regenerated in this study (14%) is relatively low in comparison with several other studies, being the same as the one obtained by Serrat et al. (2014) in Montsianell anther culture.

Regarding the plantlets' ploidy in this experiment, Montsianell and 303013 were the only varieties that regenerated green doubled haploid plantlets from the calli induced the colchicine-supplemented medium. Moreover, no regeneration of this kind of plantlets occurred in 0D1, which is not hazardous since colchicine favors the green doubled haploid production (Chen et al., 2001). Alemanno et al. (1994) described a 1.5- to 2.5-fold increase in doubled haploid green plantlets production by using a colchicine-supplemented medium in the calli induction step. Colchicine had no effect on doubled haploid obtainment in PL12, as it only regenerated haploid and mixoploid plantlets. Since no diploidization occurred in this variety during the anther culture steps, a subsequent diploidization could be forced *in vitro* with the use of antimitotic agents. Hooghvorst et al. (2018) performed diploidization in haploid plantlets proceeding from the anther culture of the temperate japonica variety Montsianell, improving the efficiency of their anther culture experiment. They found that 250 mg · L^{-1} of colchicine and 1.25 mg · L^{-1} of oryzalin treatments provided the best diploidization rates.

This study has shown the importance of the variety used in the performance of rice anther culture in combination with media additives. It has not only improved the previous anther culture protocol for the Montsianell variety, but it has also performed for the first time an essay on long grain tropical and temperate varieties. Besides, it has proved that colchicine enhances the anther culture at both induction and regeneration steps, and the usefulness of osmotic stress for plantlet regeneration.

This work contributes to the knowledge in japonica rice varieties anther culture, since few studies are yet published. Further investigations will help to elucidate the differential characteristics that anther culture of japonica rice varieties presents, meaning a substantial improvement in the obtaining of doubled haploids, and therefore, in breeding.

5.2 Experiment 2: Testing the Saltol region in hydroponic assays and in fields

In this experiment, various *Saltol*-introgressed varieties were evaluated in order to determine their response to salinity in hydroponic and field assays. This genomic region introgression will help to fight more efficiently against golden apple snails invasion in fields. After flooding the fields with saline water to greatly reduce the snails' population, the *Saltol*-introgressed varieties sown after, should be able to tolerate the residual salinity left on the soil and to develop normally.

Regarding the hydroponic assays, three analyses were performed; a SPAD analysis, a SES analysis and a Na^+/K^+ ratio analysis. A first Na^+/K^+ ratio timepoint assay was performed to determine the best unique Na^+/K^+ ratio measurement day (since this analysis is destructive), in which the sensitive and resistant parentals would act accordingly to their salt tolerance. This way, they could be taken as a reference to evaluate their *Saltol*-introgressed lines. The 7th day after salinization of the media was proved to be the one that met the evaluation criteria, being applied at the following hydroponic assays. Non-destructive assays such as SPAD and SES were performed 1, 3, 5 and 7 days after salinization of the media to obtain more information about the plants behavior and to reinforce the Na^+/K^+ ratio analyses results.

The SPAD analyses were performed with a SPAD-meter, which is a hand-held spectrophotometer that allows to measure in a non-destructive way the relative content of chlorophyll (RCC) in the plant green parts (Süß et al., 2015). This chlorophyll content can be associated with the plant photosynthetic capacity, and therefore, its physiological condition. Salinity is associated with a decrease of the chlorophyll concentration and the inhibition of photosynthetic activity (Sultana et al., 2002), therefore, RCC has been used as a measure of salinity tolerance in rice (Hussain et al., 2019; Senguttuvel et al., 2014).

The standard evaluation system (SES) was designed specifically to evaluate phenotypical salinity damage in rice, and it has been widely used in salinity tolerance studies (Batayeva et al., 2018; Ghaffari et al., 2014; Platten et al., 2013; Rahman et al., 2019; Yichie et al., 2018).

The Na^+/K^+ ratio has been shown to be the most reliable measure of salt resistance in rice (Zhu et al., 2001) and has been measured in many salt-response rice studies (Dionisio-Sese & Tobita, 1998; Krishnamurthy et al., 2016; Mel et al., 2019; Nounjan et al., 2018; Platten et al., 2013; Zhu et al., 2001). Under salinity stress, the Na^+ accumulates in the rice aerial parts can lead to cell death. An ionic stress-response activates transporters with

high affinity for K^+ and Ca^+ ions, making the Na^+ migrate from shoots to roots, to avoid further Na^+ intake. Therefore, the Na^+/K^+ ratio in shoots is a good measure of salt tolerance (Frouin et al., 2018). The lower the ratio, the better the salt resistance, since it indicates the success of the regulatory mechanisms that lower Na^+ concentration in shoots.

In terms of correlation of the data from the different types of measurements, the Spearman coefficients show a concordant relationship between parameters depending of what they measure. For both LP and MS varieties, the SES and SPAD measurements correlated in a significant, strong and negative way. This is expected since the lower the SPAD score, the more damaged is the plant, and the higher the SES score, the stronger the salinity damage. For the LP varieties, a significant positive correlation was also observed between the Na^+/K^+ ratio measurements at day 7 and the SES score at the same day. As it has been explained before, the higher the Na^+/K^+ ratio, the less tolerance to salt, so a high ratio, meaning poor salt tolerance, would match a high SES score, indicating high salinity damage. Correlation between SES scores and K^+ and/or Na^+ concentration had been described by Platten et al. (2013), Krishnamurthy et al. (2016) or Batayeva et al. (2018).

Considering all the measured parameters under salinity circumstances in hydroponic assays, and giving preference to the Na^+/K^+ ratio, the top five most tolerant LP varieties were the following: in the first place, LP9, with a Na^+/K^+ ratio of 0.504, 2.6 SES score, and a decrease 6.65% of RCC. Despite this decrease, it only meant a drop of 2 points of the RCC absolute values. Besides, LP9 presented the lowest Na^+/K^+ ratio of all the LP varieties evaluated, even less than the FL478', with a 0.591 ratio. It must be mentioned that the Na^+/K^+ ratio of the sensitive parental, PL12, was 1.229. The SES score was also good, considering that FL478, the resistant parental, presented in the final day of measurement a SES score of 4. The second best performing variety was LP7, with a Na^+/K^+ ratio of 0.661, a SES score of 4.6, and an increase of a 25% percent in the RCC. This growth is relevant and points out a good salt tolerance, although it must be taken into consideration that as Zhu et al. (2001) affirmed, "*salt resistance does not necessarily imply the ability to grow in the presence of stress*". This means that, despite of an increase on the RCC content and presumably plant growth, the most relevant in terms of salt tolerance would be that the initial RCC values would not largely drop after salinization. Following LP9 and LP7, the third best performing line was LP28, which presented a Na^+/K^+ ratio of 0.723, a SES score of 4.6 and a 17.57% of RCC increase. Next, LP18 presented a Na^+/K^+ ratio of 0.753, a SES score and a 18.22% of decrease on the SPAD

score, although the final RCC, 26.30, was similar to other varieties that experimented growth. Finally, LP16 presented a Na^+/K^+ ratio of 0.788, a SES score of 3.4 and a 3.12% RCC decrease. Although the ratio of LP16 was slightly higher than the LP18', the final RCC values are almost equal and the SES score of LP16 is remarkably better, so it could be considered better than LP18 in terms of salt resistance.

Regarding the MS varieties, a first noticeable aspect is that their general performance in the different parameters tested has been worse in terms of salt resistance, presenting higher average SES scores and higher average Na^+/K^+ ratio than the LP varieties. The MS varieties' parental IR64-*Saltol* was obtained from a FL478 x IR64 cross by Ho et al. (2016). These authors tested different parameters under salinity conditions in FL478, various IR64-*Saltol* varieties, and susceptible varieties. The performance of IR64-*Saltol* in terms of salinity tolerance was better than the susceptible varieties but did not reach the degree of tolerance that FL478 presented. This could be a possible explanation for the different performance of the LP varieties, with FL478 as a parental, and MS varieties, with IR64-*Saltol* as a parental.

It also should be mentioned the unexpected individual behavior of the resistant parental, IR64-*Saltol*, which showed worse performance in terms of salinity resistance than the sensitive parental, PM37, in our experimental conditions. One possible explanation could be that, when the plants from this variety were transplanted into the hydroponic trays after germination in a growth chamber, they were not as grown as other varieties. This was observed when performing the assay and can be confirmed with the RCC values of the 1st day after salinization. IR64-*Saltol* presented in both control and salinity conditions lower values than any other variety, indicating a smaller growth. IR64, the parental of IR64-*Saltol*, is a variety highly adapted to tropical lowland conditions (Mackill & Khush, 2018), and maybe the conditions of the growth chamber were not the ideal for the IR64-*Saltol* plants for them to grow properly. The earlier stage of development translating into a smaller size of the IR64-*Saltol* plants added to Mediterranean climatic conditions when performing the assay could be the cause for its susceptible behavior, because as Shankar et al. (2016) affirmed, the seedling stage is more susceptible to salinity than other growth stages.

Once discussed these previous aspects, it can be established that the best responsive MS varieties to salinity in the hydroponic assays were the following. In the first place, MS2, with a 1.089 Na^+/K^+ ratio, 5 SES score and a reduction of the RCC in a 22.53%, although the final value, 29.22 is a very acceptable value. The rest of the best salt-responsive MS

varieties, although far from MS2 parameters, were MS20, with a 1.219 Na^+/K^+ ratio, 5.8 SES score and a reduction of the RCC in a 39.14%, with a final RCC value of 19.50; MS11, with a 1.284 Na^+/K^+ ratio, 5.8 SES score and a reduction of the RCC in a 39.14%, with a final RCC value of 21.30; MS4, with a 1.380 Na^+/K^+ ratio, 5.8 SES score and a reduction of the RCC in a 29.65%, with a final RCC value of 21.64, and finally, MS23, with a 1.406 Na^+/K^+ ratio, 5.8 SES score and a reduction of the RCC in a 11.09%, with a final RCC value of 23.56.

Regarding the field assays, a Na^+/K^+ ratio assay was performed 15 days after transplantation in LP and MS varieties in non-salinized and salinized fields. This assay was designed to measure the initial response of the varieties after transplantation into real field conditions. The Na^+/K^+ ratio values obtained in this experiment cannot be directly compared to those of the hydroponic assays, since the varieties assayed were selected upon different criteria and not all the hydroponic varieties are present in the field assays and vice versa. Besides, the stage of plant growth in both assays was different. The field-assayed varieties had been previously germinated in soil in a greenhouse before transplanting them into the fields, as is the usual agronomical practice. Moreover, the real field assay makes much more difficult to keep controlled conditions by the nature of the assay itself, introducing more uncontrollable variables than in a greenhouse assay, like the changing climatic conditions and heterogenous patches of salinity into the fields.

Considering all these aspects, the top five best LP performing varieties in the field assays according to the Na^+/K^+ ratio were LP29, with a 0.148 ratio value; LP30, with a 0.168 ratio value; LP24, with a 0.174 ratio value; LP26, with a 0.173 ratio value and LP22, with a 0.179 ratio value. None of these scores were lower than the Na^+/K^+ ratio from the resistant parental, FL478, with a 0.123 value, but were better than the PL12 ratio, the susceptible parental, with a 0.329 value. Regarding the MS varieties, only two varieties were selected as the best-performing, due to the low number of varieties evaluated. Considering the resistant parental, IR64-*Saltol*, which presented a score of 0.165, the best performing line was MS28, with a lower ratio than its parental, 0.145. The second best performing line was MS14, with a ratio of 0.167, very close to the IR64-*Saltol* one. The ratio for the susceptible parental, PM37 in this case, is 0.203. The scale of the ratios is different from the hydroponic assay ratios due to all the different circumstances mentioned above.

Considering the yield of the LP varieties evaluated, all the varieties present a similar behavior under salinity, with the exception of LP31, whose performance is the same in

both salinized and non-salinized fields. Despite this, its yield in non-salinized fields is lower than in the rest of the varieties. The lack of statistical correlation between the Na^+/K^+ ratio and the yield values could be explained by various reasons. First, the yield data does not cover all the varieties whose Na^+/K^+ ratio was measured. A wider set of yield data would help to perform the statistical analysis with a higher sample size, which could influence the correlation coefficient. Correlation between the Na^+/K^+ ratio of the hydroponic assays and the fields yield was not performed due to the lack of common varieties assayed in both experiments.

Another reason could be the day that the samples were collected to measure the Na^+/K^+ ratio. In our experiment, samples were collected from plants previously germinated and aged 15 day-old, so approximately 30-40 days after the initial sowing. Asch et al. (2000) monitored the Na^+/K^+ ratio of local rice varieties on Senegalese salinized fields and affirmed that the days after sowing in which they found significant correlation between the yield and the ratio were around 60. Further experiments with variable sample-collecting days should be performed along various seasons to establish the ideal sampling timepoint for our varieties.

Besides the experimental design, the differences shown in yield could be due to the varieties itself. The selection of the varieties, performed outside this thesis, was made by KASP molecular markers, with a limited quantity of molecular markers that covered the *Saltol* region and punctual regions of other chromosomes. The possibility that the best performing varieties in fields could carry genes that confer better adaptation to field conditions, and not only salinity, is very high. Also, it is possible that the salt tolerance is conferred by the *Saltol* region in a large extent but not be the only QTL that provides this tolerance, specially at the seedling stage (Chattopadhyay et al., 2013).

Considering the results obtained in this experiment, the evaluated varieties can be used to further study the *Saltol* region and its function in salt tolerance. With more data collected during various seasons and thanks to the previous characterization of the salt-tolerant varieties by hydroponic assays, a further step could be taken to design a model that predicts yield from the Na^+/K^+ ratio data, see Mel et al. (2019).

5.3 Experiment 3: Studying the rice pearl in Mediterranean varieties by different approaches

This experiment has performed a multidisciplinary approach to the rice pearl in Mediterranean varieties, since it plays a very relevant gastronomical role in Spain and Italy. The disregard of the pearl character in many rice consuming countries together with the confusion of the terms pearled/chalky has limited the progress in fully understanding the causes of the pearled phenotype. During the past 10 years, studies have been published increasingly, but most of them were focused in indica varieties. This experiment is pioneering the study of the rice pearl in specific japonica Mediterranean varieties widely consumed along some European regions.

Although most of the experiment has focused in a physicochemical approach to the pearl character, an experiment was also performed at a genetical level. A sequencing study was made to check the affirmation made by Li et al. (2014) that the presence of pearl can be monogenically explained in indica varieties by the high expression of the gene *Chalk5*. This high expression would be caused by the presence of two polymorphisms in the promotor region, which allow the binding of promoter elements. The sequencing performed in our five japonica varieties confirmed that, at least, the expression of this gene is not regulated in the same way as in indica varieties. Presumably, the single expression of the *Chalk5* gene is not determinant to the presence of pearl in our studied varieties. The next steps in the study of *Chalk5* for japonica varieties could be to measure the gene expression in pearled and crystalline varieties by a quantitative PCR, and to perform a chromatin immunoprecipitation (ChIP) analysis to study the transcription factors present in the promoter region. A more ambitious and time-consuming but complete study would be to develop a RILs population from relevant pearled and crystalline japonica varieties as parentals, and look for QTLs or genes related to the pearl presence, considering the already existing knowledge.

Considering the physical measurements, they showed the variety of grain types used in this experiment regarding their classifications. Another relevant aspect about the pearled varieties was the significant differences in the percentage that the pearl takes up in their grains, showing the diversity of this character.

Regarding the amylose content, the average percentages varied for each variety and fractions, which is expected since these values are different for each variety (Wani et al., 2012). Our results showed non-significant differences in the various fractions of the pearled grains, with the pearl fraction presenting a slightly higher amylose content than

in the crystalline or whole grain part for the Montsianell and Bomba varieties. Lin et al. (2016) reported that the amylose content did not relate to the presence of pearl *per se*. A clear pattern could be found in the percentage of amylose in the chalky grains of all varieties, presenting significant lower values. This fact matches the results obtained by various authors when measuring the amylose content in normal and chalky grains of some rice varieties (Kim et al., 2000; Patindol & Wang, 2003; Singh et al., 2003). This points out to a different explanation for the formation of chalky grains and pearled grains. Patindol and Wang (2003) proposed that there is a mechanism related to chalky grains that favors glucan branching over elongation, explaining the lesser quantity of amylose, or higher of amylopectin (since the amylopectin percentage is calculated as 100 minus % amylose content).

In order to determine the degree of crystallinity of each variety, X-ray diffraction was used since is the most widespread technique to study starch structure. All diffractograms of each variety and grain type followed an A-type pattern, with peaks close to 15º, 17º, 18º and 23º, as expected for cereal starches (Li et al., 2014; Wani et al., 2012). Other authors have also found an A-type pattern when studying rice starches (Iturriaga et al., 2004; Mohan et al., 2005; Patindol & Wang, 2003). Regarding our obtained crystallinity percentages, their range seemed to vary more depending on the variety than if the grain was pearled or crystalline. A variation on the crystallinity percentage for different rice varieties had already been reported (Iturriaga et al., 2004; Patindol & Wang, 2003). The percentage of crystallinity in chalky grains did not follow a fix pattern, being for some varieties higher than the normal grains (Bomba, PL12) and lower than the values from the normal grains (Montsianell, Carnaroli, Guadiamar). Patindol and Wang (2003) studied the degree of crystallinity in normal and chalky grains of six varieties, and found that this value was always higher in the chalky grains than in the normal grains of each variety. This premise does not match our results. They also found an inverse relationship between crystallinity and amylose, result that also does not match ours, since for example, the highest amylose values belong to Carnaroli, and its crystallinity percentages are not the highest of all varieties. Nonetheless, other authors such as Wani et al. (2012) have denied a significant effect of the amylose content in the crystallinity percentage. These discrepancies can be due to further and more complex aspects that determine the degree of crystallinity besides amylose content, like the proportion of amylopectin short-side chained and long-side chained, as proposed by Singh et al. (2006).

Moving onto the SEM pictures, other authors have used this technique to study pearled or chalky areas in rice grains in comparison with crystalline fractions or grains, obtaining pictures that match the general appearance of both our pearl/chalky and crystalline pictures (Kim et al., 2000; Li et al., 2014; Lin et al., 2014, 2016; Wang et al., 2010; Xi et al., 2014; Zhu et al., 2019). In all these studies, the pearl/chalky areas present irregular and round-shaped amyloplasts and starch granules, with spaces between them and an irregular distribution, similar to our pictures. Ratanasumawong et al. (2019) studied the water diffusion in pearled and crystalline rice varieties and found a faster diffusion on the pearled grains due to the spaces between the amyloplasts and the disorganized structure in the pearled tissue. This explains why the pearl takes up the flavor of the *sofrito* or broth that the grains are cooked in. This phenomenon had already been described by Rani and Bhattacharya (1989).

Another noticeable aspect of our pictures is how the protein bodies were more visible in the crystalline tissues than in the pearl/chalky tissues. The storage protein measurements (which are contained in the protein bodies) made in this experiment cannot be considered in this issue since they are relative and not absolute measurements. Xi et al. (2014) pointed out to a weaker adhesion of the protein bodies in pearled/chalky tissues due to the many spaces between the amyloplasts. A plausible explanation could be that during the cut of the grain for the samples preparation, the protein bodies present in the surface may have fell. In our crystalline pictures, many traces of protein bodies can be observed, indicating also a loss during the sample preparation, although due to the more organized structures of the amyloplasts and the better insertion of the protein bodies between them, many of them were still observable. Lin et al. (2016) also reported observable protein bodies or traces in crystalline tissues and not in pearled tissues. Li et al. (2014) analyzed the quantity of protein bodies in a pearled and a crystalline rice variety, and found a decreased concentration of PB-Is and PB-IIs in the pearled variety in comparison to the crystalline variety. The addition of both phenomena, the poor PB adhesion and the lesser quantity, can explain the lack of visible protein bodies in our pearled/chalky pictures.

Regarding the chalky Montsianell picture, some marks of α-amylases attacks were noticed. These had already been found in other SEM studies in two pearled mutants by Lin et al. (2014, 2016) and in a heat-stressed variety by Tsutsui et al. (2013). Tsutsui et al. (2013) also mentioned that there is a relationship between chalkiness and starch degradation by α-amylases when plants are subjected to a high temperature stress. Lin et al. (2014) reported an increased expression of mRNA of various α-amylases genes and

increased α-amylase activity in a pearled mutant. On the other hand, Ishimaru et al. (2009) studied chalky and pearled grains formed under heat stress and did not found α-amylase transcripts during grain filling, suggesting that hydrolytic activity was not the cause of the pearl formation, although they did not exclude a possible α-amylase activity at late stages. These hypotheses are placed in a scenario of heat stress of the varieties studied, which besides, are mutants of crystalline varieties. Our pearled varieties showed all their grains pearled when growing under their optimal temperatures, and chalky grains occur occasionally within a panicle with normal grains. It seems that the hypothesis exposed by other authors cannot be extrapolated to our study, but a measure of free glucose and α-amylase activity within the grain would be interesting to deepen in this question.

Regarding the relative content of the different storage protein fractions, they all seemed to follow a heterogeneous distribution pattern, with some exceptions. The prolamins content in the chalky grains of all varieties (except for Bomba) were the lowest of all fractions. Lin et al. (2016) found a lesser quantity of prolamins in the pearl fraction than in the crystalline fraction of a pearled mutant. Prolamins are contained in PB-Is (Kawakatsu & Takaiwa, 2018), so a measurement of the quantity of PBs in the different fractions, as performed by Li et al. (2014), would be an interesting experiment to perform next.

Our results also showed a higher level of pro-glutelins or glutelins precursors in the chalky grains of Montsianell, Bomba, Guadiamar and PL12. Wang et al. (2010) created a rice mutant with the *OsRab5a* altered, which codifies for a GTPase responsible of the vesicle trafficking. This mutant abnormally accumulated pro-glutelins and presented a chalky grain appearance. Liu et al. (2013) created a mutant of the *OsVPS9A* gene, which codifies for an activator of *OsRab5a*, also producing an abnormal accumulation of pro-glutelins and chalky endosperm. Zhu et al. (2019) created a mutant that accumulated glutelin precursors by altering the gene *OsNHX5*, which codifies for a Na^+/H^+ antiporter and alters vesicle trafficking, originating a chalky endosperm in the grain.

Lin et al. (2014) performed an iTRAQ analysis on a pearled mutant, and found that the biggest group of differentially expressed proteins in the mutant belonged to the protein metabolism. Li et al. (2014) pointed out to an alteration of the vesicle trafficking and therefore protein trafficking to explain the rice pearl. Wada et al. (2019) affirmed that under heat stress, crystalline varieties begin to form a pearl due to the disruption of protein synthesis with the consequent PB synthesis inhibition. The different experiments cited cannot be directly correlated to our study since they are performed with mutants, some

chalky, some pearled, or from stressed crystalline varieties, although it seems clear that the protein contents in relation with their metabolism and trafficking may have a relationship with the presence of the rice pearl.

In conclusion, this study has set a first approach to dissect the causes of the rice pearl in Mediterranean japonica varieties, making a clear difference between the pearled grain and the chalky grain by evaluating the existing studies. Due to the differences in the amylose and protein measurements between the chalky and pearled grains, we can conclude that their origin or causes are not the same. On the other hand, some premises have been established in order to continue the study of the rice pearl in Mediterranean japonica varieties. A complete genomic study should be performed with RILs obtained by Mediterranean japonica varieties, considering the already detected QTLs and that these varieties have been cultivated traditionally knowing that they are pearled, and not under stresses to originate the valuated pearl. This could mean that our Mediterranean varieties have been carrying one or more mutations in one or more genes that confer the pearled phenotype. Special attention should be put in the genes involved in protein metabolism and trafficking. With the data obtained from this study, further experiments could be designed focusing in elements of interest, like expression analysis, cloning certain genes, transmission electron microscopy observations, PB studies, immunofluorescence analyses, and so on. At the end, this could lead to the creation of a marker profile useful for the genotyping and breeding of rice varieties.

5.4 Overall discussion

Nowadays, the concept of bioeconomy is resonating stronger. The Spanish Ministry of Economic Affairs and Digital Transformation (MINECO) has defined the bioeconomy as "*the whole of the economic activities that provide goods and services, and thus generate economic value, through the use, as fundamental elements, of resources of biological origin in an efficient and sustainable manner*" (Lániez & Periago, 2016). The world is facing new challenges as the increase of the population, and therefore, the food demand, as well as the emergence of climate change and the need of competitiveness of the economy. These must be faced through the empowerment of science and technologies in connection with private and public entities. According to the Knowledge Centre for Bioeconomy, agriculture is the most important bioeconomy sector in Spain, with a value of 43.8 million of euros in 2015 and coping the 50.9% employment of all bioeconomy sectors (Lániez & Periago, 2016). Rice mobilized 258.766 millions of euros in Spain in 2019 (MINECO, 2019). Thus, the studies dedicated to improving different aspects of the rice production in Spain are crucial.

This thesis has made a multidisciplinary approach to the knowledge of Mediterranean rice varieties considering the actual applicability to the Spanish rice industry through three experimental areas.

First, the application and improvement of a biotechnological tool such as anther culture to two commercial Spanish varieties, Montsianell and PL12 (commercially known as Olesa) has been performed. Anther culture presents two major advantages, which are the reduction of the culture cycles, since it allows to originate new plants *in vitro* from immature embryos and not whole mature seeds; and the production of pure homozygous lines. The results of this thesis can be applied to the acceleration and increase of the rice production with more than one generation per year, to fasten breeding processes or to save a season when the climatic circumstances (which are getting increasingly unpredictable due to climate change) force an early harvest or late sowing.

Second, the application of plant physiology to the salt-resistance testing of individuals comprehended in the NEURICE project, has allowed to select the best individuals and to collect data to be able to establish future links between yield production and hydroponic testing. The selected salt-resistant varieties will tolerate the residual salinity derived from the fields treatment with saline water to eradicate the apple snails' plagues, thanks to the successful breeding of the *Saltol* genetic region into them. Besides, a new applicability

can be given to these varieties. Due to the unfortunate recent flooding of the Ebro Delta caused by the Gloria storm, that submerged 3000 ha of fields (*La Vanguardia*, 2020), the salinity of the fields has also increased heavily. The use of these *Saltol*-introgressed commercial varieties could save this years' harvest. In a global context, salinity-tolerant crops, especially those grown near coastal areas, will be increasingly demanded due to the sea level rise caused by climate change (Mimura, 2013).

Third, the genetic and physicochemical study of the rice pearl in Mediterranean varieties, an added value to the Spanish (and Italian) gastronomy and therefore economy, has allowed to establish for the first time paths to further investigate its origin and causes. The investment in the crop research with special gastronomical value and certificate of origin will allow to increase the competitiveness of our products in a global market increasingly demanding and that considers added values.

The experiments performed in this thesis have contributed to expand the knowledge and/ or to study for the first time aspects related to Mediterranean japonica rice varieties trough plant physiology and biotechnology, with direct applicability in terms of production and market competitiveness.

6. Conclusions

- The anther culture assay has allowed to improve the performance of a previous protocol for two temperate japonica varieties (Montsianell, PL12) and has tested the performance of two tropical japonica varieties (303012 and 303013) for the first time.
- The addition of 150 mg · L^{-1} in our induction media significantly favored the calli induction for Montsianell, 303012 and 303013.
- The different regeneration media assayed significantly affected plantlet regeneration. The supplemented media improved the performance of the staple regeneration media, XACRM. Sorbitol-supplemented media (SARM) allowed regeneration in all varieties, being the highest rate for the three long-grain varieties (PL12, 30312, 30103). Gelrite-supplemented media (GERM) caused the highest regeneration for Montsianell.
- Doubled haploid plantlets only regenerated from calli obtained in the colchicine-supplemented media for Montsianell and 303013, proving that the presence of colchicine in the induction media favors green doubled haploid plantlets production.
- It is clearly shown that the variety used as well as the media additives determine the performance of rice anther culture.

- A successful salt-tolerance characterization of many *Saltol*-introgressed Mediterranean varieties was made in hydroponic and field assays.
- Regarding the hydroponic assays performed at 80 mM NaCl with LP and MS *Saltol*-introgressed varieties, the SES score and SPAD measurements correlated in a significant, strong and negative way. Furthermore, the Na^+/K^+ ratio measurements and SES score correlated in a significant and positive way.
- The top five best performing LP varieties in the hydroponic assays according to the Na^+/K^+ ratio, SES score and SPAD values were LP9, LP7, LP28, LP18 and LP16.

- The top five best performing MS varieties in the hydroponic assays according to the Na^+/K^+ ratio, SES score and SPAD values were MS2, MS20, MS11, MS4 and MS23.
- Regarding the Ebro Delta filed assays, the best performing varieties according to the Na^+/K^+ ratio were LP29, LP30, LP24, LP26 and LP22 for the LP family, and MS28 and MS14 for the MS family.

- The rice pearl study performed has set a first genetic and physicochemical approach to unravel this character in five Mediterranean japonica varieties (Montsianell, Bomba, Carnaroli, Guadiamar and PL12).
- The regulation of the expression of the *Chalk5* gene by the presence of two polymorphisms in the promoter sequence was not the same in indica varieties than in our Mediterranean japonica varieties, and therefore, it did not explain the presence of pearl.
- The amylose content did not vary significantly in the pearl fraction of the pearled varieties, so it was not determinant to the presence of pearl *per se*. On the other hand, it was significantly lower in the chalky grains of our studied varieties.
- Our Mediterranean rice varieties followed an A-type pattern in their diffractograms. Their crystallinity percentage varied in relation to the variety and not regarding the type of grain or amylose content.
- Under SEM microscopy, the pearl fractions and chalky grains presented a similar aspect, with round-shaped and disorganized amyloplasts and starch granules. The crystalline fractions or grains presented organized and polyhedral amyloplasts with visible protein bodies around them.
- The relative storage proteins content changed with the variety. The prolamin levels were generally lower and the glutelin precursors higher in the chalky grains of our varieties.

7. Bibliography

Afza, R., Shen, M., Zapata-Arias, F. J., Xie, J., Fundi, H. K., Lee, K. S., Bobadilla-Mucino, E., & Kodym, A. (2000). Effect of spikelet position on rice anther culture efficiency. *Plant Science*, *153*(2), 155–159. https://doi.org/10.1016/S0168-9452(99)00266-6

Ahmadi, N., Negrão, S., Katsantonis, D., Frouin, J., Ploux, J., Letourmy, P., Droc, G., Babo, P., Trindade, H., Bruschi, G., Greco, R., Oliveira, M. M., Piffanelli, P., & Courtois, B. (2011). Targeted association analysis identified japonica rice varieties achieving Na +/K + homeostasis without the allelic make-up of the salt tolerant indica variety Nona Bokra. *Theoretical and Applied Genetics*, *123*(6), 881–895. https://doi.org/10.1007/s00122-011-1634-4

Alcázar-Alay, S. C., & Meireles, M. A. A. (2015). Physicochemical properties, modifications and applications of starches from different botanical sources. *Food Science and Technology*, *35*(2), 215–236. https://doi.org/10.1590/1678-457X.6749

Alemanno, L., & Guiderdoni, E. (1994). Increased doubled haploid plant regeneration from rice (*Oryza sativa* L.) anthers cultured on colchicine-supplemented media. *Plant Cell Reports*, *13*(8), 432–436. https://doi.org/10.1007/BF00231961

Asch, F., Dingkuhn, M., Dörffling, K., & Miezan, K. (2000). Leaf K/Na ratio predicts salinity induced yield loss in irrigated rice. *Euphytica*, *113*(2), 109–118. https://doi.org/10.1023/A:1003981313160

Baenziger, P. S., & Meredith, C. P. (1985). *Current Plant Science and Biotechnology in Agriculture*. https://doi.org/DOI 10.1007/978-94-017-1860-8

Balsells, F. (2010). La erradicación del caracol manzana en el delta del Ebro ya es imposible. *El País*. https://elpais.com/diario/2010/12/07/catalunya/1291687645_850215.html

Bao, J., & Bergman, C. J. (2018). Rice Flour and Starch Functionality. In *Starch in Food: Structure, Function and Applications: Second Edition*. Elsevier Ltd. https://doi.org/10.1016/B978-0-08-100868-3.00010-X

Batayeva, D., Labaco, B., Ye, C., Li, X., Usenbekov, B., Rysbekova, A., Dyuskalieva, G., Vergara, G., Reinke, R., & Leung, H. (2018). Genome-wide association study of seedling stage salinity tolerance in temperate japonica rice germplasm. *BMC Genetics*, *19*(1). https://doi.org/10.1186/s12863-017-0590-7

Bernis, J. M. F., & Pamies, C. B. (2004). Variedades y mejora del arroz. In *Variedades y mejora del arroz (Oryza sativa,L).*

Bernis, J. M. F., & Pamies, C. B. (2006). *Economía del arroz: Variedades y mejora*. http://www.eumed.net/libros-gratis/2006a/fbbp/index.htm

Berthomieu, P., Conéjéro, G., Nublat, A., Brackenbury, W. J., Lambert, C., Savio, C., Uozumi, N., Oiki, S., Yamada, K., Cellier, F., Gosti, F., Simonneau, T., Essah, P. A., Tester, M., Véry, A. A., Sentenac, H., & Casse, F. (2003). Functional analysis of AtHKT1 in Arabidopsis shows that Na+ recirculation by the phloem is crucial for salt tolerance. *EMBO Journal*, *22*(9), 2004–2014. https://doi.org/10.1093/emboj/cdg207

Bonilla, P., Dvorak, J., Mackill, D., Deal, K., & Gregorio, G. (2002). RFLP and SSLP mapping of salinity tolerance genes in chromosome 1 of rice (*Oryza sativa* L.) using recombinant inbred lines. *Philippine Agricultural Scientist*, *65*(1), 68–76.

Borghini, M., Bryden, H., Schroeder, K., Sparnocchia, S., & Vetrano, A. (2014). The Mediterranean is becoming saltier. *Ocean Science*, *10*(4), 693–700. https://doi.org/10.5194/os-10-693-2014

Cai, Yicong, Li, S., Jiao, G., Sheng, Z., Wu, Y., Shao, G., Xie, L., Peng, C., Xu, J., Tang, S., Wei, X., & Hu, P. (2018). OsPK2 encodes a plastidic pyruvate kinase involved in rice endosperm starch synthesis, compound granule formation and grain filling. *Plant Biotechnology Journal*, 1878–1891. https://doi.org/10.1111/pbi.12923

Cai, Yue, Zhang, W., Jin, J., Yang, X., You, X., Yan, H., Wang, L., Chen, J., Xu, J., Chen, W., Chen, X., Ma, J., Tang, X., Kong, F., Zhu, X., Wang, G., Jiang, L., Terzaghi, W., Wang, C., & Wan, J. (2018). *OsPKpα1* encodes a plastidic pyruvate kinase that affects starch biosynthesis in the rice endosperm. *Journal of Integrative Plant Biology*. https://doi.org/10.1111/jipb.12692

Calleberg, E. K., & Johansson, L. B. (1996). Effect of gelling agents on anther cultures. In *In Vitro Haploid Production in Higher Plants* (pp. 189–203). Springer, Dordrecht. https://doi.org/10.1007/978-94-017-1860-8_11

Chang, T., & Bardenas, E. O. (1965). The morphology and varietal characteristics of the rice plant. In *The morphology and varital characteristics of the rice plant*.

Chattopadhyay, K., Nath, D., Das, G., Mohanta, R. L., Marndi, B. C., Singh, D. P., Sarkar, R. K., & Singh, O. N. (2013). Phenotyping and QTL-linked marker-based genotyping of rice lines with varying level of salt tolerance at flowering stage. *Indian Journal of Genetics and Plant Breeding*, *73*(4), 434–437. https://doi.org/10.5958/j.0975-6906.73.4.065

Chen, C.-C., Tsay, H.-S., & Huang, C.-R. (1991). Factors Affecting Androgenesis in Rice (*Oryza sativa* L.). In Y. P. S. Bajaj (Ed.), *Rice* (pp. 193–215). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-83986-3_14

Chen, C. C., Tsay, H. S., & Huang, C. R. (1991). Factors Affecting Androgenesis in Rice (*Oryza sativa* L.). In *Rice* (Issue 14, pp. 193–215). Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-83986-3_14

Chen, C., Chen, H., Lin, Y. S., Shen, J. B., Shan, J. X., Qi, P., Shi, M., Zhu, M. Z., Huang, X. H., Feng, Q., Han, B., Jiang, L., Gao, J. P., & Lin, H. X. (2014). A two-locus interaction causes interspecific hybrid weakness in rice. *Nature Communications*, *5*, 3357. https://doi.org/10.1038/ncomms4357

Chen, Q. F., Wang, C. L., Lu, Y. M., Shen, M., Afza, R., Duren, M. V, & Brunner, H. (2001). Anther culture in connection with induced mutations for rice improvement. *Euphytica*, *120*(3), 401–408. https://doi.org/10.1023/A:1017518702176

Chen, Y. (1986). The inheritance of rice pollen plants and its applications in crop improvement. *Haploids of Higher Plants In Vitro*, 118–136. http://ci.nii.ac.jp/naid/10006702206/en/

Cheng, F. M., Zhong, L. J., Wang, F., & Zhang, G. P. (2005). Differences in cooking and eating properties between chalky and translucent parts in rice grains. *Food Chemistry*, *90*(1–2), 39–46. https://doi.org/10.1016/j.foodchem.2004.03.018

Chu, C. (1981). The N6 medium and its application to anther culture of cereal crops, in Plant Tissue Culture. *Plant Tissue Culture. Proceedings of the Peking Symposium*, 43–50. https://ci.nii.ac.jp/naid/10003229689/

Chun, A., Song, J., Kim, K.-J., & Lee, H.-J. (2009). Quality of head and chalky rice and deterioration of eating quality by chalky rice. *Journal of Crop Science and Biotechnology*, *12*(4), 239–244. https://doi.org/10.1007/s12892-009-0142-4

Chung, G. S. (1987). Application of anther culture technique for rice (*Oryza sativa* L.) improvement. In *Proc Korea-China Plant tissue Cult Symposium* (pp. 35–56).

Claes, B., Dekeyser, R., Villarroel, R., Van Den Bulcke, M., Bauw, G., Van Montagu, M., & Caplan, A. (1990). Characterization of a rice gene showing organ-specific expression in response to salt stress and drought. *Plant Cell*, *2*(1), 19–27. https://doi.org/10.2307/3869047

Counce, P. A., Keisling, T. C., & Mitchell, A. J. (2000). A uniform, objectives, and adaptive system for expressing rice development. *Crop Science*, *40*(2), 436–443. https://doi.org/10.2135/cropsci2000.402436x

Council Regulation (EC) No 1234/2007 of 22 October 2007 establishing a common organisation of agricultural markets and on specific provisions for certain agricultural products (Single CMO Regulation), L299 Official Journal of the European Union 1 (2007). http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2007:299:0001:0149:EN:PDF

CRRI. (2008). *Central Rice Research Institute (CRRI) Annual Report*.

CRRI. (2010). *Central Rice Research Institute (CRRI) Annual Report*.

Davey, M. R. (2009). Advances in haploid production in higher plants. In *Annals of Botany* (Vol. 104, Issue 7). https://doi.org/10.1093/aob/mcp248

Del Rosario, A. R., Briones, V. P., & Vidal, A. J. (1968). Composition and endosperm structure of developing and mature rice kernel. *Cereal Chemistry*, *45*(3), 225–235. http://www.aaccnet.org/publications/cc/backissues/1968/Documents/CC1968a26.html

Dionisio-Sese, M. L., & Tobita, S. (1998). Antioxidant responses of rice seedlings to salinity stress. *Plant Science*, *135*, 1–9. https://eurekamag.com/pdf/003/003043875.pdf

Dwivedi, S. L., Britt, A. B., Tripathi, L., Sharma, S., Upadhyaya, H. D., & Ortiz, R. (2015). Haploids: Constraints and opportunities in plant breeding. In *Biotechnology Advances* (Vol. 33, Issue 6, pp. 812–829). https://doi.org/10.1016/j.biotechadv.2015.07.001

EFSA. (2012). *Scientific Opinion on the evaluation of the pest risk analysis on Pomacea insularum, the island apple snail, prepared by the Spanish Ministry of Environment and Rural and Marine Affairs*.

EFSA. (2016). Scientific Opinion on the environmental risk assessment of the apple snail for the EU. *EFSA Journal*, *12*(4). https://doi.org/10.2903/j.efsa.2014.3641

EPO. (2018). *First report of Pomacea sp. in France*.

Fan, C. C., Yu, X. Q., Xing, Y. Z., Xu, C. G., Luo, L. J., & Zhang, Q. (2005). The main effects, epistatic effects and environmental interactions of QTLs on the cooking and eating quality of rice in a doubled-haploid line population. *Theoretical and Applied Genetics*, *110*(8), 1445–1452. https://doi.org/10.1007/s00122-005-1975-y

FAOSTAT. (2020). *FAOSTAT*. http://www.fao.org/faostat/en/#data/QC

Faure, J., & Mazaud, F. (1995). Rice quality in the European Union. *Agriculture et Developpement (France)*, *December*.

Ferreres, I., Ortega, M., López-Cristoffanini, C., Nogués, S., & Serrat, X. (2019). Colchicine and osmotic stress for improving anther culture efficiency on long grain temperate and tropical japonica rice genotypes. *Plant Biotechnology*, *36*(4), 269–273. https://doi.org/10.5511/plantbiotechnology.19.1022a

Ferrero, A. (2007). Rice scenario in the European Union. *Cahiers Agricultures*, *16*(4), 272–277. https://doi.org/10.1684/agr.2007.0108

Forster, B. P., & Thomas, W. T. B. (2010). Doubled Haploids in Genetics and Plant Breeding. In *Plant Breeding Reviews* (Vol. 25). https://doi.org/10.1002/9780470650301.ch3

Frouin, J., Languillaume, A., Mas, J., Mieulet, D., Boisnard, A., Labeyrie, A., Bettembourg, M., Bureau, C., Lorenzini, E., Portefaix, M., Turquay, P., Vernet, A., Périn, C., Ahmadi, N., & Courtois, B. (2018). Tolerance to mild salinity stress in japonica rice: A genome-wide association mapping study highlights calcium signaling and metabolism genes. *PLoS ONE*, *13*(1), 1–27. https://doi.org/10.1371/journal.pone.0190964

Furuta, T., Komeda, N., Asano, K., Uehara, K., Gamuyao, R., Angeles-Shim, R. B., Nagai, K., Doi, K., Wang, D. R., Yasui, H., Yoshimura, A., Wu, J., McCouch, S. R., & Ashikari, M. (2015). Convergent loss of awn in two cultivated rice species *Oryza sativa* and *Oryza glaberrima* is caused by mutations in different loci. *G3: Genes, Genomes, Genetics*, *5*(11), 2267–2274. https://doi.org/10.1534/g3.115.020834

Gao, Y., Liu, C., Li, Y., Zhang, A., Dong, G., Xie, L., Zhang, B., Ruan, B., Hong, K., Xue, D., Zeng, D., Guo, L., Qian, Q., & Gao, Z. (2016). QTL analysis for chalkiness of rice and fine mapping of a candidate gene for qACE9. *Rice*, *9*(1). https://doi.org/10.1186/s12284-016-0114-5

Garris, A. J., Tai, T. H., Coburn, J., Kresovich, S., & McCouch, S. (2005). Genetic structure and diversity in *Oryza sativa* L. *Genetics*, *169*(3), 1631–1638. https://doi.org/10.1534/genetics.104.035642

Germanà, M. A. (2011). Anther culture for haploid and doubled haploid production. In *Plant Cell, Tissue and Organ Culture* (Vol. 104, Issue 3, pp. 283–300). https://doi.org/10.1007/s11240-010-9852-z

Ghaffari, A., Gharechahi, J., Nakhoda, B., & Salekdeh, G. H. (2014). Physiology and proteome responses of two contrasting rice mutants and their wild type parent under salt stress conditions at the vegetative stage. *Journal of Plant Physiology*, *171*(1), 31–44. https://doi.org/10.1016/j.jplph.2013.07.014

Ghesquière, A., Albar, L., Lorieux, M., Ahmadi, N., Fargette, D., Huang, N., McCouch, S. R., & Notteghem, J. L. (1997). A major quantitative trait locus for rice yellow mottle virus resistance maps to a cluster of blast resistance genes on chromosome 12. *Phytopathology*, *87*(12), 1243–1249. https://doi.org/10.1094/PHYTO.1997.87.12.1243

GISD. (2019). *Species profile: Pomacea canaliculata.* Global Invasive Species Database. http://www.iucngisd.org/gisd/species.php?sc=135

He, T., Yang, Y., Tu, S. B., Yu, M. Q., & Li, X. F. (2006). Selection of interspecific hybrids for anther culture of indica rice. *Plant Cell, Tissue and Organ Culture*, *86*(2), 271–277. https://doi.org/10.1007/s11240-006-9117-z

Ho, V. T., Thomson, M. J., & Ismail, A. M. (2016). Development of salt tolerant IR64 near isogenic lines through marker-assisted breeding. *Journal of Crop Science and Biotechnology*, *19*(5), 373–381. https://doi.org/10.1007/s12892-016-0049-9

Hooghvorst, I., Ramos-Fuentes, E., López-Cristofannini, C., Ortega, M., Vidal, R., Serrat, X., & Nogués, S. (2018). Antimitotic and hormone effects on green double haploid plant production through anther culture of Mediterranean japonica rice. *Plant Cell, Tissue and Organ Culture*, *134*(2), 205–215. https://doi.org/10.1007/s11240-018-1413-x

Hooghvorst, I., Torrico, O., Hooghvorst, S., & Nogués, S. (2020). In situ parthenogenetic doubled haploid production in melon ‘Piel de Sapo’ for breeding purposes. *Frontiers in Plant Science*, *11*, 378. https://doi.org/10.3389/fpls.2020.00378

Hu, H., & Zeng, J. (1984). Development of new varieties via anther culture. In *Handbook of Plant Cell Culture: III. Crop Species* (pp. 65–90). http://www.sidalc.net/cgi-bin/wxis.exe/?IsisScript=CIMMYT.xis&method=post&formato=2&cantidad=1&expresion=mfn=015226

Huang, X., Kurata, N., Wei, X., Wang, Z. X., Wang, A., Zhao, Q., Zhao, Y., Liu, K., Lu, H., Li, W., Guo, Y., Lu, Y., Zhou, C., Fan, D., Weng, Q., Zhu, C., Huang, T., Zhang, L., Wang, Y., … Han, B. (2012). A map of rice genome variation reveals the origin of cultivated rice. *Nature*, *490*(7421), 497–501. https://doi.org/10.1038/nature11532

Hussain, S., Bai, Z., Huang, J., Cao, X., Zhu, L., Zhu, C., Khaskheli, M. A., Zhong, C., Jin, Q., & Zhang, J. (2019). 1-Methylcyclopropene Modulates Physiological, Biochemical, and Antioxidant Responses of Rice To Different Salt Stress Levels. *Frontiers in Plant Science*, *10*. https://doi.org/10.3389/fpls.2019.00124

International Rice Research Institute. (2002). *Rice Standard Evaluation System 2002*. 1–56. http://www.knowledgebank.irri.org/images/docs/rice-standard-evaluation-system.pdf

IRRI. (2002). A Proposal for IRRI to Establish a Grain Quality and Nutrition Research Center. *IRRI Discussion Paper Series No. 44*, *44*, 7. www.riceweb.org

Ishimaru, T., Horigane, A. K., Ida, M., Iwasawa, N., San-oh, Y. A., Nakazono, M., Nishizawa, N. K., Masumura, T., Kondo, M., & Yoshida, M. (2009). Formation of grain chalkiness and changes in water distribution in developing rice caryopses grown under high-temperature stress. *Journal of Cereal Science*, *50*(2), 166–174. https://doi.org/10.1016/j.jcs.2009.04.011

Islam, S. M. S., & Tuteja, N. (2012). Enhancement of androgenesis by abiotic stress and other pretreatments in major crop species. *Plant Science*, *182*(1), 134–144. https://doi.org/10.1016/j.plantsci.2011.10.001

Iturriaga, L., Lopez, B., & Añon, M. (2004). Thermal and physicochemical characterization of seven argentine rice flours and starches. *Food Research International*, *37*(5), 439–447. https://doi.org/10.1016/j.foodres.2003.12.005

IUCN. (2001). *100 of the world's worst invasive species: a selection from the global invasive species database by IUCN.* www.issg.org/booklet.pdf

Jiang, G. H., Xu, C. G., Li, X. H., & He, Y. Q. (2004). Characterization of the Genetic Basis for Yield and Its Component Traits of Rice Revealed by Doubled Haploid Population. *Acta Genetica Sinica*, *31*(1), 63–72.

Kang, H. G., Park, S., Matsuoka, M., & An, G. (2005). White-core endosperm floury endosperm-4 in rice is generated by knockout mutations in the C4-type pyruvate orthophosphate dikinase gene (OsPPDKB). *Plant Journal*, *42*(6), 901–911. https://doi.org/10.1111/j.1365-313X.2005.02423.x

Kasha, K. J., Hu, T. C., Oro, R., Simion, E., & Shim, Y. S. (2001). Nuclear fusion leads to chromosome doubling during mannitol pretreatment of barley (Hordeum vulgare L.) microspores. *Journal of Experimental Botany*, *52*(359), 1227–1238. https://doi.org/10.1093/jxb/52.359.1227

Kato, Y., Sakaguchi, M., Mori, Y., Saito, K., Nakamura, T., Bakker, E. P., Sato, Y., Goshima, S., & Uozumi, N. (2001). Evidence in support of a four transmembrane-pore-transmembrane topology model for the Arabidopsis thaliana Na + /K + translocating AtHKT1 protein, a member of the superfamily of K + transporters. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(11), 6488–6493. https://doi.org/10.1073/pnas.101556598

Kaushal, L., Balachandran, S. M., Ulaganathan, K., & Shenoy, V. (2014). Effect of culture media on improving anther culture response of rice (*Oryza sativa* L.). *International Journal of Agriculture Innovations and Research*, *3*(1), 218–224.

Kawakatsu, T., & Takaiwa, F. (2018). Rice proteins and essential amino acids. In *Rice*. AACCI. Published by Elsevier Inc. in cooperation with AACC International. https://doi.org/10.1016/b978-0-12-811508-4.00004-6

Khanna, H. . K., & Raina, S. . K. (1998). Genotype and culture media interaction effects on regeneration response of three indica rice cultivars. *Plant Cell, Tissue and Organ Culture*, *52*(3), 145–153. https://doi.org/10.1023/A:1006032303195

Kim, K. M., Kwon, Y. S., Lee, J. J., Eun, M. Y., & Sohn, J. K. (2004). QTL mapping and molecular marker analysis for the resistance of rice to ozone. *Molecules and Cells*, *17*(1), 151–155. http://www.ncbi.nlm.nih.gov/pubmed/15055542

Kim, S. S., Lee, S. E., Kim, O. W., & Kim, D. C. (2000). Physicochemical characteristics of chalky kernels and their effects on sensory quality of cooked rice. *Cereal Chemistry*, *77*(3), 376–379. https://doi.org/10.1094/CCHEM.2000.77.3.376

Kim, T. H., Hur, Y. J., Han, S. I., Cho, J. H., Kim, K. M., Lee, J. H., Song, Y. C., Kwon, Y. U., & Shin, D. (2017). Drought-tolerant QTL qVDT11 leads to stable tiller formation under drought stress conditions in rice. *Plant Science*, *256*, 131–138. https://doi.org/10.1016/j.plantsci.2016.11.008

Kovach, M. J., Sweeney, M. T., & McCouch, S. R. (2007). New insights into the history of rice domestication. *Trends in Genetics*, *23*(11), 578–587. https://doi.org/10.1016/j.tig.2007.08.012

Kraehmer, H., Thomas, C., & Vidotto, F. (2017). Rice production in Europe. In *Rice Production Worldwide*. https://doi.org/10.1007/978-3-319-47516-5_4

Krishnamurthy, S. L., Sharma, P. C., Sharma, S. K., Batra, V., Kumar, V., & Rao, L. V. S. (2016). Effect of salinity and use of stress indices of morphological and physiological traits at the seedling stage in rice. *Indian Journal of Experimental Biology*, *54*(12), 843–850.

Kubo, M., & Purevdorj, M. (2004). The Future of Rice Production and Consumption. *Journal of Food Distribution Research*, *35*(1), 128–142. https://doi.org/10.22004/ag.econ.27145

Kumar, V., Singh, A., Mithra, S. V. A., Krishnamurthy, S. L., Parida, S. K., Jain, S., Tiwari, K. K., Kumar, P., Rao, A. R., Sharma, S. K., Khurana, J. P., Singh, N. K., & Mohapatra, T. (2015). Genome-wide association mapping of salinity tolerance in rice (Oryza sativa). *DNA Research*, *22*(2), 133–145. https://doi.org/10.1093/dnares/dsu046

Kumari, M., Clarke, H. J., Small, I., & Siddique, K. H. M. (2009). Albinism in plants: A major bottleneck in wide hybridization, androgenesis and doubled haploid culture. *Critical Reviews in Plant Sciences*, *28*(6), 393–409. https://doi.org/10.1080/07352680903133252

Lániez, M., & Periago, M. J. (2016). The Bioeconomy: An Opportunity for the Spanish Economy. *Intech*, *i*(tourism), 13. https://doi.org/http://dx.doi.org/10.5772/57353

Lee, G. H., Kang, I. K., & Kim, K. M. (2016). Mapping of novel QTL regulating grain shattering using doubled haploid population in rice (*Oryza sativa* L.). *International Journal of Genomics*, *2016*. https://doi.org/10.1155/2016/2128010

Lee, Y., Lim, M., Kim, H., Shin, H., … C. K.-R. R. of, & 1989, U. (1989). An anther-derived new high quality rice variety with disease and insect resistance "Hwacheongbyeo." *Research Reports of the Rural Development Administration, Rice*, *31*(2), 27–34. https://www.cabdirect.org/cabdirect/abstract/19911620993

Li, H., Chen, Z., Hu, M., Wang, Z., Hua, H., Yin, C., & Zeng, H. (2011). Different effects of night versus day high temperature on rice quality and accumulation profiling of rice grain proteins during grain filling. *Plant Cell Reports*, *30*(9), 1641–1659. https://doi.org/10.1007/s00299-011-1074-2

Li, W., Xiao, X., Zhang, W., Zheng, J., Luo, Q., Ouyang, S., & Zhang, G. (2014). Compositional, morphological, structural and physicochemical properties of starches from seven naked barley cultivars grown in China. *Food Research International*, *58*, 7–14. https://doi.org/10.1016/j.foodres.2014.01.053

Li, Y., Fan, C., Xing, Y., Yun, P., Luo, L., Yan, B., Peng, B., Xie, W., Wang, G., Li, X., Xiao, J., Xu, C., & He, Y. (2014). *Chalk5* encodes a vacuolar H + -translocating pyrophosphatase influencing grain chalkiness in rice. *Nature Genetics*, *46*(4), 398–404. https://doi.org/10.1038/ng.2923

Li, Z., Mu, P., Li, C., Zhang, H., Li, Z., Gao, Y., & Wang, X. (2005). QTL mapping of root traits in a doubled haploid population from a cross between upland and lowland japonica rice in three environments. *Theoretical and Applied Genetics*, *110*(7), 1244–1252. https://doi.org/10.1007/s00122-005-1958-z

Lin, Z., Zhang, X., Yang, X., Li, G., Tang, S., Wang, S., Ding, Y., & Liu, Z. (2014). Proteomic analysis of proteins related to rice grain chalkiness using iTRAQ and a novel comparison system based on a notched-belly mutant with white-belly. *BMC Plant Biology*, *14*(1), 163. https://doi.org/10.1186/1471-2229-14-163

Lin, Z., Zheng, D., Zhang, X., Wang, Z., Lei, J., Liu, Z., Li, G., Wang, S., & Ding, Y. (2016). Chalky part differs in chemical composition from translucent part of japonica rice grains as revealed by a notched-belly mutant with white-belly. *Journal of the Science of Food and Agriculture*, *96*(11), 3937–3943. https://doi.org/10.1002/jsfa.7793

Liu, F., Ren, Y., Wang, Y., Peng, C., Zhou, K., Lv, J., Guo, X., Zhang, X., Zhong, M., Zhao, S., Jiang, L., Wang, H., Bao, Y., & Wan, J. (2013). OsVPS9A functions cooperatively with OsRAB5A to regulate post-golgi dense vesicle-mediated storage protein trafficking to the protein storage vacuole in rice endosperm cells. *Molecular Plant*, *6*(6), 1918–1932. https://doi.org/10.1093/mp/sst081

Liu, G. F., Li, M., Wen, J., Du, Y., & Zhang, Y. M. (2010). Functional mapping of quantitative trait loci associated with rice tillering. *Molecular Genetics and Genomics*, *284*(4), 263–271. https://doi.org/10.1007/s00438-010-0566-z

Liu, G., Yang, J., Xu, H., & Zhu, J. (2007). Influence of Epistasis and QTL × Environment Interaction on Heading Date of Rice (*Oryza sativa* L.). *Journal of Genetics and Genomics*, *34*(7), 608–615. https://doi.org/10.1016/S1673-8527(07)60069-1

Liu, X., Wang, Y., & Wang, S. W. (2012). QTL analysis of percentage of grains with chalkiness in Japonica rice (Oryza sativa). *Genetics and Molecular Research : GMR*, *11*(1), 717–724. https://doi.org/10.4238/2012.March.22.1

Liu, Xiaolu, Guo, T., Wan, X., Wang, H., Zhu, M., Li, A., Su, N., Shen, Y., Mao, B., Zhai, H., Mao, L., & Wan, J. (2010). Transcriptome analysis of grain-filling caryopses reveals involvement of multiple regulatory pathways in chalky grain formation in rice. *BMC Genomics*, *11*(1), 730. https://doi.org/10.1186/1471-2164-11-730

Loo, S., & Xu, Z. (1986). Rice: Anther Culture for Rice Improvement in China. In *Biotechnology in Agriculture and Forestry* (pp. 139–156). https://doi.org/10.1007/978-3-642-61625-9_8

López, J., Quiñonero, S., & Tarruella, A. (2009). Presencia masiva de *Pomacea* cf . *canaliculata* (Lamarck , 1822) (Gastropoda : Ampullariidae) en el Delta del Ebro (Cataluña , España). *Spira*, *3*(1–2), 117–121.

López, M. A., Altaba, C. R., Andree, K. B., & López, V. (2010). First invasion of the apple snail Pomacea insularum in Europe. *Tentacle*, *18*(18), 26–28.

Lu, C., Shen, L., Tan, Z., Xu, Y., He, P., Chen, Y., & Zhu, L. (1997). Comparative mapping of QTLs for agronomic traits of rice across environments by using a doubled-haploid population. *Theoretical and Applied Genetics*, *94*(1), 145–150. https://doi.org/10.1007/s001220050393

Ma, L., Bao, J., Guo, L., Zeng, D., Li, X., Ji, Z., Xia, Y., Yang, C., & Qian, Q. (2009). Quantitative trait loci for panicle layer uniformity identified in doubled haploid lines of rice in two environments. *Journal of Integrative Plant Biology*, *51*(9), 818–824. https://doi.org/10.1111/j.1744-7909.2009.00854.x

Ma, L., Yang, C., Zeng, D., Cai, J., Li, X., Ji, Z., Xia, Y., Qian, Q., & Bao, J. (2009). Mapping QTLs for heading synchrony in a doubled haploid population of rice in two environments. *Journal of Genetics and Genomics*, *36*(5), 297–304. https://doi.org/10.1016/S1673-8527(08)60118-6

Maas, E. V, & Hoffman, G. J. (1977). Crop salt tolerance - Current assessment. *ASCE J Irrig Drain Div*, *103*(2), 115–134.

Mackill, D. J., & Khush, G. S. (2018). IR64: a high-quality and high-yielding mega variety. In *Rice* (Vol. 11, Issue 1). https://doi.org/10.1186/s12284-018-0208-3

Maheshwari, S. C., Rashid, A., & Tyagi, A. K. (1982). Haploids from pollen grains - retrospect and prospect. *American Journal of Botany*, *69*(5), 865–879. https://doi.org/10.2307/2442979

Makowska, K., & Oleszczuk, S. (2014). Albinism in barley androgenesis. In *Plant Cell Reports* (Vol. 33, Issue 3, pp. 385–392). https://doi.org/10.1007/s00299-013-1543-x

Maraschin, S. F., De Priester, W., Spaink, H. P., & Wang, M. (2005). Androgenic switch: An example of plant embryogenesis from the male gametophyte perspective. In *Journal of Experimental Botany* (Vol. 56, Issue 417, pp. 1711–1726). https://doi.org/10.1093/jxb/eri190

Masayoshi Tsukahara, T. H. (1992). Simple dehydration treatment promotes plantlet regeneration of rice (*Oryza sativa* L.) callus. *Plant Cell Reports*, *11*(11), 550–553. https://doi.org/10.1007/BF01690259

McLean, J., Hardy, B., & Hettel, G. (2013). Rice Almanac, 4th edition. In *IRRI, Los Baños, Philippines*. https://doi.org/10.1093/aob/mcg189

Mel, V. C., Bado, V. B., Ndiaye, S., Djaman, K., Aissata Bama Nati, D., Manneh, B., & Futakuchi, K. (2019). Predicting rice yield under salinity stress using K/Na ratio variable in plant tissue. *Communications in Soil Science and Plant Analysis*, *0*(0), 1–9. https://doi.org/10.1080/00103624.2019.1614605

Mimura, N. (2013). Sea-level rise caused by climate change and its implications for society. In *Proceedings of the Japan Academy Series B: Physical and Biological Sciences* (Vol. 89, Issue 7, pp. 281–301). Japan Academy. https://doi.org/10.2183/pjab.89.281

MINECO. (2018). *Caracol Manzana (Pomacea maculata) Memoria de actuaciones*. https://www.mapa.gob.es/es/agricultura/temas/sanidad-vegetal/caracol-manzana/

MINECO. (2019). *Cuentas Económicas de la Agricultura*.

Mishra, R., & Rao, G. J. N. (2013). Effect of cold pretreatment and phytohormones on anther culture efficiency of two indica rice (Oryza sativa L.) hybrids-Ajay and Rajalaxmi. *Journal of Experimental Biology and Agricultural Sciences*, *1*(2), 69–76. http://www.jebas.org/vol1i2/Rukmini et al JEBAS.pdf

Mishra, R., & Rao, G. J. N. (2016). In-vitro Androgenesis in Rice: Advantages, Constraints and Future Prospects. *Rice Science*, *23*(2), 57–68. https://doi.org/10.1016/j.rsci.2016.02.001

Mohan, B. H., Gopal, A., Malleshi, N. G., & Tharanathan, R. N. (2005). Characteristics of native and enzymatically hydrolyzed ragi (*Eleusine coracana*) and rice (*Oryza sativa*) starches. *Carbohydrate Polymers*, *59*(1), 43–50. https://doi.org/10.1016/j.carbpol.2004.08.022

Mueck, K., Deaton, L. E., Lee, A., & Guilbeaux, T. (2018). Physiology of the apple snail *Pomacea maculata*: Aestivation and overland dispersal. *Biological Bulletin*, *235*(1), 43–51. https://doi.org/10.1086/698817

Munns, R. (2002). Comparative physiology of salt and water stress. *Plant, Cell and Environment*, *25*(2), 239–250. https://doi.org/10.1046/j.0016-8025.2001.00808.x

Munns, R., & Tester, M. (2008). Mechanisms of Salinity Tolerance. *Annual Review of Plant Biology*, *59*(1), 651–681. https://doi.org/10.1146/annurev.arplant.59.032607.092911

Murashige, T., & Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum*, *15*(3), 473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Negrão, S., Courtois, B., Ahmadi, N., Abreu, I., Saibo, N., & Oliveira, M. M. (2011). Recent updates on salinity stress in rice: From physiological to molecular responses. *Critical Reviews in Plant Sciences*, *30*(4), 329–377. https://doi.org/10.1080/07352689.2011.587725

Niizeki, H. ., & Oono, K. . (1968). Induction of Haploid Rice Plant from Anther Culture. *Proceedings of the Japan Academy*, *44*, 554–557.

Nounjan, N., Chansongkrow, P., Charoensawan, V., Siangliw, J. L., Toojinda, T., Chadchawan, S., & Theerakulpisut, P. (2018). High Performance of Photosynthesis and Osmotic Adjustment Are Associated With Salt Tolerance Ability in Rice Carrying Drought Tolerance QTL: Physiological and Co-expression Network Analysis. *Frontiers in Plant Science*, *9*, 1135. https://doi.org/10.3389/fpls.2018.01135

Nutan, K. K., Kushwaha, H. R., Singla-Pareek, S. L., & Pareek, A. (2017). Transcription dynamics of Saltol QTL localized genes encoding transcription factors, reveals their differential regulation in contrasting genotypes of rice. *Functional and Integrative Genomics*, *17*(1), 69–83. https://doi.org/10.1007/s10142-016-0529-5

Obert, B., & Barnabas, B. (2004). Colchicine induced embryogenesis in maize. *Plant Cell, Tissue and Organ Culture*, *77*(3), 283–285. https://doi.org/10.1023/B:TICU.0000018399.60106.33

Ogawa, T., Fukuoka, H., & Ohkawa, Y. (1995). Plant regeneration through direct culture of isolated pollen grains in rice. *Breeding Science*, *45*(3), 301–307. https://doi.org/10.1270/jsbbs1951.45.301

Patil, V. D., Nerkar, Y. S., Misal, M. B., & Harkal, S. R. (1997). Parag 401, a semidwarf rice variety developed through anther culture. *International Rice Research Notes*, *22*(2), 58025.

Patindol, J., & Wang, Y. J. (2003). Fine structures and physicochemical properties of starches from chalky and translucent rice kernels. *Journal of Agricultural and Food Chemistry*, *51*(9), 2777–2784. https://doi.org/10.1021/jf026101t

Pauk, J., Jancsó, M., & Simon-Kiss, I. (2009). Rice Doubled Haploids and Breeding. *Advances in Haploid Production in Higher Plants*, 189–197. https://doi.org/10.1007/978-1-4020-8854-4_16

Plan, M. R. R., Saska, I., Cagauan, A. G., & Craik, D. J. (2008). Backbone cyclised peptides from plants show molluscicidal activity against the rice pest *Pomacea canaliculate* (golden apple snail). *Journal of Agricultural and Food Chemistry*, *56*(13), 5237–5241. https://doi.org/10.1021/jf800302f

Platten, J. Damien, Cotsaftis, O., Berthomieu, P., Bohnert, H., Davenport, R. J., Fairbairn, D. J., Horie, T., Leigh, R. A., Lin, H. X., Luan, S., Mäser, P., Pantoja, O., Rodríguez-Navarro, A., Schachtman, D. P., Schroeder, J. I., Sentenac, H., Uozumi, N., Véry, A. A., Zhu, J. K., … Tester, M. (2006). Nomenclature for HKT transporters, key determinants of plant salinity tolerance. In *Trends in Plant Science* (Vol. 11, Issue 8, pp. 372–374). https://doi.org/10.1016/j.tplants.2006.06.001

Platten, John Damien, Egdane, J. A., & Ismail, A. M. (2013). Salinity tolerance, Na+ exclusion and allele mining of HKT1;5 in *Oryza sativa* and *O. glaberrima*: Many sources, many genes, one mechanism? *BMC Plant Biology*, *13*(1). https://doi.org/10.1186/1471-2229-13-32

Plaza, T., & Galimany, G. (2013). El caracol manzana, una nueva plaga del cultivo del arroz. *VidaRURAL*, *370*, 50–56.

Powell, W. (1988). The influence of genotype and temperature pre-treatment on anther culture response in barley (*Hordeum vulgare* L.). *Plant Cell, Tissue and Organ Culture*, *12*(3), 291–297. https://doi.org/10.1007/BF00034371

Rahman, M. A., Thomson, M. J., De Ocampo, M., Egdane, J. A., Salam, M. A., Shah-E-Alam, M., & Ismail, A. M. (2019). Assessing trait contribution and mapping novel QTL for salinity tolerance using the Bangladeshi rice landrace Capsule. *Rice*, *12*(1). https://doi.org/10.1186/s12284-019-0319-5

Rakshit, S., Rakshit, A., Matsumura, H., Takahashi, Y., Hasegawa, Y., Ito, A., Ishii, T., Miyashita, N. T., & Terauchi, R. (2007). Large-scale DNA polymorphism study of *Oryza sativa* and *O. rufipogon* reveals the origin and divergence of Asian rice. *Theoretical and Applied Genetics*, *114*(4), 731–743. https://doi.org/10.1007/s00122-006-0473-1

Ramakrishnan, V. (2007). Salinity, ph, temperature, desiccation and hypoxia tolerance in the invasive freshwater apple snail Pomacea Insularum. In *Response* (Issue May).

Ranathunge, K., Kotula, L., Steudle, E., & Lafitte, R. (2004). Water permeability and reflection coefficient of the outer part of young rice roots are differently affected by closure of water channels (aquaporins) or blockage of apoplastic pores. *Journal of Experimental Botany*, *55*(396), 433–447. https://doi.org/10.1093/jxb/erh041

Rani, M. R. S., & Bhattacharya, K. R. (1989). Slurry Viscosity As a Possible Indicator of Rice Quality. *Journal of Texture Studies*, *20*(2), 139–149. https://doi.org/10.1111/j.1745-4603.1989.tb00428.x

Ratanasumawong, S., Seesuk, P., & Sirichayakornkun, U. (2019). Microstructure, water migration and texture of Thai chalky rice varieties. *Journal of Nutritional Science and Vitaminology*, *65*, S188–S191. https://doi.org/10.3177/jnsv.65.S188

Ren, D., Rao, Y., Huang, L., Leng, Y., Hu, J., Lu, M., Zhang, G., Zhu, L., Gao, Z., Dong, G., Guo, L., Qian, Q., & Zeng, D. (2016). Fine Mapping Identifies a New QTL for Brown Rice Rate in Rice (*Oryza Sativa* L.). *Rice*, *9*(1), 1–10. https://doi.org/10.1186/s12284-016-0076-7

Ren, Z. H., Gao, J. P., Li, L. G., Cai, X. L., Huang, W., Chao, D. Y., Zhu, M. Z., Wang, Z. Y., Luan, S., & Lin, H. X. (2005). A rice quantitative trait locus for salt tolerance encodes a sodium transporter. *Nature Genetics*, *37*(10), 1141–1146. https://doi.org/10.1038/ng1643

Rodríguez-Serrano, M., Bárány, I., Prem, D., Coronado, M. J., Risueño, M. C., & Testillano, P. S. (2012). NO, ROS, and cell death associated with caspase-like activity increase in stress-induced microspore embryogenesis of barley. *Journal of Experimental Botany*, *63*(5), 2007–2024. https://doi.org/10.1093/jxb/err400

Senadhira, D., Zapata-Arias, F. J., Gregorio, G. B., Alejar, M. S., De la Cruz, H. C., Padolina, T. F., & Galvez, A. M. (2002). Development of the first salt-tolerant rice cultivar through indica/indica anther culture. *Field Crops Research*, *76*(2–3), 103–110. https://doi.org/10.1016/S0378-4290(02)00032-1

Senguttuvel, P., Vijayalakshmi, C., Thiyagarajan, K., Kannanbapu, J. R., Kota, S., Padmavathi, G., Geetha, S., Sritharan, N., & Viraktamath, B. C. (2014). Changes in photosynthesis, chlorophyll fluorescence, gas exchange parameters and osmotic potential to salt stress during early seedling stage in rice (*Oryza sativa* L.). *Sabrao Journal of Breeding and Genetics*, *46*(1), 120–135.

Serrat, X., Cardona, M., Gil, J., Brito, A. M., Moysset, L., Nogués, S., & Lalanne, E. (2014). A Mediterranean japonica rice (*Oryza sativa*) cultivar improvement through anther culture. *Euphytica*, *195*(1), 31–44. https://doi.org/10.1007/s10681-013-0955-6

Shahnewaz, S., Bari, M. A., Siddique, N. A., & Rahman, M. H. (2004). Effects of Genotype on Induction of Callus and Plant Regeneration Potential *in vitro* Anther Culture of Rice (*Oryza sativa L.*). In *Pakistan Journal of Biological Sciences* (Vol. 7, Issue 2, pp. 235–237).

Shankar, R., Bhattacharjee, A., & Jain, M. (2016). Transcriptome analysis in different rice cultivars provides novel insights into desiccation and salinity stress responses. *Scientific Reports*, *6*. https://doi.org/10.1038/srep23719

Shouyi, L., & Shouyin, H. (1991). Huayu 15, a High-Yielding Rice Variety Bred by Anther Culture. In *Biotechnology in Agriculture and Forestry* (pp. 230–247). https://doi.org/10.1007/978-3-642-83986-3_16

Silva, T. D. (2010). Indica rice anther culture: Can the impasse be surpassed? In *Plant Cell, Tissue and Organ Culture* (Vol. 100, Issue 1, pp. 1–11). Springer Netherlands. https://doi.org/10.1007/s11240-009-9616-9

Singh, M. K., & Singh, H. B. (2011). Effect of Cold Pretreatment on Anther culture of Boro Rice Hybrids. *Reproductive Biology*, *3*(1), 69–73.

Singh, N., Inouchi, N., & Nishinari, K. (2006). Structural, thermal and viscoelastic characteristics of starches separated from normal, sugary and waxy maize. *Food Hydrocolloids*, *20*(6), 923–935. https://doi.org/10.1016/j.foodhyd.2005.09.009

Singh, N., Sodhi, N. S., Kaur, M., & Saxena, S. K. (2003). Physico-chemical, morphological, thermal, cooking and textural properties of chalky and translucent rice kernels. *Food Chemistry*, *82*(3), 433–439. https://doi.org/10.1016/S0308-8146(03)00007-4

Ślusarkiewicz-Jarzina, A., Pudelska, H., Woźna, J., & Pniewski, T. (2017). Improved production of doubled haploids of winter and spring triticale hybrids via combination of colchicine treatments on anthers and regenerated plants. *Journal of Applied Genetics*, *58*(3), 287–295. https://doi.org/10.1007/s13353-016-0387-9

Sreenivasulu, N. (2019). Rice Grain Quality. *Methods in Molecular Biology*, *1892*(06), 253–264. https://doi.org/10.1007/978-1-4939-8914-0

Sreenivasulu, N., Butardo, V. M., Misra, G., Cuevas, R. P., Anacleto, R., & Kishor, P. B. K. (2015). Designing climate-resilient rice with ideal grain quality suited for high-temperature stress. In *Journal of Experimental Botany* (Vol. 66, Issue 7, pp. 1737–1748). https://doi.org/10.1093/jxb/eru544

Sultana, N., Ikeda, T., & Kashem, M. A. (2002). Effect of NaCl salinity on photosynthesis and dry matter accumulation in developing rice grains. *Photosynthetica*, *40*(1), 115–119. https://doi.org/10.1023/A:1020175130290

Sun, S., Wu, Y., Lin, X., Wang, J., Yu, J., Sun, Y., Miao, Y., Li, Q., Sanguinet, K. A., & Liu, B. (2017). Hybrid weakness in a rice interspecific hybrid is nitrogen-dependent, and accompanied by changes in gene expression at both total transcript level and parental allele partitioning. *PLoS ONE*, *12*(3). https://doi.org/10.1371/journal.pone.0172919

Süß, A., Danner, M., Obster, C., Locherer, M., Hank, T., & Richter, K. (2015). Measuring leaf chlorophyll content with the Konica Minolta SPAD-502Plus - Theory, Measurements, Problems, Interpretation. *EnMAP Field Guides Technical Report, GFZ Data Services*, 1–4. https://doi.org/10.2312/enmap.2015.010

Swamy, B. P. M., Descalsota, G. I. L., Nha, C. T., Amparado, A., Inabangan-Asilo, M. A., Manito, C., Tesoro, F., & Reinke, R. (2018). Identification of genomic regions associated with agronomic and biofortification traits in DH populations of rice. *PLoS ONE*, *13*(8). https://doi.org/10.1371/journal.pone.0201756

Szarejko, I., & Forster, B. P. (2007). Doubled haploidy and induced mutation. In *Euphytica* (Vol. 158, Issue 3, pp. 359–370). https://doi.org/10.1007/s10681-006-9241-1

Talebi, R., Rahemi, M. R., Arefi, H., Nourozi, M., & Bagheri, N. (2007). In vitro plant regeneration through anther culture of some Iranian local rice (*Oryza sativa* L.) cultivars. *Pakistan Journal of Biological Sciences*, *10*(12), 2056–2060. https://doi.org/10.3923/pjbs.2007.2056.2060

Thomson, M. J., de Ocampo, M., Egdane, J., Rahman, M. A., Sajise, A. G., Adorada, D. L., Tumimbang-Raiz, E., Blumwald, E., Seraj, Z. I., Singh, R. K., Gregorio, G. B., & Ismail, A. M. (2010). Characterizing the Saltol quantitative trait locus for salinity tolerance in rice. *Rice*, *3*(2–3), 148–160. https://doi.org/10.1007/s12284-010-9053-8

Tsutsui, K., Kaneko, K., & Hanashiro, I. (2013). Characteristics of Opaque and Translucent Parts of High Temperature Stressed Grains of Rice. *Journal of Applied Glycoscience*, *67*, 61–67. https://doi.org/10.5458/jag.jag.JAG-2012

Ul Haq, T., Gorham, J., Akhtar, J., Akhtar, N., & Steele, K. A. (2010). Dynamic quantitative trait loci for salt stress components on chromosome 1 of rice. *Functional Plant Biology*, *37*(7), 634–645. https://doi.org/10.1071/FP09247

Uozumi, N., Kim, E. J., Rubio, F., Yamaguchi, T., Muto, S., Tsuboi, A., Bakker, E. P., Nakamura, T., & Schroeder, J. I. (2000). The Arabidopsis HKT1 gene homolog mediates inward Na+ currents in *Xenopus laevis* oocytes and Na+ uptake in *Saccharomyces cerevisiae*. *Plant Physiology*, *122*(4), 1249–1259. https://doi.org/10.1104/pp.122.4.1249

Vanguardia, L. (2020). *El mar inunda arrozales y mejilloneras y llega a 3 kilómetros tierra adentro en el Delta del Ebro*. https://www.lavanguardia.com/vida/20200121/473036355698/delta-ebro-inundaciones-arrozales-borrasca-gloria.html

Wada, H., Hatakeyama, Y., Onda, Y., Nonami, H., Nakashima, T., Erra-Balsells, R., Morita, S., Hiraoka, K., Tanaka, F., & Nakano, H. (2019). Multiple strategies for heat adaptation to prevent chalkiness in the rice endosperm. *Journal of Experimental Botany*, *70*(4), 1299–1311. https://doi.org/10.1093/jxb/ery427

Wan, X. Y., Wan, J. M., Weng, J. F., Jiang, L., Bi, J. C., Wang, C. M., & Zhai, H. Q. (2005). Stability of QTLs for rice grain dimension and endosperm chalkiness characteristics across eight environments. *Theoretical and Applied Genetics*, *110*(7), 1334–1346. https://doi.org/10.1007/s00122-005-1976-x

Wang, E., Wang, J., Zhu, X., Hao, W., Wang, L., Li, Q., Zhang, L., He, W., Lu, B., Lin, H., Ma, H., Zhang, G., & He, Z. (2008). Control of rice grain-filling and yield by a gene with a potential signature of domestication. *Nature Genetics*, *40*(11), 1370–1374. https://doi.org/10.1038/ng.220

Wang, T. B., Gassmann, W., Rubio, F., Schroeder, J. I., & Glass, A. D. M. (1998). Rapid up-regulation of HKT1, a high-affinity potassium transporter gene, in roots of barley and wheat 4 following withdrawal of potassium. *Plant Physiology*, *118*(2), 651–659. https://doi.org/10.1104/pp.118.2.651

Wang, X. S., Zhu, J., Mansueto, L., & Bruskiewich, R. (2005). Identification of candidate genes for drought stress tolerance in rice by the integration of a genetic (QTL) map with the rice genome physical map. *Journal of Zhejiang University: Science*, *6 B*(5), 382–388. https://doi.org/10.1631/jzus.2005.B0382

Wang, Y., Ren, Y., Liu, X., Jiang, L., Chen, L., Han, X., Jin, M., Liu, S., Liu, F., Lv, J., Zhou, K., Su, N., Bao, Y., & Wan, J. (2010). OsRab5a regulates endomembrane organization and storage protein trafficking in rice endosperm cells. *Plant Journal*, *64*(5), 812–824. https://doi.org/10.1111/j.1365-313X.2010.04370.x

Wang, Z., Taramino, G., Yang, D., Liu, G., Tingey, S. V., Miao, G. H., & Wang, G. L. (2001). Rice ESTs with disease-resistance gene or defense-response gene-like sequences mapped to regions containing major resistance genes or QTLs. *Molecular and General Genetics*, *265*(2), 302–310. https://doi.org/10.1007/s004380000415

Wani, A. A., Singh, P., Shah, M. A., Schweiggert-Weisz, U., Gul, K., & Wani, I. A. (2012). Rice Starch Diversity: Effects on Structural, Morphological, Thermal, and Physicochemical Properties-A Review. *Comprehensive Reviews in Food Science and Food Safety*, *11*(5), 417–436. https://doi.org/10.1111/j.1541-4337.2012.00193.x

Wei, X., & Huang, X. (2019). Origin, taxonomy, and phylogenetics of rice. In *Rice*. AACCI. Published by Elsevier Inc. in cooperation with AACC International. https://doi.org/10.1016/b978-0-12-811508-4.00001-0

Wei, X., Qiao, W. H., Chen, Y. T., Wang, R. S., Cao, L. R., Zhang, W. X., Yuan, N. N., Li, Z. C., Zeng, H. L., & Yang, Q. W. (2012). Domestication and geographic origin of *Oryza sativa* in China: Insights from multilocus analysis of nucleotide variation of *O. sativa* and *O. rufipogon*. *Molecular Ecology*, *21*(20), 5073–5087. https://doi.org/10.1111/j.1365-294X.2012.05748.x

Wetherell, D. F. (1984). Enhanced adventive embryogenesis resulting from plasmolysis of cultured wild carrot cells. *Plant Cell, Tissue and Organ Culture*, *3*(3), 221–227. https://doi.org/10.1007/BF00040341

Woo, M. O., Ham, T. H., Ji, H. S., Choi, M. S., Jiang, W., Chu, S. H., Piao, R., Chin, J. H., Kim, J. A., Park, B. S., Seo, H. S., Jwa, N. S., McCouch, S., & Koh, H. J. (2008). Inactivation of the UGPase1 gene causes genic male sterility and endosperm chalkiness in rice (*Oryza sativa* L.). *Plant Journal*, *54*(2), 190–204. https://doi.org/10.1111/j.1365-313X.2008.03405.x

Xi, M., Lin, Z., Zhang, X., Liu, Z., Li, G., Wang, Q., Wang, S., & Ding, Y. (2014). Endosperm Structure of White-Belly and White-Core Rice Grains Shown by Scanning Electron Microscopy. *Plant Production Science*, *17*(4), 285–290. https://doi.org/10.1626/pps.17.285

Xie, W., Wang, G., Yuan, M., Yao, W., Lyu, K., Zhao, H., Yang, M., Li, P., Zhang, X., Yuan, J., Wang, Q., Liu, F., Dong, H., Zhang, L., Li, X., Meng, X., Zhang, W., Xiong, L., He, Y., … Zhang, Q. (2015). Breeding signatures of rice improvement revealed by a genomic variation map from a large germplasm collection. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(39), E5411–E5419. https://doi.org/10.1073/pnas.1515919112

Xu, J., Feng, D., Li, X., Chang, T., & Zhu, Z. (2002). Cloning of genomic DNA of rice 5-enolpyruvylshikimate 3-phosphate synthase gene and chromosomal localization of the gene. *Science in China, Series C: Life Sciences*, *45*(3), 251–259. https://doi.org/10.1360/02yc9028

Yamagishi, M., Otani, M., Higashi, M., Fukuta, Y., Fukui, K., Yano, M., & Shimada, T. (1998). Chromosomal regions controlling anther culturability in rice (*Oryza sativa* L.). *Euphytica*, *103*(2), 227–234. https://doi.org/10.1023/A:1018328708322

Yang, X R; Fu, H. H. (1989). A high protein indica rice. *Intl Rice Res Nwsl*, *14*(3), 14–15.

Yang, C. C., Yoshihiro Kawahara, Mizuno, H., Wu, J., Matsumoto, T., & Itoh, T. (2012). Independent domestication of asian rice followed by gene flow from japonica to indica. *Molecular Biology and Evolution*, *29*(5), 1471–1479. https://doi.org/10.1093/molbev/msr315

Yang, C., Tian, Y., Lv, T., Chang, X., Zhang, M., Gong, G., Zhao, L., Yang, S., & Chen, H. (2018). Histopathological effects of Pedunsaponin A on *Pomacea canaliculata*. *Pesticide Biochemistry and Physiology*, *148*, 151–158. https://doi.org/10.1016/j.pestbp.2018.04.010

Yang, Q. Q., Liu, S. W., Song, F., Liu, G. F., & Yu, X. P. (2018). Comparative mitogenome analysis on species of four apple snails (Ampullariidae: *Pomacea*). *International Journal of Biological Macromolecules*, *118*, 525–533. https://doi.org/10.1016/j.ijbiomac.2018.06.092

Yichie, Y., Brien, C., Berger, B., Roberts, T. H., & Atwell, B. J. (2018). Salinity tolerance in Australian wild *Oryza* species varies widely and matches that observed in *O. sativa*. *Rice*, *11*(1). https://doi.org/10.1186/s12284-018-0257-7

Yoshida, K. T., Fujii, S., Sakata, M., & Takeda, G. (1994). Control of Organogenesis and Embryogenesis in Rice Calli. *Ikushugaku Zasshi*, *44*(4), 355–360. https://doi.org/10.1270/jsbbs1951.44.355

Yoshida, Shinya, Ikegami, M., Kuze, J., Sawada, K., Hashimoto, Z., Ishii, T., Nakamura, C., & Kamijima, O. (2002). QTL analysis for plant and grain characters of Sake-brewing rice using a doubled haploid population. *Breeding Science*, *52*(4), 309–317. https://doi.org/10.1270/jsbbs.52.309

Yoshida, Shouichi, Forno, D. A., & Cock, J. (1976). *Laboratory Manual for Physiological Studies of Rice*.

Zhang, J., Jia, W., Yang, J., & Ismail, A. M. (2006). Role of ABA in integrating plant responses to drought and salt stresses. *Field Crops Research*, *97*(1 SPEC. ISS.), 111–119. https://doi.org/10.1016/j.fcr.2005.08.018

Zhou, L., Chen, L., Jiang, L., Zhang, W., Liu, L., Liu, X., Zhao, Z., Liu, S., Zhang, L., Wang, J., & Wan, J. (2009). Fine mapping of the grain chalkiness QTL qPGWC-7 in rice (*Oryza sativa* L.). *Theoretical and Applied Genetics*, *118*(3), 581–590. https://doi.org/10.1007/s00122-008-0922-0

Zhou, Z., Robards, K., Helliwell, S., & Blanchard, C. (2002). Composition and functional properties of rice. *International Journal of Food Science and Technology*, *37*(8), 849–868. https://doi.org/10.1046/j.1365-2621.2002.00625.x

Zhu, D., & Pan, X. (1990). *Rice (*Oryza sativa *L.): Guan 18 - an Improved Variety Through Anther Culture* (pp. 204–211). https://doi.org/10.1007/978-3-642-61499-6_8

Zhu, G. Y., Kinet, J. M., & Lutts, S. (2001). Characterization of rice (*Oryza sativa* L.) F3 populations selected for salt resistance. I. Physiological behaviour during vegetative growth. *Euphytica*, *121*(3), 251–263. https://doi.org/10.1023/A:1012016431577

Zhu, J., Ren, Y., Wang, Y., Liu, F., Teng, X., Zhang, Y., Duan, E., Wu, M., Zhong, M., Hao, Y., Zhu, X., Lei, J., Wang, Y., Yu, Y., Pan, T., Bao, Y., Wang, Y., & Wan, J. (2019). OsNHX5-mediated pH homeostasis is required for post-Golgi trafficking of seed storage proteins in rice endosperm cells. *BMC Plant Biology*, *19*(1). https://doi.org/10.1186/s12870-019-1911-y

Zubko, M., & Day, A. (2002). Differential regulation of genes transcribed by nucleus-encoded plastid RNA polymerase, and DNA amplification, within ribosome-deficient plastids in stable phenocopies of cereal albino mutants. *Molecular Genetics and Genomics*, *267*(1), 27–37. https://doi.org/10.1007/s00438-001-0627-4

8. Supplementary material

Experiment 1: Anther culture of two temperate and two tropical japonica varieties

Table SM 1. Chi-square test for calli induction / medium association ($p < 0.05$).

Medium	Statistic	p-value
0D1	197.326	0.000
150D1	927.093	0.000

Table SM 2. Chi-square test for plantlet regeneration / medium association ($p < 0.05$).

Medium	Statistic	p-value
XACRM	17.732	0.000
SARM	20.083	0.000
GERM	42.852	0.000

Experiment 2: Testing the Saltol region in hydroponic assays and in fields

Table SM 3. Yoshida solution composition.

	Element	Reagent	Final concentration
Macronutrients	N	NH_4NO_3	14.28 mM
	S	K_2SO_4	5.13 mM
	P	KH_2PO_4	8.49 mM
		K_2HPO_4	1.23 mM
	Ca	$CaCl_2 \cdot 2 H_2O$	7.54 mM
	Mg	$MgSO_4 \cdot 7 H_2O$	16.44 mM
Micronutrients	Fe	Na_2EDTA $FeSO_4 \cdot 7 H_2O$	37.75 μM
	Mn	$MnCl_2 \cdot 4 H_2O$	9.5 μM
	Mo	$(NH_4)_6Mo_7O_{24} \cdot 4 H_2O$	0.075 μM
	B	H_3BO_3	18.89 μM
	Zn	$ZnSO_4 \cdot 7 H_2O$	0.152 μM
	Cu	$CuSO_4 \cdot 5 H_2O$	0.156 μM

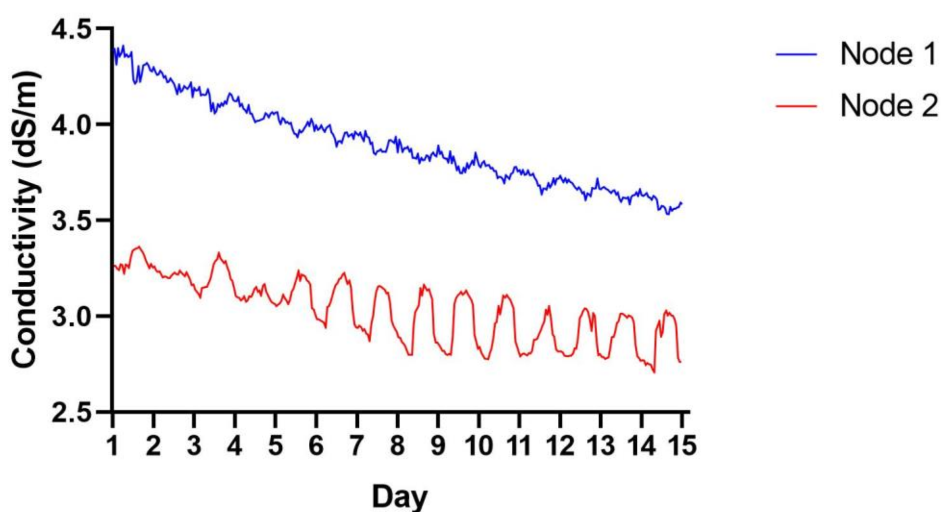

Figure SM 1. Conductivity in the Vascos field taken by two low-cost salinity sensors (i.e. nodes) developed by IRIS Technology Solutions (Castelldefels, Barcelona) during the 15 days of the plants' exposure to salinity.

Table SM 4. Two sample t-test to compare control (c) and salinity (s) Na^+/K^+ ratio values for all the varieties in different measurement days ($p < 0.05$).

	Variety	μ (c)	μ (s)	SEM (c)	SEM (s)	p-value
Day 1	PL12	0.03560	0.1233	0.0058	0.0080	0.001
	FL478	0.02200	0.1512	0.0065	0.0150	0.001
	LP5	0.02747	0.2058	0.0005	0.0400	0.011
	LP8	0.03187	0.1620	0.0055	0.1240	0.145
Day 2	PL12	0.02395	0.4000	0.0037	0.063	0.004
	FL478	0.02040	0.1978	0.0069	0.042	0.014
	LP5	0.01562	0.3520	0.0039	0.100	0.031
	LP8	0.15100	0.5040	0.0710	0.083	0.032
Day 4	PL12	0.01360	0.6379	0.0006	0.037	0.000
	FL478	0.02520	0.8751	0.0081	0.055	0.000
	LP5	0.02051	0.7730	0.0043	0.180	0.013
	LP8	0.01649	0.7630	0.0034	0.073	0.001
Day 7	PL12	0.01231	1.0380	0.0029	0.098	0.000
	FL478	0.00459	0.3349	0.0008	0.055	0.004
	LP5	0.01068	1.1132	0.0022	0.036	0.000
	LP8	0.00902	1.1000	0.0036	0.200	0.006

Table SM 5. Two sample t-test to compare salinity (s) Na^+/K^+ ratio values between parentals PL12 and FL478 in different measurement days ($p < 0.05$).

	μ (PL12 s)	μ (FL478 s)	SEM (PL12 s)	SEM (FL478 s)	p-value
Day 1	0.1233	0.1512	0.008	0.015	0.179
Day 2	0.4000	0.1978	0.063	0.042	0.056
Day 4	0.6397	0.8751	0.037	0.055	0.023
Day 7	1.038	0.3349	0.098	0.055	0.003

Table SM 6. Two sample t-test to compare SPAD values for all LP varieties and their parentals in different measurement days ($p < 0.05$).

	Day 1					Day 3					Day 5					Day 7				
Variety	μ (c)	μ (s)	SEM (c)	SEM (s)	p-value	μ (c)	μ (s)	SEM (c)	SEM (s)	p-value	μ (c)	μ (s)	SEM (c)	SEM (s)	p-value	μ (c)	μ (s)	SEM (c)	SEM (s)	p-value
PL12	24.86	24.93	2.9	3.1	0.987	23.53	28.56	2.9	2.2	0.189	22.72	18.3	2.1	5.9	0.534	28.56	13.0	2.0	4.8	0.022
FL478	25.05	27.88	2.7	1.4	0.369	29.32	28.75	2.0	0.88	0.802	32.55	33.15	2.5	2.5	0.868	39.8	37.3	4.9	6.1	0.756
LP1	29.22	25.16	0.59	2.10	0.097	43.5	30.70	5.3	3.7	0.085	29.86	3.09	1.4	6.3	0.335	38.7	21.2	6.0	7.6	0.107
LP2	24.24	29.74	1.8	2.8	0.138	32.8	29.00	5.3	4.1	0.582	29.80	26.10	0.73	6.6	0.593	31.16	28.6	1.6	11	0.826
LP3	27.08	26.56	1.8	2.4	0.868	29.76	21.3	4.3	5.6	0.264	31.66	23.8	1.8	6.0	0.244	34.9	25.3	5.3	9.0	0.386
LP4	25.62	25.32	2.2	2.2	0.929	38.5	26.64	6.9	4.4	0.187	25.88	9.9	2.5	6.4	0.049	35.25	11.3	1.8	7.1	0.022
LP6	30.80	23.18	2.1	2.6	0.054	38.8	29.02	4.8	2.02	0.098	31.94	31.42	0.63	2.1	0.820	30.78	28.9	1.7	9.5	0.849
LP7	24.24	28.08	3.3	1.7	0.330	32.6	33.20	4.7	1.9	0.906	28.32	32.06	0.86	2.10	0.135	40.1	35.1	6.0	8.0	0.628
LP9	34.7	27.06	5.8	2.3	0.260	31.42	34.8	3.0	9.7	0.750	27.62	18.3	2.1	7.5	0.269	37.6	25.3	4.5	7.3	0.190
LP10	24.48	25.46	3.2	1.9	0.763	30.72	27.38	4.1	1.1	0.457	25.94	28.72	1.2	2.3	0.311	35.62	30.9	3.0	11	0.688
LP11	30.30	24.60	1.3	3.1	0.165	22.36	37.3	2.1	5.8	0.061	27.16	21.9	3.3	5.9	0.460	42.6	14.5	6.5	9.7	0.320
LP12	32.12	33.60	1.4	1.8	0.534	35.7	31.4	5.2	7.8	0.653	33.78	27.8	1.7	7.2	0.462	33.1	24.1	5.3	7.2	0.345
LP14	29.54	25.28	3.2	2.0	0.299	30.1	32.1	7.2	4.9	0.823	32.54	19.9	3.9	6.6	0.151	35.20	17.5	3.9	7.8	0.098
LP15	22.54	23.14	3.1	1.8	0.872	36.2	26.9	6.1	7.9	0.387	27.32	17.9	0.7	7.3	0.272	23.62	21.9	1.7	13	0.903
LP16	30.26	26.92	2.0	2.4	0.325	29.60	25.78	6.6	1.1	0.602	24.40	19.60	3.3	6.2	0.518	35.02	26.1	4.3	9.0	0.413
LP17	20.35	26.08	3.0	3.7	0.274	24.76	34.80	3.1	4.3	0.099	21.84	22.82	2.7	3.0	0.813	32.2	22.8	6.2	5.9	0.311
LP18	26.24	32.2	2.5	6.5	0.435	22.42	27.52	1.3	2.9	0.176	31.98	29.18	3.0	3.4	0.558	32.0	26.3	6.6	4.5	0.497
LP20	30.5	33.26	4.9	3.9	0.678	33.1	35.5	5.5	6.3	0.785	27.88	22.3	1.8	6.2	0.439	35.3	21.3	4.8	7.1	0.146
LP21	23.94	28.14	1.7	1.5	0.103	32.3	27.10	6.2	0.59	0.445	29.00	14.60	1.5	6.3	0.090	32.65	22.9	1.2	11	0.412
LP23	25.30	29.38	4.3	1.3	0.432	23.90	33.3	0.94	6.2	0.205	27.08	25.1	1.6	4.8	0.720	34.9	26.2	5.8	7.9	0.401
LP24	22.38	22.85	3.8	3.8	0.934	23.56	21.4	0.94	5.4	0.713	25.80	18.3	1.8	6.5	0.333	41.3	14.7	5.7	10	0.062
LP26	23.48	28.42	2.6	1.7	0.166	29.56	23.4	1.6	5.0	0.307	28.90	24.9	1.5	6.4	0.577	32.36	16.6	1.5	4.6	0.030
LP28	25.04	22.42	3.0	2.8	0.541	29.40	35.1	3.9	6.4	0.474	29.22	20.4	2.8	5.2	0.184	36.5	26.4	6.2	7.0	0.314
LP29	25.74	25.78	2.0	1.6	0.988	23.70	29.38	2.3	4.0	0.266	27.14	26.02	1.8	2.4	0.719	36.6	22.9	4.9	9.4	0.253
LP35	23.00	28.55	2.1	2.9	0.180	24.38	24.96	3.0	2.0	0.878	26.78	18.6	3.0	7.7	0.369	32.9	20.1	6.3	9.8	0.310

Table SM 7. Two sample t-test to compare SPAD values for all MS varieties and their parentals in different measurement days ($p < 0.05$).

Variety	Day 1					Day 3					Day 5					Day 7				
	μ (c)	μ (s)	SEM (c)	SEM (s)	p-value	μ (c)	μ (s)	SEM (c)	SEM (s)	p-value	μ (c)	μ (s)	SEM (c)	SEM (s)	p-value	μ (c)	μ (s)	SEM (c)	SEM (s)	p-value
PM37	34.10	30.44	4.1	1.9	0.434	28.28	25.75	1.1	1.8	0.251	37.26	19.4	2.5	4.7	0.005	36.2	13.2	4.4	5.1	0.004
IR64	21.93	23.15	2.0	1.8	0.655	25.14	16.95	1.3	3.2	0.034	26.40	5.7	1.1	3.8	0.000	31.55	5.7	2.1	3.7	0.000
MS2	34.16	37.72	1.9	4.3	0.467	29.68	33.34	0.66	3.5	0.331	33.72	31.80	1.2	1.1	0.273	38.1	29.22	5.3	1.9	0.150
MS3	29.00	27.30	2.8	0.86	0.579	24.88	23.30	1.3	3.4	0.675	27.70	17.1	1.5	7.1	0.184	29.6	6.5	6.0	4.6	0.016
MS4	28.02	30.76	2.2	2.6	0.436	28.64	26.4	0.69	4.7	0.643	33.74	23.5	0.24	6.3	0.141	39.4	21.6	5.5	5.8	0.057
MS5	32.28	27.56	2.3	1.2	0.107	25.96	24.78	0.9	1.0	0.409	35.1	14.6	5.6	6.9	0.049	29.98	13.8	1.4	5.9	0.028
MS6	33.16	31.98	2.4	2.9	0.762	25.24	27.38	1.0	0.74	0.132	29.78	24.3	1.0	6.1	0.398	32.2	18.6	5.4	6.4	0.143
MS7	31.30	25.16	1.3	1.7	0.022	27.84	23.0	0.99	5.0	0.365	26.84	5.68	1.7	3.8	0.001	34.0	9.1	4.7	5.6	0.010
MS8	30.60	32.08	3.2	1.5	0.689	28.64	28.74	1.1	1.5	0.958	31.38	24.3	0.75	6.2	0.291	39.3	9.35	8.2	5.0	0.022
MS9	31.22	30.44	3.4	1.6	0.840	30.00	29.44	1.2	1.9	0.806	31.52	24.98	1.4	2.6	0.060	37.4	9.6	4.8	4.9	0.004
MS11	32.22	32.96	2.1	1.2	0.767	29.26	23.44	0.91	3.1	0.078	39.4	22.5	4.9	5.7	0.055	33.88	21.3	3.1	6.1	0.105
MS12	26.86	30.28	2.6	2.0	0.349	31.96	30.2	2.9	5.3	0.761	25.08	11.8	1.0	6.8	0.066	25.64	6.83	1.4	4.7	0.004
MS14	34.16	34.60	0.67	1.1	0.748	28.90	24.66	1.3	2.0	0.116	30.34	11.5	1.4	7.1	0.032	31.0	4.84	4.7	3.7	0.002
MS16	31.42	29.17	2.5	0.48	0.452	31.0	28.0	5.4	4.5	0.678	31.80	17.2	1.2	7.3	0.082	31.26	17.6	2.7	7.5	0.124
MS17	27.14	32.5	2.4	4.7	0.334	29.42	21.0	2.7	5.3	0.192	38.88	6.60	3.5	4.2	0.000					
MS18	33.62	27.82	1.9	1.7	0.054	30.92	23.8	2.7	4.8	0.233	30.6	11.5	0.39	4.7	0.004	35.44	10.1	3.4	6.3	0.007
MS20	30.18	32.04	2.1	1.8	0.520	30.94	28.0	1.1	4.5	0.550	32.26	26.74	2.1	0.74	0.037	41.6	19.5	5.0	6.2	0.023
MS21	36.9	35.2	4.8	4.6	0.807	30.28	34.88	2.9	2.4	0.256	33.20	29.80	1.8	3.3	0.395	35.12	14.1	2.7	4.5	0.004
MS23	25.88	26.50	4.3	0.47	0.889	23.66	33.48	0.57	4.1	0.044	31.74	26.88	3.1	1.5	0.192	33.8	23.56	4.7	2.8	0.097
MS25	32.18	29.40	2.8	1.2	0.439	26.96	25.52	0.82	4.3	0.753	35.1	8.3	5.6	5.6	0.009					
MS26	29.60	36.16	2.9	1.8	0.071	27.58	26.46	1.2	2.6	0.707	37.94	23.5	2.9	5.9	0.059	46.5	13.4	6.6	10	0.025

Table SM 8. One-way ANOVA for the SPAD measurements of the LP varieties under different treatments and days after salinization ($p < 0.05$).

		F value	p-value
Day 1	Control	1.44	0.106
	Salinity	1.18	0.278
Day 3	Control	1.62	0.047
	Salinity	0.83	0.697
Day 5	Control	1.81	0.020
	Salinity	1.05	0.416
Day 7	Control	0.75	0.793
	Salinity	0.57	0.944

Table SM 9. One-way ANOVA for the SPAD measurements of the MS varieties under different treatments and days after salinization ($p < 0.05$).

		F value	p-value
Day 1	Control	1.63	0.062
	Salinity	2.74	0.001
Day 3	Control	1.55	0.083
	Salinity	1.63	0.063
Day 5	Control	2.53	0.002
	Salinity	2.57	0.001
Day 7	Control	1.18	0.292
	Salinity	2.09	0.010

Table SM 10. Two sample t-test to compare Na^+/K^+ ratio values for all LP varieties and their parentals in the hydroponic assay ($p < 0.05$).

Variety	μ (c)	μ (s)	SEM (c)	SEM (s)	p-value
PL12	0.00555	1.229	0.00089	0.18	0.003
FL478	0.001727	0.591	0.000075	0.28	0.101
LP1	0.005269	0.923	0.00053	0.12	0.002
LP2	0.003873	0.874	0.00045	0.25	0.026
LP3	0.003467	1.148	0.00038	0.29	0.018
LP4	0.003630	1.307	0.00025	0.24	0.005
LP6	0.003488	0.929	0.00042	0.38	0.072
LP7	0.00440	0.661	0.0011	0.22	0.039
LP9	0.003486	0.504	0.00040	0.24	0.104
LP10	0.004114	0.839	0.00035	0.20	0.013
LP11	0.003815	0.852	0.00021	0.20	0.012
LP12	0.003341	1.034	0.00012	0.43	0.076
LP14	0.004162	0.832	0.00039	0.34	0.074
LP15	0.00695	0.926	0.00058	0.23	0.015
LP16	0.007382	0.788	0.00043	0.18	0.013
LP17	0.00670	0.759	0.00091	0.29	0.059
LP18	0.006713	0.754	0.00053	0.14	0.006
LP20	0.00848	0.836	0.0016	0.21	0.016
LP21	0.00772	1.026	0.0020	0.28	0.023
LP23	0.00912	1.067	0.0032	0.058	0.000
LP24	0.00534	0.968	0.00078	0.15	0.003
LP26	0.004834	0.882	0.00066	0.25	0.069
LP28	0.004749	0.728	0.00028	0.24	0.038
LP29	0.00680	1.034	0.0016	0.12	0.001
LP35	0.00671	1.009	0.0010	0.34	0.041

Table SM 11. Two sample t-test to compare Na^{+}/K^{+} ratio values for all MS varieties and their parentals in the hydroponic assay ($p < 0.05$).

Variety	μ (c)	μ (s)	SEM (c)	SEM (s)	p-value
PM37	0.00766	1.697	0.00098	0.19	0.001
IR64	0.005933	1.995	0.00031	0.36	0.005
MS2	0.005770	1.0894	0.00031	0.029	0.000
MS3	0.00914	1.804	0.0021	0.29	0.004
MS4	0.004772	1.383	0.00038	0.22	0.003
MS5	0.00819	1.628	0.0031	0.29	0.005
MS6	0.004669	1.557	0.00038	0.40	0.018
MS7	0.005815	1.709	0.000083	0.38	0.011
MS8	0.004757	1.570	0.00017	0.41	0.018
MS9	0.00743	1.772	0.00072	0.083	0.000
MS11	0.007541	1.284	0.00037	0.48	0.057
MS12	0.00559	1.979	0.0014	0.35	0.005
MS14	0.006398	1.144	0.00043	0.41	0.050
MS16	0.00596	1.351	0.00097	0.38	0.025
MS17	0.004800	2.272	0.00025	0.23	0.001
MS18	0.00776	1.762	0.00084	0.37	0.009
MS20	0.0036527	1.220	0.00003	0.26	0.010
MS21	0.004052	1.280	0.00032	0.14	0.001
MS23	0.007249	1.4064	0.00023	0.037	0.000
MS25	0.005778	2.350	0.00021	0.60	0.017
MS26	0.005213	1.744	0.00032	0.073	0.000

Table SM 12. Two sample t-test to compare Na^+/K^+ ratio values for all LP varieties and their parentals in the field assay ($p < 0.05$).

Variety	μ (c)	μ (s)	SEM (c)	SEM (s)	p-value
PL12	0.09216	0.3292	0.0028	0.024	0.000
FL478	0.03050	0.1230	0.0023	0.018	0.002
LP1	0.05592	0.2116	0.0020	0.013	0.000
LP2	0.0659	0.2410	0.0060	0.022	0.000
LP3	0.07862	0.2149	0.0025	0.030	0.004
LP4	0.05668	0.2663	0.0030	0.040	0.002
LP5	0.0509	0.1901	0.0052	0.022	0.001
LP6	0.05944	0.2759	0.0021	0.050	0.005
LP7	0.05604	0.2516	0.0034	0.015	0.000
LP8	0.06396	0.2012	0.0025	0.020	0.000
LP11	0.06678	0.2281	0.0043	0.024	0.001
LP12	0.05360	0.2435	0.0023	0.030	0.001
LP13	0.05210	0.1828	0.0024	0.025	0.002
LP14	0.05668	0.2477	0.0038	0.042	0.004
LP16	0.06276	0.2550	0.0041	0.017	0.000
LP17	0.05323	0.2407	0.0012	0.020	0.000
LP20	0.0682	0.3004	0.0052	0.022	0.000
LP21	0.0738	0.3419	0.0067	0.022	0.000
LP22	0.05831	0.1795	0.0048	0.020	0.001
LP24	0.05525	0.17464	0.0035	0.0032	0.000
LP25	0.06329	0.316	0.0028	0.063	0.007
LP26	0.05834	0.1739	0.0024	0.014	0.000
LP27	0.0708	0.342	0.0062	0.053	0.002
LP28	0.05985	0.1882	0.0039	0.035	0.011
LP29	0.04876	0.1484	0.0043	0.0081	0.000
LP30	0.05582	0.1681	0.0035	0.014	0.000
LP31	0.04005	0.1984	0.0013	0.016	0.000

Table SM 13. Two sample t-test to compare Na^+/K^+ ratio values for all MS varieties and their parentals in the field assay ($p < 0.05$).

Variety	μ (c)	μ (s)	SEM (c)	SEM (s)	p-value
PM37	0.07580	0.2032	0.0150	0.0098	0.000
IR64	0.03882	0.1657	0.0039	0.011	0.000
MS3	0.05230	0.2151	0.0025	0.010	0.000
MS12	0.07510	0.2900	0.0083	0.025	0.000
MS14	0.04042	0.1672	0.0030	0.020	0.001
MS17	0.07080	0.2131	0.0120	0.032	0.005
MS21	0.04187	0.2202	0.0038	0.011	0.000
MS23	0.05111	0.2268	0.0038	0.013	0.000
MS25	0.04138	0.2110	0.0032	0.019	0.000
MS28	0.03927	91.450	0.0023	0.0098	0.000

Experiment 3: Studying the rice pearl in Mediterranean varieties by multiple approaches

Table SM 14. Comparison tests for the physical measurements ($p < 0.05$). One-way ANOVA with post-hoc Tukey test (A / T) was used when the data presented homoscedasticity, and a Welch-ANOVA with a post-hoc Games-Howell test (WA / G-H) was applied when the data presented heteroscedasticity. M= Montsianell, B= Bomba, C= Carnaroli, G = Guadiamar, P =PL12.

Measurement	Test	F value	p-value	post-hoc				
				M	B	C	G	P
Area	WA / G-H	210.33	0.000	b	d	a	c	cd
Pearl area	A / T	228.34	0.000	b	c	a		
Perimeter	WA / G-H	295.11	0.000	b	d	a	c	a
Length	WA / G-H	463.96	0.000	b	c	a	b	a
Width	WA / G-H	1325.29	0.000	a	b	c	d	e
Length/width	WA / G-H	1299.65	0.000	d	e	b	c	a
Weight	A / T	185.51	0.000	a	b	a	b	c

Table SM 15. One-way ANOVA with a Tukey post-hoc analysis for the crystallinity measurements of the different types of grain and varieties ($p < 0.05$). LP= large pearl fraction, SP= small pearl fraction, WG= whole grain, CG= chalky grain.

Variety	F value	p-value	post-hoc			
			LP	SP	WG	CG
Montsianell	2.84	0.135	a	a		a
Bomba	1.64	0.270	a	a		a
Carnaroli	0.33	0.731	a	a		a
Guadiamar	0.60	0.480			a	a
PL12	13.17	0.022			b	a

Table SM 16. One-way ANOVA with a Tukey post-hoc analysis for the amylose content measurements of the different types or fractions of grains for each variety ($p < 0.05$). WG= whole grain, PF= pearl fraction, CF=crystalline fraction, CG= chalky grain.

Variety	F value	p-value	post-hoc			
			WG	PF	CF	CG
Montsianell	20.30	0.000	a	a	a	b
Bomba	24.80	0.000	a	a	a	b
Carnaroli	0.88	0.492	a	a	a	a
Guadiamar	28.29	0.006	a			b
PL12	50.11	0.002	a			b

Figure SM 2. Five pictures of 20 grains were taken for each variety in order to calculate the physical measurements. Each row belongs to a variety. A= Montsianell. B= Bomba. C= Carnaroli. D= Guadiamar. E= PL12.

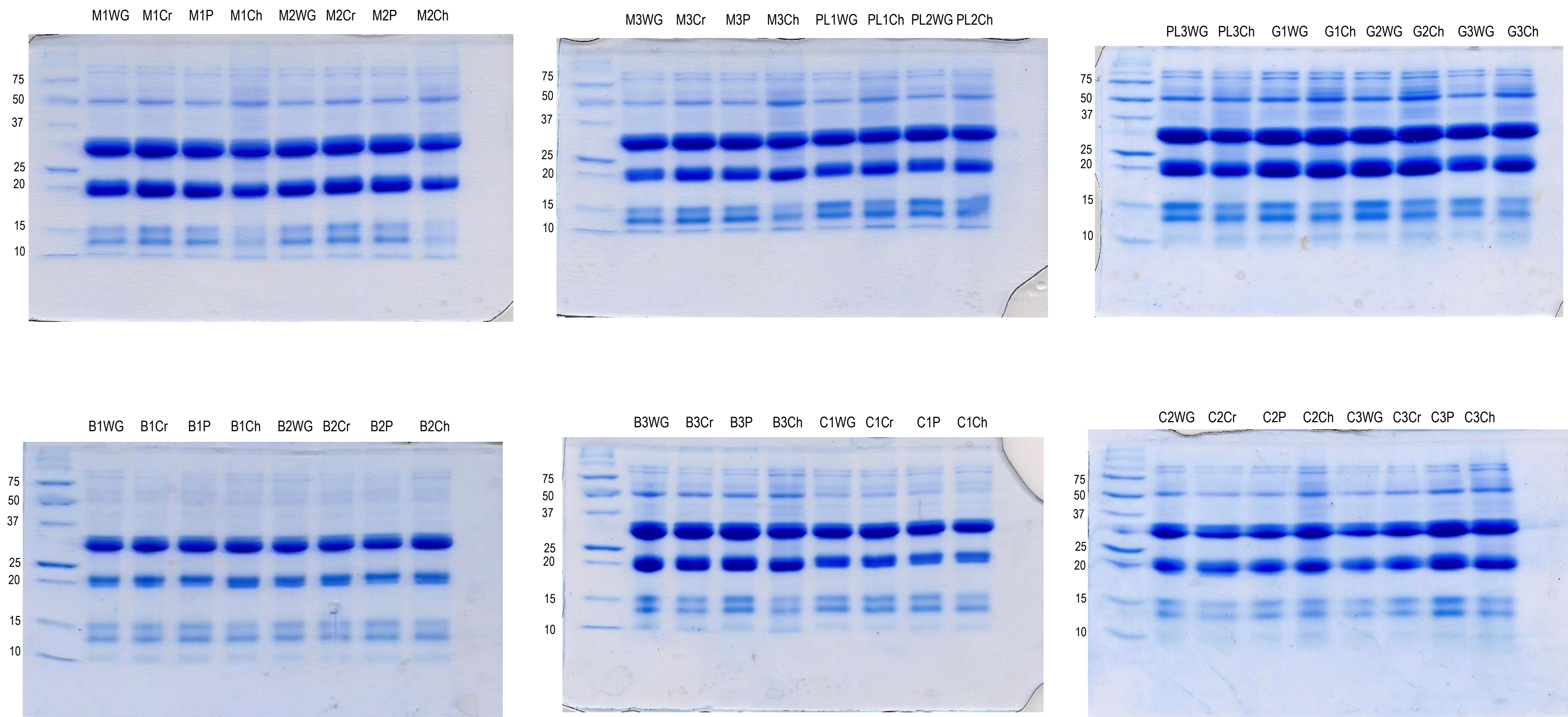

Figure SM 3. 15% polyacrylamide gels of three replicas for each fraction and variety. Molecular weights expressed on kilodaltons (kDa) of the protein ladder are shown at the left of each gel. M= Montsianell. PL= PL12. G= Guadiamar. B= Bomba. C= Carnaroli. 1, 2 or 3= number of replica. WG= whole grain. Cr= crystalline fraction. P= pearl fraction. Ch= Chalky grain.

Table SM 17. One-way ANOVA with a Tukey post-hoc analysis for the storage proteins content of the different grains and fractions for each variety ($p < 0.05$). WG= whole grain, PF= pearl fraction, CF=crystalline fraction, CG= chalky grain.

Variety	Protein fraction	F value	p-value	post-hoc			
				WG	PF	CF	CG
Montsianell	Glutelin precursor	29.80	0.000	b	b	b	a
	Glutelin acidic subunit	1.10	0.402	a	a	a	a
	Glutelin basic subunit	2.20	0.166	a	a	a	a
	Prolamins	19.11	0.001	a	a	a	b
Bomba	Glutelin precursor	2.04	0.187	a	a	a	a
	Glutelin acidic subunit	1.22	0.362	a	a	a	a
	Glutelin basic subunit	2.76	0.112	a	a	a	a
	Prolamins	0.83	0.515	a	a	a	a
Carnaroli	Glutelin precursor	0.06	0.981	a	a	a	a
	Glutelin acidic subunit	0.27	0.846	a	a	a	a
	Glutelin basic subunit	0.20	0.891	a	a	a	a
	Prolamins	0.30	0.822	a	a	a	a
Guadiamar	Glutelin precursor	16.37	0.016	b			a
	Glutelin acidic subunit	4.76	0.095	a			a
	Glutelin basic subunit	2.20	0.212	a			a
	Prolamins	41.90	0.003	a			b
PL12	Glutelin precursor	3.84	0.122	a			a
	Glutelin acidic subunit	5.14	0.086	a			a
	Glutelin basic subunit	0.22	0.663	a			a
	Prolamins	8.20	0.046	a			b