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New advances in the control of brown rot in stone fruit using the biocontrol agent *Bacillus amyloliquefaciens* CPA-8

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Ph.D. THESIS RESEARCH

New advances in the control of brown rot in stone fruit using the biocontrol agent *Bacillus amyloliquefaciens* CPA-8

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To my parents, Demetrio and Amparo, for having endured me during the thesis and the previous 28 years. This is not over and you know it.

To Edi. Ask my parents how to put up with me. Maybe they can help you.

Enormously grateful.

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Hoy puede ser un gran día, date una oportunidad

Hoy puede ser un gran día, y mañana también.

J.M. Serrat, 1981

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Abbreviations

AFLP	Amplified Fragment Length Polymorphism
AMP	Ampicillin
a_w	Water activity, water availability
BCA	BioControl Agent
BLAST	Basic Local Alignment Search Tool
CAS	Chrome Azurol S
CHL	Chloramphenicol
DE	Dextrose Equivalent
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
DSF	Defatted Soy Flour
EC	European Commission
EC_{50}	Half Maximal Effective Concentration
EDTA	Ethylenediaminetetraacetic Acid
ERIC	Enterobacterial Repetitive Intergenic Consensus
EU	European Union
FBSD	Fluid-Bed Spray-Drying
FD	Freeze-Drying
FDA	Fluorescein Diacetate
FEM	Field Emission Microscopy
GC-MS	Gas Chromatography- Mass Spectrometry
GEN	Gentamicin
HBL	Hemolysin BL
HPMC	Hydroxypropyl Methylcellulose
HS-SPME	Head Space-Solid Phase MicroExtraction
HYG	Hygromycin
ISR	Induced Systemic Resistance
ITS	Internal Transcribed Spacer
LQF	Liquid Formulation
MEA	Malt Extract Agar
MW	Molecular Weight
NA	Nutrient Agar
NAL	Nalidixic Acid
NCBI	National Centre of Biotechnology Information

NHE	Non Haemolytic Enterotoxin
NYDA	Nutrient Yeast Dextrose Agar
NYDB	Nutrient Yeast Dextrose Broth
PB	Phosphate Buffer
PCR	Polymerase Chain Reaction
PCR-rep	PCR Repetitive Element PCR fingerprinting
PDA	Potato Dextrose Agar
PGPR	Plant Growth Promoting Rhizobacteria
qPCR	Quantitative PCR
RA	Relative Area
RAPD	Randomly Amplified Polymorphism DNA
rDNA	Ribosomal DNA
REP	Repetitive Extragenic Palindromic
RFLP	Restriction Fragment Length Polymorphism
RH	Relative Humidity
RH $_{max, min}$	Relative Humidity maximum, minimum
RODAC	Replicate Organism Detection And Counting
ROS	Reactive Oxygen Species
RT	Retention Time
SCAR	Sequence Characterised Amplified Region
SDS	Sodium Dodecyl Sulphate
SM	Skimmed Milk
STR	Streptomycin
T $_{max, min,}$	Temperature maximum, minimum
TBE	Tris-Borate-EDTA
TRIS-HCl	Hydroxymethyl-aminomethane- hydrochloride
TSA	Tryptone Soya Agar
VOC	Volatile Organic Compound
WSP	Water Sensitive Paper

SUMMARY/RESUMEN/RESUM

Summary

Brown rot caused by the wound-invading fungus *Monilinia* spp. is specially responsible for substantial postharvest losses of stone fruit, reaching even as high as 80 % of the production in years when the conditions of temperature and wetness are favourable for the development of the disease, especially in late-ripening varieties. The use of synthetic fungicides is still the most effective way for preventing yield losses caused by most fungal diseases in different crops. However, the use of chemical applications in stone fruit after harvesting is hardly authorized in Europe. In addition, public concerns on the sustainability of agricultural practices, an increasing importance of organic agriculture, and the prevention of acquired fungicide resistance in target pathogens have promoted the search for alternative methods that involve a reduction in the number of field chemical applications.

Among the control strategies, the application of environment-friendly strategies such as those using biological control agents (BCAs), either alone or in combination with physico-chemical treatments, has been strongly considered over the last decades by many scientists and several commercial companies worldwide, although it is not already routinely applied in fruit industry.

Recently, detailed studies have shown that the BCA *Bacillus amyloliquefaciens* CPA-8, which is a common constituent of the resident microbiota on peaches, is an effective antagonist to control brown rot in peach caused by *Monilinia* spp., (alone or with hot water and curing), based on its capability of production of fengycin-like lipopeptides. However, while an abundance of reports exists describing beneficial microorganisms in controlling postharvest diseases, little success rate of BCA-based products are already available in the market. Therefore, the main goal of this thesis is to complete de development of the BCA *B. amyloliquefaciens* CPA-8 to obtain efficacious CPA-8-based products that provide a plausible commercial strategy to control brown rot in stone fruit.

Reliable markers were developed to molecularly detect and identify the BCA CPA-8. In a first approach, the SCAR-4 marker (based on RAPD methodology),

amplified a semi-specific fragment of 665 bp not only for the strain CPA-8 but also for other 12 strains, morphologically different to CPA-8. Consequently, a strain-specific genomic marker for CPA-8 was obtained from the *RBAM 007760* gene, gene mainly involved in bacterial surface adhesion. These insights into CPA-8 genetics allowed to demonstrate that this strain is closely related to *B. amyloliquefaciens* species rather than *B. subtilis* (which was its first name in the literature).

This work also focused on the wide tolerance of CPA-8 to grow under different pH-temperature and water activity (a_w)-temperature conditions. While CPA-8 could not grow at pH lower than 4.5, the optimum growth was observed at 37 ºC and pH between 7 and 5. Moreover, the type of solute used to reduce a_w had a great influence on the minimum a_w at which the bacterium was able to grow. The lowest a_w for CPA- 8 growth in media modified with glycerol and glucose was 0.950 and 0.960, respectively. However, at 20 ºC, CPA-8 was not able to grow at less than 0.990 a_w, regardless of the type of solute. Antibiotic susceptibility tests were also carried out to determine which antibiotic could affect the behaviour of this bacterium, indicating that CPA-8 was clearly resistant to hygromycin. Besides that, a PCR amplification assay to detect the presence of enterotoxigenic genes from *Bacillus cereus* in CPA-8 revealed that, although CPA-8 amplified for *nheA* gene, it seems to be not enough for the toxicity expression, demonstrating that this strain would not be a potential vehicle for foodborne illnesses.

Antagonists can display a wide range of modes of action, at different stages of their activity, relating to different hosts and pathogens. Therefore, fengycin production should not be considered the unique mechanism of action for strain CPA-8. In this work, the antifungal effect of the volatile organic compounds (VOCs) emitted by CPA-8 was demonstrated against *Monilinia laxa*, *Monilinia fructicola* and *Botrytis cinerea*. VOCs were evaluated with a double petri dish assay against mycelial and colony growth of target pathogens (*in vitro* conditions) and on artificially inoculated cherry fruits (*in vivo* conditions). Moreover, the main VOCs emitted by CPA-8 were identified by solid-phase microextraction (SPME)-gas chromatography as 1,3 pentadiene, acetoin (3-hydroxy-2-butanone) and thiophene.

However, one of the major bottlenecks in the commercialisation of biocontrol products is the development of shelf-stable formulations. A useful microbial formulation

should be economical to produce, easy to distribute to the intended environment, contain enough CFU, and provide a long shelf-life (preferentially stored at room temperatures and maintained for at least 6-24 months). As part of the production process improvement, three different nitrogen sources were evaluated. Low-cost culture medium based on the isolated soy protein PROSTAR 510A at 20 g L^{-1} provided the best CPA-8 growth curves compared to the boiled extract from Defatted Soy Flour (DSF) and protein PROSTAR 510A at 10 g L^{-1}, thus avoiding the hurdles caused by the raw DSF medium (microbial contamination and low solubility). Furthermore, CPA-8 endospores significantly increased from 24 to 72 h of culture (medium containing protein PROSTAR 510A at 20 g L^{-1}), suggesting that the ability of this strain to survive high temperatures is highly related with the age of the culture. With the optimised medium, a high concentration of endospores was achieved (>10^8 CFU mL^{-1}).

The next step was focused on the assessment and comparison of three different CPA-8 formulation approaches: liquid, freeze-drying and fluid-bed spray-drying. Moreover, the effect of protectants on cell viability, storage stability and antagonistic activity of CPA-8 was evaluated. Although satisfactory concentrations were finally obtained for both, liquid ($1.9 \cdot 10^9$ to $2.9 \cdot 10^9$ CFU mL^{-1}) and dry products ($4.8 \cdot 10^9$ to $1.0 \cdot 10^{10}$ CFU g^{-1}), the fluid-bed spray-drying technology was considered the most suitable one to develop stable and effective CPA-8 products for practical applications. Once the best drying process was defined for CPA-8 formulation, different processing parameters to obtain high performance need to be optimised. Specifically, this work focussed on the effect of protectants and carrier materials. The use of the protectants 20 % sucrose plus 10 % skimmed milk resulted in the best formulations when either carrier material, maltodextrin or potato starch, was used. These two products (that hereafter will be called BA3 -formulated with maltodextrin, and BA4 -formulated with potato starch-) were selected for assays of shelf-life and efficacy. The results revealed that CPA-8 viability was unchanged after 15 months of storage at 4 and 22 ^oC, maintaining concentrations between $7.8 \cdot 10^9$ and $1.2 \cdot 10^{10}$ CFU g^{-1}. Moreover, the efficacy of these two CPA-8-formulated products against *Monilinia* spp. was confirmed on peaches, nectarines, flat peaches, cherries, apricots and plums.

Subsequently, this thesis aimed to describe the population dynamics of CPA-8 on the surface of nectarines and peaches after being treated with two CPA-8-formulated products (BA3 and BA4) and exposed to different environmental conditions. CPA-8

demonstrated wide tolerance to different factors such as temperature, relative humidity and simulated rainfall (being the last the most constricting factor). The minimal antagonist population obtained after exposure was generally higher than 10^4 CFU cm^{-2} of fruit surface, thus ensuring high treatment coverture and therefore, efficacy. The results also indicated that peaches were, in general, more suitable for the CPA-8 survival than nectarines. Moreover, the properties of the two CPA-8-formulated products influenced the population dynamics of this bacterium, suggesting that the BA4 CPA-8-formulated product provided higher degree of ecological fitness of CPA-8 over the fruit than the BA3 CPA-8-formulated product.

Finally, the step wise screening of microorganisms for commercial use in biological control requires full field trials, including disease control in crops with complete common plant protection schedules. In order to do this, the last part of this thesis aimed to assess the potential of different formulations of CPA-8 for brown rot control under commercial peach production. Specifically, the CPA-8 dose of treatment, the CPA-8 population dynamics once applied in the orchard and the CPA-8 efficacy in controlling *Monilinia* spp. incidence at harvest and postharvest, were studied. Different degree of biocontrol activity was obtained depending on the disease pressure, which was associated with meteorological conditions. Under drastic pathogen pressure in the field (>50 %), only the chemical treatment reduced brown rot incidence at postharvest (and any treatment at harvest). However, when disease pressure was in the range of the standard levels recorded in the area (5.3 and 17.3 % *Monilinia* spp. incidence at harvest and postharvest, respectively), treatments based on CPA-8 formulations proved to be efficacious. At harvest, BA3 and BA4 treatments reduced *Monilinia* spp. incidence compared to the control, although less than the chemicals. Otherwise, the BA4 treatment effectively reduced brown rot disease at postharvest, statistically similar to chemical applications. CPA-8 was also evaluated combined with another BCA, *Penicillium frequentans*, which had already demonstrated efficacy against *Monilini*a spp. However, such combination did not improve CPA-8's efficacy. Moreover, the capability of colonisation of CPA-8 on fruit surface after treatment application until harvest and after postharvest conditions was largely demonstrated.

In conclusion, the data obtained in this thesis confirm the potential of the BCA *B. amyloliquefaciens* CPA-8 as an effective alternative in the control of *Monilinia* spp.

Summary, Resumen, Resum

Two products based on CPA-8 have been obtained, which are in the last stage of their commercial development, ready to the registration process. The integration of these products into the usual cropping systems can be a promising strategy to achieve a high level of control of brown rot, thus contributing to the management of post-harvest diseases in stone fruit in the framework of a sustainable and/or organic agriculture.

Resumen

La podredumbre marrón causada por el hongo *Monilinia* spp. es responsable de importantes pérdidas en la poscosecha de la fruta de hueso, alcanzando el 80 % de la producción cuando las condiciones de temperatura y humedad son favorables para el desarrollo de la enfermedad, especialmente en variedades de maduración tardía. Hasta la fecha, el uso de fungicidas sintéticos sigue siendo la forma más eficaz para prevenir enfermedades fúngicas, causantes de tan graves pérdidas en la producción de diferentes cultivos. Sin embargo, la lista de productos autorizados en Europa para uso en poscosecha es muy limitada. Además, existe una gran conciencia social respecto a la sostenibilidad de las prácticas agrícolas, un incremento de la producción ecológica y la necesidad de prevenir la aparición de cepas fúngicas resistentes a fungicidas. Todo ello ha promovido la búsqueda de métodos alternativos que impliquen una reducción en el número de aplicaciones químicas en el campo.

Entre los métodos de control, la aplicación de estrategias respetuosas con el medio ambiente, tales como las que utilizan agentes de biocontrol (ABCs) solos o en combinación con tratamientos físico-químicos, se ha tenido enormemente en cuenta durante las últimas décadas por científicos y empresas de todo el mundo. Sin embargo, tales prácticas aún no se aplican de manera rutinaria en la industria frutícola.

Recientemente se ha demostrado la capacidad antagonista de la cepa CPA-8 de *Bacillus amyloliquefaciens* (aislado de la microbiota del melocotón), el cual es capaz de controlar la podredumbre marrón del melocotón causada por el hongo *Monilinia* spp. Dicha habilidad se debe principalmente a su capacidad de producir lipopéptidos del grupo de las fengicinas. No obstante, aunque existe una gran variedad de trabajos que describen diferentes microorganismos eficaces en el control de enfermedades de poscosecha, la cantidad de productos basados en ABCs disponibles en el mercado es considerablemente baja. Por lo tanto, el objetivo principal de esta tesis es completar el desarrollo del ABC *B. amyloliquefaciens* CPA-8 para así obtener un producto eficaz que proporcione una estrategia comercialmente viable para el control de la podredumbre marrón de la fruta de hueso.

En primer lugar, se diseñaron dos marcadores para detectar e identificar molecularmente el ABC CPA-8. En un primer enfoque, el marcador SCAR-4 (basado en la metodología RAPD) amplificó un fragmento semiespecífico de 665 pb, el cual no sólo dio positivo para la cepa CPA-8, sino también para otras 12 cepas (todas ellas morfológicamente diferentes a CPA-8 y fácilmente distinguibles previamente a la amplificación del ADN). En consecuencia, se desarrolló un segundo marcador mucho más preciso a partir del gen *RBAM 007760*. Dicho gen está implicado en la adaptación ecológica de la bacteria, principalmente en la adhesión superficial bacteriana. A raíz de dicho estudio, los conocimientos adquiridos en la genética de CPA-8 permitieron demostrar que esta cepa está estrechamente relacionada con especies del grupo *B. amyloliquefaciens* en lugar de *B. subtilis* (el cual fue su primer nombre en la literatura).

En este trabajo también se describió la capacidad de CPA-8 para crecer en diferentes condiciones de temperatura- pH y de temperatura-actividad de agua (a_w). Mientras que CPA-8 no pudo crecer a pH inferior a 4.5, el crecimiento óptimo se observó a 37 °C y pH entre 7 y 5. Además, el tipo de soluto utilizado para reducir la a_w tuvo una gran influencia en la mínima a_w en la cual la bacteria pudo crecer. La a_w más baja para el crecimiento de CPA-8 en medios modificados con glicerol y glucosa fue de 0.950 y 0.960, respectivamente. Sin embargo, a 20 ºC, CPA-8 no fue capaz de crecer a menos de 0.990 a_w, independientemente del tipo de soluto utilizado para modificar el medio. Continuando con la caracterización de CPA-8, también se realizaron pruebas para determinar su susceptibilidad y/o resistencia a diferentes antibióticos. Los resultados indicaron que CPA-8 era claramente resistente a la higromicina. Además, se realizó un ensayo (basado en amplificación por PCR) para detectar la presencia de genes enterotóxicos propios de *Bacillus cereus* en CPA-8. Dicho estudio sugirió que, pese a que el gen *nheA* amplificó en CPA-8, esto no es suficiente para la expresión de toxicidad, sugiriendo así que esta cepa no es un patógeno de transmisión alimentaria.

Los antagonistas pueden mostrar una amplia gama de modos de acción, en diferentes etapas de su actividad y dependiendo de los diferentes huéspedes y patógenos. Por lo tanto, la producción de fengicinas no debe considerarse el único mecanismo de acción de la cepa CPA-8. En este trabajo se demostró el efecto

antifúngico de los compuestos orgánicos volátiles emitidos por CPA-8 contra *Monilinia laxa*, *Monilinia fructicola* y *Botrytis cinerea*. Dichos compuestos se evaluaron con un ensayo de doble placa petri para analizar el crecimiento micelial y el número de colonias en cada patógeno diana (ensayos *in vitro*) y también en cerezas inoculadas artificialmente con los patógenos mencionados (ensayos *in vivo*). Además, los principales volátiles emitidos por CPA-8 se identificaron mediante cromatografía de gases en fase sólida de microextracción (SPME) como 1,3 pentadieno, acetoina (3-hidroxi-2-butanona) y tiofeno.

Sin embargo, uno de los principales cuellos de botella en la comercialización de productos de biocontrol es el desarrollo de formulaciones que puedan ser almacenadas de manera estable. La formulación de un microrganismo debe ser económica de producir, fácil de distribuir, contener suficientes UFC y tener una larga vida útil (preferentemente almacenados a temperatura ambiente durante al menos 6-24 meses). Como parte de la mejora del proceso de producción, se evaluaron tres fuentes de nitrógeno diferentes. El medio de cultivo basado en el aislado proteico de soja PROSTAR 510A (20 g L^{-1}) proporcionó la mejor curva de crecimiento de CPA-8, en comparación con el extracto hervido de harina de soja desengrasada (DSF) y el extracto proteico PROSTAR 510A (10 g L^{-1}). De esta forma se evitaron los inconvenientes que causaba el medio DSF crudo (contaminación por alta carga microbiana de la harina y baja solubilidad). Además, la producción de endosporas de CPA-8 aumentó considerablemente a medida que el tiempo de cultivo era más largo (de 24 a 72 h), lo que sugirió que la capacidad de esta cepa para sobrevivir a altas temperaturas está altamente relacionada con la edad del cultivo. Con el medio de cultivo optimizado, finalmente se obtuvo una alta concentración de endosporas (>10^8 UFC mL^{-1}).

La siguiente etapa se centró en la evaluación y comparación de tres tecnologías diferentes para la formulación de CPA-8: líquida, liofilización y secado por lecho fluido-atomización. Además, se evaluó el efecto de diferentes protectores en la viabilidad, la estabilidad y la actividad antagonista de CPA-8. Aunque finalmente se obtuvieron concentraciones satisfactorias tanto para los productos líquidos ($1.9 \cdot 10^9$-$2.9 \cdot 10^9$ UFC mL^{-1}) como para los productos secos ($4.8 \cdot 10^9$-$1.0 \cdot 10^{10}$ UFC g^{-1}), se consideró que la tecnología de secado por lecho fluido-atomización era la más

adecuada para el desarrollo de productos basados en CPA-8 estables y eficaces para posteriores aplicaciones prácticas. Una vez que se definió el mejor proceso de secado para la formulación de CPA-8, fue necesaria una posterior optimización de algunos parámetros y así obtener un mayor rendimiento. En particular, este trabajo se centró en el efecto de los protectores y de los materiales de soporte empleados durante la fluidificación. El uso de los protectores 20 % de sacarosa más 10 % de leche en polvo resultó ser la mejor combinación para formular CPA-8 tanto cuando se usó maltodextrina como substrato como cuando se usó el almidón de patata. Estos dos productos (que de ahora en adelante se denominarán BA3 -formulados con maltodextrina, y BA4 -formulados con almidón de patata-) fueron seleccionados para posteriores ensayos de vida útil y eficacia. Los resultados revelaron que la viabilidad de CAP-8 se mantuvo estable después de 15 meses de almacenamiento a 4 y 22 °C, obteniendo concentraciones entre $7.8 \cdot 10^9$ y $1.2 \cdot 10^{10}$ UFC g^{-1}. Además, la eficacia de estos dos productos formulados frente a *Monilinia* spp. se demostró en melocotones, nectarinas, paraguayos, cerezas, albaricoques y ciruelas.

Posteriormente, se estimó la dinámica poblacional de CPA-8 en la superficie de nectarinas y melocotones una vez tratados con los productos formulados de CPA-8 (BA3 y BA4) y expuestos a diferentes condiciones ambientales. CPA-8 demostró una amplia tolerancia a diferentes valores extremos de temperatura, humedad relativa y lluvia (siendo éste último el factor más restrictivo). La población mínima de CPA-8 obtenida después de la exposición fue en la mayoría de los casos superior a 10^4 UFC cm^{-2} de superficie del fruto, garantizando así una buena cobertura del tratamiento. Los resultados también indicaron que los melocotones proporcionaron, por lo general, un mejor nicho para el mantenimiento de CPA-8 sobre la superficie del fruto que las nectarinas. Además, las propiedades de los dos productos formulados influyeron enormemente en la dinámica poblacional de CPA-8 sobre la fruta, sugiriendo que el producto formulado BA4 favoreció una mayor supervivencia de CPA-8.

Por último, para el desarrollo de ABCs comercialmente funcionales, es necesario realizar ensayos en condiciones reales de campo, incluyendo experimentos en cultivo completo (y no sólo en frutos). Para ello, la última parte de esta tesis tuvo como objetivo evaluar el potencial de diferentes formulaciones de CPA-8 en el control de la

podredumbre marrón en campos comerciales de melocotón. Específicamente se estudió la dosis de tratamiento de CPA-8, la dinámica poblacional de CPA-8 una vez aplicado en campo y la eficacia de CPA-8 en el control de *Monilinia* spp., tanto en la cosecha como en la poscosecha. Los resultados demostraron que, dependiendo de la presión de la enfermedad (principalmente condicionada por la meteorología) se obtuvo un grado variable de control biológico. Bajo una alta incidencia de *Monilinia* spp. en campo (>50 %), sólo el tratamiento químico pudo controlar la enfermedad en poscosecha (en cosecha ni siquiera el químico demostró efecto comparado con el control). Sin embargo, cuando la presencia del patógeno se engloba dentro de los límites estándares registrados en la zona (5.3 y 17.3 % de incidencia de *Monilinia* spp. en cosecha y poscosecha, respectivamente), los tratamientos basados en formulaciones de CPA-8 demostraron ser eficaces. En la cosecha, los productos BA3 y BA4 redujeron considerablemente la incidencia de *Monilinia* spp. (aunque menos que los productos químicos). Además, el tratamiento con el producto BA4 redujo eficazmente la enfermedad en poscosecha e incluso estadísticamente similar a las aplicaciones químicas. También se evaluó la eficacia de CPA-8 una vez aplicado junto con otros ABCs. En este caso se escogió *Penicillium frequentans,* el cual ya había sido descrito como antagonista de *Monilinia* spp. Sin embargo, tal combinación no aumentó la eficacia de CPA-8. Además, se demostró la persistencia de CPA-8 en la superficie del fruto desde que se aplicó el tratamiento hasta la cosecha y posterior poscosecha.

En conclusión, los datos obtenidos en esta tesis permiten confirmar el potencial del ABC *B. amyloliquefaciens* CPA-8 como una alternativa eficaz en el control de *Monilinia* spp. Se han obtenido dos productos basados en CPA-8, los cuales se encuentran en la última etapa de su desarrollo comercial, a falta de la fase de registro. La integración de estos productos en los sistemas de cultivo habituales puede ser una estrategia prometedora para conseguir un mayor nivel de control de la podredumbre marrón, contribuyendo así en el manejo de las enfermedades de poscosecha en fruta de hueso en el marco de una agricultura sostenible y/o ecológica.

Resum

La podridura marró causada pel fong *Monilinia* spp. és responsable d'importants pèrdues en la postcollita de la fruita de pinyol, assolint el 80 % de la producció quan les condicions de temperatura i humitat són favorables pel desenvolupament de la malaltia, especialment en varietats de maduració tardana. Fins a la data, l'ús de fungicides sintètics segueix essent la forma més eficaç per prevenir malalties fúngiques, causants de tan greus pèrdues en la producció de diferents cultius. No obstant això, la llista de productes autoritzats a Europa per a ús en postcollita és molt limitada. A més a més, hi ha una gran consciència social respecte a la sostenibilitat de les pràctiques agrícoles, un increment en l'agricultura ecològica i la necessitat de prevenir l'aparició de soques fúngiques resistents a fungicides. Tot això ha promogut la recerca de mètodes alternatius que impliquin una reducció en el nombre d'aplicacions químiques en el camp.

Entre els mètodes de control, l'aplicació d'estratègies respectuoses amb el medi ambient, com ara les que utilitzen agents de biocontrol (ABCs) sols o en combinació amb tractaments fisicoquímics, s'ha tingut enormement en compte durant les últimes dècades per científics i empreses de tot el món. No obstant això, aquesta pràctica no s'aplica actualment de manera rutinària en la indústria fructícola.

Recentment s'ha demostrat la capacitat antagonista de la soca CPA-8 de *Bacillus amyloliquefaciens* (aïllat de la microbiota del préssec), el qual és capaç de controlar la podridura marró del préssec causada pel fong *Monilinia* spp. Aquesta habilitat es deu principalment a la seva capacitat de produir lipopèptids del grup de les fengicines. No obstant això, encara que existeix una gran varietat de treballs que descriuen diferents microorganismes eficaços en el control de malalties de postcollita, la quantitat de productes basats en ABCs disponibles al mercat, és considerablement baixa. Per tant, l'objectiu principal d'aquesta tesi és completar el desenvolupament de l'ABC *B. amyloliquefaciens* CPA-8 per així obtenir un producte eficaç que proporcioni una estratègia comercialment viable en el control de la podridura marró de la fruita de pinyol.

En primer lloc es van dissenyar dos marcadors per detectar i identificar molecularment l'ABC CPA-8. En una primera aproximació, el marcador SCAR-4 (basat en la metodologia RAPD) va amplificar un fragment semi-específic de 665 pb, el qual no només va donar positiu per la soca CPA-8, sinó també per unes altres 12 soques (totes elles morfològicament diferents a CPA-8 i fàcilment distingibles prèviament a l'amplificació de l'ADN). En conseqüència, es va desenvolupar un segon marcador molt més precís a partir del gen *RBAM 007760*. Aquest gen està implicat en l'adaptació ecològica del bacteri, principalment en l'adhesió superficial bacteriana. Arran d'aquest estudi, els coneixements adquirits en la genètica de CPA- 8 van permetre demostrar que aquesta soca està estretament relacionada amb espècies del grup *B. amyloliquefaciens* en lloc de *B. subtilis* (el qual va ser el seu primer nom en la literatura).

En aquest treball també es va descriure la capacitat de CPA-8 per créixer en diferents condicions de temperatura-pH i de temperatura-activitat d'aigua (a_w). Mentre que CPA-8 no va poder créixer a pH inferior a 4.5, el creixement òptim es va observar a 37 ° C i pH entre 7 i 5. A més a més, el tipus de solut utilitzat per reduir l' a_w va tenir una gran influència en la a_w mínima en la qual el bacteri va poder créixer. L' a_w més baixa per al creixement de CPA-8 en medi modificat amb glicerol i glucosa va ser de 0.950 i 0.960, respectivament. No obstant això, a 20 ^{o}C, CPA-8 no va ser capaç de créixer a menys de 0.990 a_w, independentment del tipus de solut utilitzat per modificar el medi. Continuant amb la caracterització de CPA-8, també es van realitzar proves per determinar la seva susceptibilitat i/o resistència a diferents antibiòtics. Els resultats van indicar que CPA-8 era clarament resistent a la higromicina. A més a més, es va realitzar un assaig (basat en amplificació per PCR) per detectar la presència de gens enterotòxics propis de *Bacillus cereus* en CPA-8. Aquest estudi va suggerir que, tot i que el gen *nheA* va amplificar en CPA-8, això no és suficient per a l'expressió de toxicitat, suggerint així que aquesta soca no és un patogen de transmissió alimentària.

Els antagonistes poden mostrar una àmplia gamma de modes d'acció, en diferents etapes de la seva activitat i depenent dels diferents hostes i patògens. Per tant, la producció de fengicines no s'ha de considerar l'únic mecanisme d'acció de la soca CPA-8. En aquest treball, es va demostrar l'efecte antifúngic dels compostos

orgànics volàtils emesos per CPA-8 contra *Monilinia laxa*, *Monilinia fructicola* i *Botrytis cinerea*. Aquests compostos es van avaluar amb un assaig de doble placa petri per analitzar el creixement micelial i el nombre de colònies de cada patogen diana (assajos *in vitro*) i també en cireres inoculades artificialment amb els patògens esmentats (assajos *in vivo*). A més a més, els principals volàtils emesos per CPA-8 es van identificar mitjançant cromatografia de gasos en fase sòlida de microextracció (SPME) com 1,3 pentadiè, acetoina (3-hidroxi-2-butanona) i tiofè.

No obstant això, un dels principals colls d'ampolla en la comercialització de productes de biocontrol és el desenvolupament de formulacions que puguin ser emmagatzemades de manera estable. La formulació d'un microorganisme ha de ser econòmica de produir, fàcil de distribuir, contenir suficients UFC i tenir una llarga vida útil (preferentment emmagatzemats a temperatura ambient i durant almenys 6-24 mesos). Com a part de l'optimització del procés de producció, es van avaluar tres fonts de nitrogen diferents. El medi de cultiu basat en l'aïllat proteic de soja PROSTAR 510A (20 g L^{-1}) va proporcionar la millor corba de creixement de CPA-8, en comparació amb l'extracte bullit de farina de soja desgreixada (DSF) i l'extracte proteic PROSTAR 510A (10 g L^{-1}). D'aquesta manera es van evitar els inconvenients que causava el medi DSF cru (contaminació per alta càrrega microbiana de la farina i baixa solubilitat). A més a més, la producció d' endòspores de CPA-8 va augmentar considerablement a mesura que el temps de cultiu era més llarg (de 24 a 72 h), la qual cosa va suggerir que la capacitat d'aquesta soca per sobreviure a altes temperatures està altament relacionada amb l'edat del cultiu. Amb el medi de cultiu optimitzat, finalment es va obtenir una elevada concentració d'endospores (>10^8 UFC mL^{-1}).

El proper pas es va centrar en l'avaluació i comparació de tres tecnologies diferents per a la formulació de CPA-8: líquida, liofilització i assecat per llit fluïditzat-atomització. A més a més, es va avaluar l'efecte de diferents protectors en la viabilitat, l'estabilitat i l'activitat antagonista de CPA-8. Tot i que finalment es van obtenir concentracions satisfactòries tant per als productes líquids ($1.9 \cdot 10^9$–$2.9 \cdot 10^9$ UFC mL^{-1}) com per als productes sòlids ($4.8 \cdot 10^9$–$1.0 \cdot 10^{10}$ UFC g^{-1}), es va considerar que la tecnologia d'assecat per llit fluid-atomització era la més adequada pel desenvolupament de productes basats en CPA-8 estables i eficaços per a posteriors aplicacions pràctiques. Una vegada que es va definir el millor procés d'assecatge per a la formulació de CPA-8, va ser necessària una posterior optimització d'alguns

paràmetres i així obtenir un major rendiment. En particular, aquest treball es va centrar en l'efecte dels protectors i dels materials de suport emprats durant la fluïdificació. L'ús dels protectors 20 % de sacarosa més 10 % de llet en pols va resultar ser la millor combinació per a formular CPA-8 tant quan es va emprar maltodextrina com a substrat com quan es va utilitzar el midó de patata. Aquests dos productes (que d'ara endavant es denominaran BA3 -formulats amb maltodextrina, i BA4 -formulats amb midó de patata-) van ser seleccionats per a posteriors assajos de vida útil i eficàcia. Els resultats van revelar que la viabilitat del CPA-8 es va mantenir estable després de 15 mesos d'emmagatzematge a 4 i 22 ° C, obtenint concentracions entre $7.8 \cdot 10^9$ i $1.2 \cdot 10^{10}$ UFC g^{-1}. D' altra banda, l'eficàcia d'aquests dos productes formulats davant de *Monilinia* spp. va ser demostrada en préssecs, nectarines, paraguaians, cireres, albercocs i prunes.

Posteriorment, es va estimar la dinàmica poblacional de CPA-8 a la superfície de nectarines i préssecs un cop tractats amb els productes formulats de CPA-8 (BA3 i BA4) i exposats a diferents condicions ambientals. CPA-8 va demostrar una àmplia tolerància a diferents valors extrems de temperatura, humitat relativa i pluja (essent aquest últim factor el més restrictiu). La població mínima de CPA-8 obtinguda després de l'exposició va ser gairebé sempre superior a 10^4 UFC cm^{-2} de superfície del fruit, garantint així una gran cobertura del tractament. Els resultats també van indicar que els préssecs proporcionaven, generalment, un nínxol més adequat pel manteniment de CPA-8 sobre la superfície de la fruita que les nectarines. A més a més, les propietats dels dos productes formulats van influir enormement en la dinàmica poblacional de CPA-8 sobre la fruita, suggerint que el producte formulat BA4 va afavorir una major supervivència de CPA-8.

Finalment, pel desenvolupament d'ABCs comercialment funcionals, cal realitzar assajos en condicions reals de camp, incloent experiments amb el cultiu complet (i no només en fruits). Per això, l'última part d'aquesta tesi va tenir com a objectiu avaluar el potencial de diferents formulacions de CPA-8 en el control de la podridura marró en camps comercials de préssec. Específicament es va estudiar la dosi de tractament de CPA-8, la dinàmica poblacional de CPA-8 un cop aplicat en camp i l'eficàcia de CPA-8 en el control de *Monilinia* spp., tant en la collita com en la postcollita. Els resultats van demostrar que, depenent de la pressió de la malaltia

(principalment condicionada per la meteorologia) es va obtenir un grau variable de control biològic. Sota una alta incidència de *Monilinia* spp. a camp (>50 %), només el tractament químic va controlar la malaltia a postcollita (a collita ni tan sols el químic demostrà gairebé efecte). Altrament, quan la presencia del patogen s'engloba dins dels límits estàndards registrats a la zona (5.3 i 17.3 % d' incidència de *Monilinia* spp. en collita i postcollita, respectivament), els tractaments basats en formulacions de CPA-8 demostraren ser eficaços. A la collita, els productes BA3 i BA4 van reduir considerablement la incidència de *Monilinia* spp. (encara que menys que els productes químics). D'altra banda, el tractament amb el producte BA4 va reduir eficaçment la malaltia en postcollita i fins i tot estadísticament similar a les aplicacions químiques. També es va avaluar l'eficàcia de CPA-8 un cop aplicat juntament amb altres ABCs. En aquest cas es va escollir *Penicillium frequentans*, el qual ja havia estat descrit com antagonista de *Monilinia* spp. No obstant això, aquesta combinació no va augmentar l'eficàcia de CPA-8. A més a més, es va demostrar la persistència de CPA- 8 a la superfície del fruit des que s'aplica el tractament fins a la collita i posterior postcollita.

En conclusió, les dades obtingudes en aquesta tesi permeten confirmar el potencial de l'ABC *B. amyloliquefaciens* CPA-8 com una alternativa eficaç en el control de *Monilinia* spp. S'han obtingut dos productes basats en CPA-8, els quals es troben en l'última etapa del seu desenvolupament comercial, a falta de la fase de registre. La integració d'aquests productes en els sistemes de cultiu habituals pot ser una estratègia prometedora per aconseguir un major nivell de control de la podridura marró, contribuint així en el maneig de les malalties de postcollita en fruita de pinyol en el marc d'una agricultura sostenible i/o ecològica.

Theoretical Framework

Theoretical Framework

1. BROWN ROT

Brown rot in stone fruit essentially caused by *Monilinia* spp. is one of the major factors that affect quality of fruit during storage, causing important losses that could reach even as high as 80 % in years when the climate conditions are favourable for the development of the disease, especially in late-ripening varieties (Usall *et al.*, 2015). The genus *Prunus*, which has hundreds of species with some economically important members including the cultivated almond, peach, nectarine, plum, cherry, and apricot is widely affected by this fungus. *Monilinia* spp. are able to infect various plant organs, causing blossom blight, twig blight, and brown rot in immature and mature fruit, the latter being the most sensitive host phenological phase. The relatively long period of incidence (extended from bloom to postharvest), and the multiplicity of climatic factors favouring the disease spread usually result in severe, unavoidable, and even unpredictable losses in the fruit market (Lino *et al.*, 2016).

1.1 Monilinia spp., the brown rot causative agent

The causative agents of brown rot are polytrophic fungi belonging to the phylum Ascomycota, class Leotiomycotes, order Helioteliales, family Sclerotiniaceae and genus *Monilinia***.** The generic name *Monilinia* also includes those members of *Sclerotinia* that produce moniloid conidia and pseudosclerotia (Holst-Jensen *et al.*, 1998; Lino *et al.*, 2016). According to phylogenetic analysis based on rDNA sequences, the separation of the genus in two sections is consistent: junctoriae or disjunctoriae, attacking hosts members of the Rosaceae and Ericaceae families, respectively. However, partial congruence found in the branching topologies of host and pathogen phylogenies led to the suggestion of cospeciation between them (Holst-Jensen *et al.*, 1998; Lino *et al.*, 2016).

The main species that are pathogenic to stone fruit (among the extended genus *Monilinia* Honey) are *Monilinia laxa* (Aderhold & Ruhland) and *Monilinia fructicola* (G. Winter). *M. laxa* has been historically associated with European blossom blight and brown rot of fruit (Rungjindamai *et al.*, 2014). Otherwise, *M. fructicola* is described as the most widely distributed species (Fan *et al.*, 2010). In Europe, it was a quarantine pathogen until early 2014, when it was considered as a current pathogen due to its

spread across the continent, including Mediterranean countries such as Italy, France and Spain, mainly producers of stone fruit in Europe (De Cal *et al.*, 2009; Villarino *et al.*, 2013). Although *Monilinia fructigena* (Aderhold & Ruhland), has been associated with pome fruit, its occurrence in stone fruit has also been recently documented in Europe, Brazil and China (Lino *et al.*, 2016; Villarino *et al.*, 2013). A fourth specie, *Monilinia polystroma*, native from Japan, has been recently reported to occur in China, Hungary, Czech Republic and Switzerland (Petroczy & Palkovics, 2009).

Few studies have already characterized *M. laxa*, *M. fructicola* and *M. fructigena* species on agar media, describing differences on conidial size, the pigmentation of the mycelia, growth rate estimations and germ tub morphologies (Byrde & Willetts, 1977; Rungjindamai *et al.*, 2014). However, the major distinguishing characters among these species are the natural occurrence, colours, and appearance of conidial pustules on the hosts and plant parts that they infect (figure 1) (Byrde & Willetts, 1977; Lino *et al.*, 2016; Mercier *et al.*, 2009). The colour of *M. fructigena* ranges from white to light beige and it is deposited in the fruit in concentric circles with large conidiospore tufts (1.5 mm on average). *M. fructicola* is brown-coloured, medium size (1 mm on average conidiospore tufts) and presents black spots. Similarly, *M. laxa* can be distinguished by greenish-grey conidiospore tufts (<0.5 mm on average) that cover the whole infected surface. However, such differentiation may sometimes be difficult and molecular methods are needed to successfully identify these species (Hughes *et al.*, 2000). DNA techniques based on sequence analysis of the internal transcribed spacer (ITS) region from the rDNA are generally used (Rungjindamai *et al.*, 2014).

Figure 1. Peaches affected by different species of brown rot.

Theoretical Framework

The disease cycle of *Monilinia* spp. is represented in the figure 2. Primary inoculum sources in the spring are overwintering decayed fruit mummies either on the tree, which produce asexual fruiting structures (sporodochia) and spores (conidia), or on the orchard floor, which produce sexual fruiting structures (apothecia) and spores (ascospores) (Lino *et al.*, 2016). These primary infections can remain latent in the blossoms and immature fruit when the weather conditions are unfavourable, and persist as latent infections during the fruit growing season until the weather conditions become conducive to disease expression (Villarino *et al.*, 2012). Since brown rot is a polycyclic disease, secondary inoculum is of great importance in its incidence and severity within each growing season. Conidia formed on infected tissue serve thus as secondary inoculum for infection to immature and mature fruit. Moreover, climatic factors are critical for *Monilinia* spp. infection in stone fruit. Temperature and wetness duration probed to be the most relevant in the penetration, infection and latent infection of *Monilinia* spp. (Gell *et al.*, 2008; Luo & Michailides, 2001). Particularly, these conditions provide the highest potential for explosive spread of brown rot during the preharvest period.

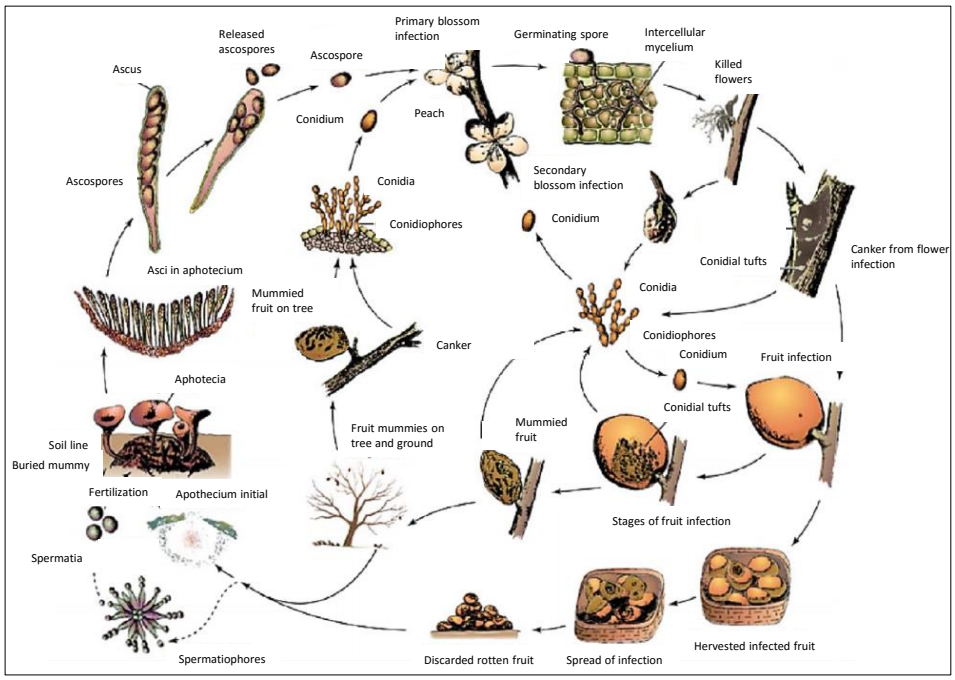

Figure 2. *Monilinia spp.* life cycle. Reprinted from Plant Pathology. Copyright 2005 (Lino *et al.*, 2016).

1.2 Brown rot control

The current strategy to control postharvest diseases caused by a large number of fungal pathogens, including brown rot caused by *Monilinia* spp., employs disease programs in the field using conventional synthetic fungicides (e.g. cyprodinil, fenhexamid, fludioxinil, boscalid and triazole-like compounds). Its use is relatively inexpensive, easy to apply and have both curative and preventive action against established and new infections, respectively (Usall *et al.*, 2016a). However, the use of chemical applications in fruit after harvesting is hardly authorized in Europe due to EU regulations. For instance, fludioxonil has been used commercially in Spain after harvest to treat stone fruit under exceptional authorization in 2012-2015 (Usall *et al.*, 2015) until its final registration in 2016. Moreover, the appearance of fungicide-resistant population of pathogens against these compounds and public concerns about health risk and environmental contamination have promoted the research over the last three decades

for alternative methods which could be widely accepted and commercially viable (Droby *et al.*, 2016; Usall *et al.*, 2015; Wisniewski *et al.*, 2016). Few examples will be given below:

Preharvest disease management

Since brown rot is a polycyclic disease, overwintering sources of inoculum can be reduced by removing diseased parts, including mummies, chancres, branches, and fruit, preferably before bloom. Moreover, the careful handling of the fruit at harvest to minimise damage and an appropriate management of irrigation to prevent trees receiving excess of water that increases fruit split are also crucial factors to reduce the risk of infections (Byrde & Willetts, 1977; Usall *et al.*, 2015).

Alternative chemical compounds

Chemical alternatives to conventional fungicides for postharvest disease control are natural or synthetic compounds with minimal toxicological effects on mammals and the environment (Palou *et al.*, 2016; Usall *et al.*, 2015). According to their origin, these alternatives have been classified as food additives (organic and inorganic salts such as calcium, potassium sorbate, sodium benzoate and peracetic acid) (Elmer *et al.*, 2007; Mari *et al.*, 2004; Palou *et al.*, 2009), volatile and essential oils including biodegradable compounds that leave minimal residues in fruit (Mari *et al.*, 2016; Sivakumar & Bautista-Baños, 2014; Spadaro & Gullino, 2014) and plant extracts (De Corato *et al.*, 2010) or natural edible coatings. Among such substances, the antifungal properties of chitosan (the deacetylated soluble form of chitin), have been reported on a variety of postharvest pathogens (Feliziani *et al.*, 2013; Romanazzi, 2010).

Postharvest physical treatments

Physical treatments have gained great interest in recent years to control many postharvest diseases because the total absence of residues in the treated product and minimal environmental impact (Usall *et al.*, 2016a). The most well-known physical treatment is the heat. It has been traditionally applied in the form of hot water dipping, hot water rinsing and brushing, vapour, hot air and curing (Casals *et al.*, 2010a; Fallik, 2004; Karabulut *et al.*, 2010). More recently, the interest in the use of the radio frequency or microwave energy to heat fruit has increased (Casals *et al.*, 2010c; Sisquella *et al.*, 2013). Other promising technologies are hypobaric and hyperbaric pressure (Thompson, 2015)

and especially far ultraviolet radiation (UV-C light) due to the direct activity against the pathogens and the induction of resistance in the host (Romanazzi *et al.*, 2016; Stevens *et al.*, 2005). In addition, cold storage and controlled and modified atmospheres are complementary physical tools to reduce or delay the development of postharvest pathogens (Tian *et al.*, 2001) although they are mainly used to maintain fruit quality after harvest. Whilst these physical treatments have shown potential to inhibit *Monilinia* spp., their use has been limited in commercial fruit production due to various factors, including injuries caused in the fruit surface, labour costs, and the time constraint during postharvest handling (Casals *et al.*, 2010c).

Induction of resistance

Fruits defend themselves from attack by pathogenic microorganisms by a wide range of strategies, including accumulation of phytoalexins, modification of the cell walls, and synthesis of antifungal hydrolases, which can be induced by both physical and biological agents that elicit resistance in harvested fruit (Usall *et al.*, 2015). For instance, phenolic compounds such as, caffeic acid and chlorogenic acid, which are abundant in the exocarp of peach fruit, did not inhibit spore germination of *M. fructicola* but inhibited appressorium formation thus reducing subsequent lesion size (Lee & Bostock, 2007).

Biological control

Of various biological approaches, the use of antagonistic microorganisms is becoming popular during the last decades. Such a naturally occurring, interference between beneficial microorganisms and plant pathogens contributes to the natural buffering of cropping systems, thus preventing or limiting disease development (Köhl *et al.*, 2011). The mechanism(s) by which microbial antagonists suppress the postharvest diseases mainly involve competition for nutrients and space, production of antibiotics, direct parasitism, and possibly, induced resistance (Droby *et al.*, 2009; Sharma *et al.*, 2009).

2. BIOLOGICAL CONTROL

The concept of biological control has been gradually incorporated into the development of alternative methods of postharvest disease management. The expanded definition of biological control was conceived by Dr. Charles Wilson -discussed in Droby *et al.* (2009) and Wisniewski *et al.* (2007)-, who defined it as "the management of a plant disease by a biological process or the product of a biological process"(Wisniewski *et al.*, 2016).

While an abundance of research articles exists describing beneficial microorganisms with effective antagonistic activity against postharvest diseases, little success of biocontrol products has been reported. The first commercial products for use by growers was introduced in the market in France in 1976 'BINAP T', (Köhl *et al.*, 2011; Ricard & Ricard, 1997). Since then, the biocontrol industry is dealing with biological products development to include them into a professional business.

Screening of new microorganisms for commercial purposes in biocontrol of plant pathogens is a complex process. Different categories of criteria are distinguished besides the antagonistic efficacy, ranging from ecological characteristics needed for good field performances to toxicological profiles, growth in bioreactors for mass production, successful formulation, protection of intellectual property rights, and marketing (Droby *et al.*, 2016; Köhl *et al.*, 2011; Usall *et al.*, 2016b). Such criteria may be considered when industries and scientific institutions are initiating new programs for biological control development. The figure 3 provides an example of the key decisions needed for selecting the most promising candidates.

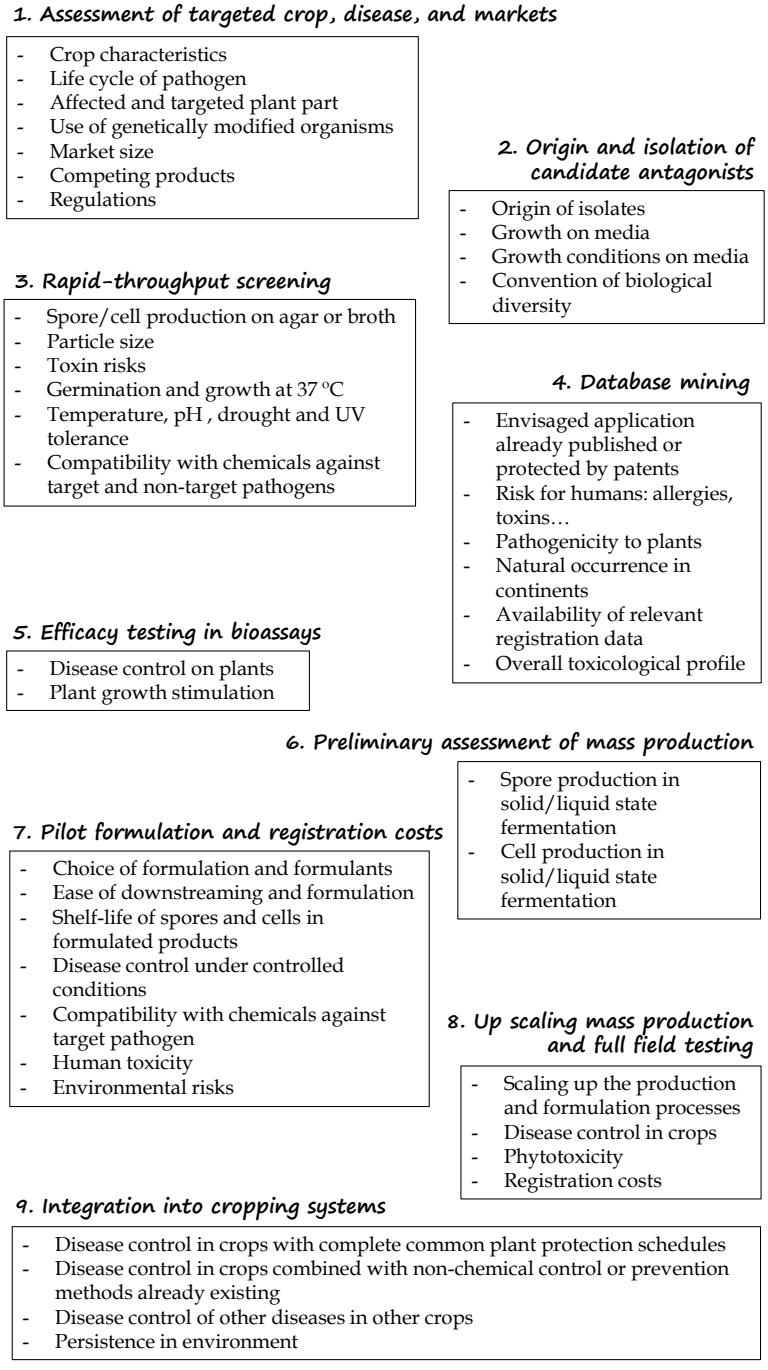

Figure 3. Stepwise screening of microorganism for commercial use in biological control. Adapted from Köhl *et al.* (2011).

Over recent years, significant progress has been made on epidemiology and management of brown rot caused by *Monilinia* spp. in stone fruit, particularly using biological control methods. However, an exhaustive research on microorganism-based product development is needed before it could be successfully implemented in the market. In general, much effort has been directed to find efficacious antagonists. However, many potential microorganisms have not been taken forward into further development due to problem in scaling-up production and formulation trials (Droby *et al.*, 2016; Teixidó *et al.*, 2011; Usall *et al.*, 2016b). Even for the few commercial products on the market, there is insufficient ecological knowledge for their effective use in fruit production. Moreover and unlike fungicides, biological control products are based on living microorganisms and hence biocontrol efficacy will be influenced by environmental conditions (Rungjindamai *et al.*, 2014). It then goes on to briefly describe some relevant considerations that should be taken into account in the development of microorganism-based products.

3. BIOLOGICAL CONTROL AGENT(s) (BCA) DEVELOPMENT

Several antagonists of postharvest pathogens (mostly yeasts and bacteria) have already been reached advanced levels of development and commercialisation. Among the first generation of biocontrol products registered and made commercially available were *Candida oleophila* (Aspire, Ecogen, Langhorne, PA, USA), *Cryptococcus albidus* (YieldPlus, Lallemand, Montreal, Canada), *Candida sake* (Candifruit, IRTA, Lleida, Spain), *Pseudomonas syringae* Van Hall (BioSave, first Ecosciences Inc., PA, USA; and later JET Harvest Solutions, Longwood, FL, USA) (Droby *et al.*, 2016; Wisniewski *et al.*, 2016) and Shemer *Metschnikowia fructicola*, firstly introduced in Israel, then acquired by Bayer CropScience, Germany; and currently pending of registration by Koppert, The Netherlands. More recently, *C. oleophila*, (Nexy, Leasafre, Lille, France), developed in Belgium, was submitted for regulatory approval in 2005 for postharvest application against wound pathogens on pome fruit, citrus, and banana (Lahlali *et al.*, 2011), receiving registration approval throughout the EU in 2013 (Massart & Jijakli, 2014). *Aureobasidium pullulans* (BoniProtect, Bio-Ferm, Tulln,

Austria) has also been suggested for using as a preharvest application to control wounding pathogens developed on pome fruit during storage (Lima *et al.*, 2015). Regarding brown rot control in stone fruit, *Bacillus subtilis* strain QST 713 (Serenade Max, Bayer CropScience, Germany) is a wettable product effective against *Monilinia* spp. currently used during preharvest applications.

As it has been mentioned before, the low success rate of postharvest biocontrol products has been attributed to several problems, mainly evidenced because of its inconsistent performance once applied under commercial conditions (Droby *et al.*, 2016; Usall *et al.*, 2016b). In this regard, different approaches should be developed taken into account all the components of the biocontrol system. Information on the BCA biology and ecology, mode of action, mass production and formulation, and population dynamics of the applied microorganism may be useful to develop a new strategy to achieve higher levels of disease control and to contribute to a successful handling of postharvest diseases.

3.1. BCA characterisation

Before BCAs can be commercialised, the microorganisms need to be accurately identified at the specie and strain levels (Alabouvette & Cordier, 2011). Monitoring methods can be grouped into cultivation-based and DNA-based techniques. The first technique consists of growing the colonies on a selective or semi-selective medium. However, this classic microbiological methodology generally lacks specificity, hence the BCA could not be distinguished from non-target microorganisms present in the natural microbiota (Montesinos, 2003; Nunes *et al.*, 2008). The availability of more specific DNA-based techniques has greatly facilitated the surveying and identification of candidate organisms, providing valuable data for identification purposes (Droby *et al.*, 2009). They are based on the amplification of gene sequences by means of the polymerase chain reaction (PCR; e.g. RAPD, REP, ERIC, AFLP) or by means of DNA digestion with restriction enzymes (RFLP). SCAR markers have been commonly used for developing monitoring methods for BCAs because they are natural sequences present in the genome which allow a simple specific detection and do not require any prior knowledge of the strain genome (Pujol *et al.*, 2005). Carrying out RAPD and then designing more repeatable SCAR markers is suggested as a

necessary approach that will enable the BCA to be traced and distinguished from other strains of the same specie (Alabouvette & Cordier, 2011; Nunes *et al.*, 2008; Schena *et al.*, 2000). However, recent studies have revealed that many strains belonging to complex genus with genetically similar species can be easily confused (Fritze, 2004; Reva *et al.*, 2004). In this case, sequencing housekeeping genes has proven to be useful for taxonomic classification and therefore, useful for molecular marker design. These genes are essential and therefore are not lost from genome, but evolve more quickly than 16S rDNA, which often offers insufficient or unsatisfactory taxonomical information within related species (Fritze, 2004; Maughan & Van der Auwera, 2011). Currently, the emergence of new techniques that can quantify nucleic acids *in vitro* (qPCR) using different fluorometric detection systems (such as the DNA-binding dye SYBR Green or the TaqMan hydrolysis probe) proved to be more specific and sensitive, thus providing a fast and accurate tool for the detection and quantification of the BCAs (Soto-Muñoz *et al.*, 2014).

In order to deeply characterise the desired microorganisms, a database mining regarding the safety and ecology (temperature, pH, water availability (a_w) etc.) of each candidate should be conducted. Findings on the most favourable environmental conditions in which the BCA is able to grow are particularly relevant for predicting biocontrol responses. Moreover, a toxicological profile concerning the risks for humans and the plant pathogenicity of the microorganism is considered crucial to thus preferring species and strains expected to fulfil the criteria of low-risk substances (Köhl *et al.*, 2011).

3.2. Mode of action

It is generally considered that there are three main ways for a BCA to control a plant pathogen (Alabouvette *et al.*, 2009; Bardin *et al.*, 2015; Jacobsen, 2006): first by acting directly on the plant pathogen, through antibiosis, competition for nutrients or space, or parasitism; secondly, by interfering with the mechanisms of pathogenesis of the plant pathogen; and thirdly by modifying the interaction of the plant pathogen through the induction of local or systemic resistance. Sometimes, different modes act simultaneously and it is therefore difficult to establish which individual mechanism has contributed to a specific antagonistic function (Di Francesco *et al.*, 2016). To clarify

the mechanism of action, as well as the understanding of biocontrol systems, it is crucial to know the interactions among environment, pathogen, and the BCA (Parafati *et al.*, 2015).

Understanding the mode of action facilitates the assessment of some requirements related to further steps on the BCA development. As more information is accumulated, it may be possible to express desirable biocontrol traits by manipulating production and formulation processes (Fravel *et al.*, 1998; Jones & Burges, 1998).

3.3. Mass production

For the commercial development of a BCA, the microorganism should be mass-produced. In general, and depending on the agent's nature (bacteria, filamentous fungi, yeast, nematodes or viruses), the methods used for industrial scale-up are solid- or liquid- phase fermentation, which take profit from the advanced technology in the pharmaceutical and food industries. Bacteria and yeasts are usually produced by liquid fermentation using continuously stirred tank bioreactors, but many fungi are fermented in a solid state (Teixidó *et al.*, 2011). The table 1 compares the advantages and disadvantages of these methods of mass production, using either, liquid or solid substrates.

Table 1. Advantages and disadvantages of solid and liquid BCAs production (Jones & Burges, 1998).

	Advantages	**Disadvantages**
Solid	Low capital cost Small scale Simple formulation	Difficult scaling up Control is complex Can be difficult to make addition during the process
Liquid	High density Simple to control: pH, T, O_2, etc. Simple to add nutrients	Need to harvest and formulate Can be difficult to get correct forms, e.g. spores Capital cost might be high

Independently of the method used for mass-production, the aim is to achieve the highest yield possible with the lowest cost of culture medium. To economically

produce large quantities of microorganisms, the culture medium requires the use of commercial products and by-products (such as, molasses, flours, and whey) combined with specific components like mineral trace supplements. Moreover, an exhaustive research is needed to optimise the growth conditions (temperature, agitation, aeration and pH) that ensures a high, stable and effective microbial population prior to the formulation process (Hynes & Boyetchko, 2006). The figure 4 below illustrates the large-scale production of microorganisms in both, liquid and solid state.

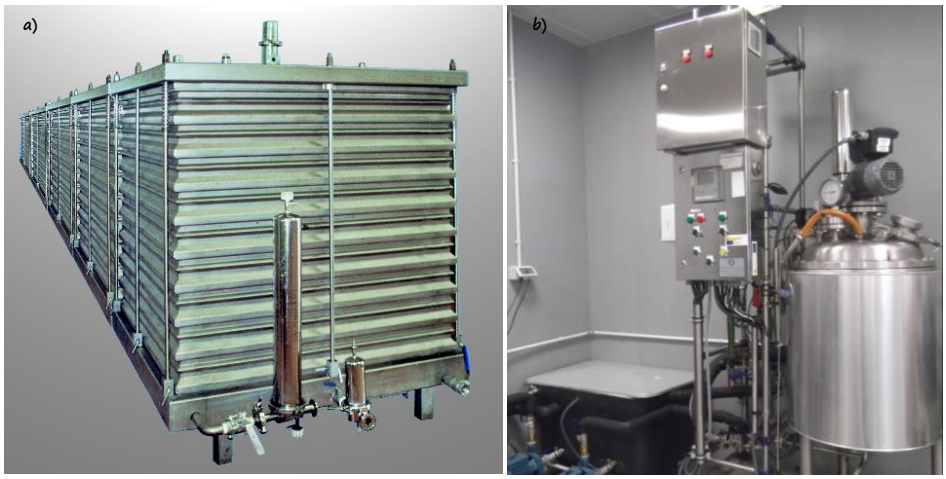

Figure 4. Pilot plant equipment commonly used in producing BCAs in a) solid (Bayer CropSience, Germany) and b) liquid state.

Once the microorganism has been successfully produced, downstream processing protocols have to be designed in order to separate the biomass from the spent medium. Depending on the BCA, the procedure will be focused on obtaining cells (or spores), the supernatant with the secreted metabolites or even both. Such processes include filtration (pressure filtration o rotary vacuum drum filtration), centrifugation or, in some cases, flocculation (Teixidó *et al.*, 2011).

3.4. Formulation

Independently of the method used for the production of microbial cells, the final product should be formulated before using. Formulation affects many aspects of the success of a BCA, including the shelf-life of the final product and the ability of the microorganism to proliferate and survive in the intended environment. Moreover, it should maintain biocontrol efficacy similarly to fresh cells and must be both convenient to use and safe to handle (Fravel *et al.*, 1998; Teixidó *et al.*, 2011). Beyond these considerations, there are several other desirable characteristics of a formulation such as, compatibility with agricultural machinery, ease of integration into a pest management system and cost to produce and purchase (Fravel *et al.*, 1998). Due to the above mentioned, the formulation process is often considered as the major bottleneck in the commercialisation of BCAs (Droby *et al.*, 2016; Navarta *et al.*, 2011; Usall *et al.*, 2016b).

Formulation technology must be considered at all stages from production of an organism to its eventual action on the target. There is a wide variety of formulation types, both liquid and solid. The main types currently used for organisms have been classified by Jones & Burges (1998) and Rhodes (1993) into dry products (dusts, granules and briquettes) and suspensions (oil or water-based and emulsions). Liquid formulations often consist of separating the biomass from the growth media and then suspending it in an aqueous medium containing stabilisers, surfactants, colorants or additional nutrients to extend the viability of the product as much as possible (Teixidó *et al.*, 2011).

Compared to liquid forms, dried products obtained by spray-drying, drying with fluidised-bed dryers or freeze and vacuum-drying are more feasible due to the storage capability, transportation and their ability to produce large amounts of dried product at low cost. However, dried products frequently show low viability rates because of the thermal and dehydration stress suffered during the drying process (Abadias *et al.*, 2005; Melin *et al.*, 2007).

Nevertheless, the microbial cell viability and efficacy resulting after the formulation process can be improved by the addition of certain stabilising/protecting substances (e.g.,

polymers, sugars, albumin, milk, salts, honey, polyols or aminoacids) to the formulation medium. Components of the formulation media have two main functions in preserving the viability of formulated cells: to provide a dry residue and thus acting as a receptor in the rehydration process and also to protect biochemically the cells against damage during the drying process (Abadias *et al.*, 2001; Melin *et al.*, 2011; Sabuquillo *et al.*, 2010). Some of these products also help the microorganism to survive under exposure to variable and frequently hostile environmental conditions such as ultraviolet light (UV), low water potential, nutrient limitation, extreme temperatures, and rainfall (Cañamás *et al.*, 2008a; Köhl & Fokkema, 1998; Lahlali *et al.*, 2008). Eventually, since beneficial organisms are regarded as environmentally friendly, it is desirable that any formulation additive also maintains such characteristics.

A general recipe does not exist and different methods are established empirically for each strain. Critical formulation requirements are determined by features of the organisms themselves and of their environments. An overriding feature is the mode of action (it dictates the formulator's ultimate objectives) together with the needs of the environments to which the organism are applied and the purposes for which they are used (Burges & Jones, 1998a). The concept, as well as some concerns and interests of the technologies most commonly used for drying biocontrol microorganisms, are briefly described below. In the figure 5, the laboratory scale equipment mostly used in formulating BCAs is shown.

Spray-drying

The industrial scale of spray-drying dates back to the 1920s, when it was firstly used to produce powdered milk and washing powder. Since then, its use has been widely disseminated and applied in the food, pharmaceutical and chemical industries (Bhandari *et al.*, 2008; Costa *et al.*, 2015). This process consists in transforming a product from fluid to a solid state in the form of powder by the removal of the moisture from the liquid droplets. Inside the chamber, the low-humidity hot air is mixed with the dispersed droplets, mainly produced by the rotary wheel/disc atomisers, pressure nozzle or pneumatic-type atomiser. Then, the moisture, in the form of vapour, quickly evaporates from the suspended droplets due to simultaneous and fast heat and mass transfer processes. Drying of the droplets continues inside the drying chamber until the desired particle characteristics are

achieved. Finally, the separation of the dried particles from the drying air and their subsequent collection take place in external equipment such as cyclones and/or bag-filtre houses (Bhandari *et al.*, 2008; Costa *et al.*, 2015).

Formulation of many BCAs has been reported by using spray-drying. However, the procedure of this technology involves poor survival rates in many microorganisms (but those capable to produce endospores such as the genus *Bacillus*), due to the elevated temperatures applied.

Freeze-drying

Freeze-drying (or lyophilisation), can be traced back toward the end of the 1880s, when the process was used on a laboratory scale and to the 1930s when it developed sufficiently to be used in food and pharmaceutical industries. Operationally, we could define freeze-drying as a controllable method of dehydrating labile products by vacuum desiccation. Briefly, the liquid sampled must be cooled until the final conversion of the freezable solution water into ice, the crystallisation of the solutes, and the formation of an amorphous matrix comprising the non-crystallising solutes. Then, the ice is sublimated under vacuum controlled conditions and the water is evaporated from the amorphous matrix. Finally, the moisture content of the product is desorpted (Adams, 2007).

While this technology is being widely used in formulating BCAs, it involves severe damages in the microorganisms during the whole process. For avoiding the undesirable effects, such as denaturation of proteins and decrease in cell viability of many cell types, some cryoprotecting agents have been added to the medium. Moreover, this process is highly time-consuming which means elevated costs in massive production (Strasser *et al.*, 2009).

Fluid-bed drying

The fluidised-bed process was primarily developed in the 1950s by the pharmaceutical industry. Other process industries, including agrochemicals, have adopted this technology to utilise its advantages in preservation and product formulation. Fluidised-bed technology is traditionally used for drying, granulation and

coating of powders, granules, and spheres (Strasser, 2008). It is based on the intense interactions between particles. Filtered and optionally dehumidified inlet air enters the product through a perforated plate, thereby compensating the gravitational force and fluidising the product previously loaded or sucked into the granulator. Such product, however, requires to be previously extruded. Small particles carried by the air flow to the cylindrical expansion chamber are held back by filtres and then falling back into the conical product chamber. This cycle continues through the process, primarily ruled by variation of the product temperature and fluidisation air flow (Strasser, 2008).

Fluid-bed spray-drying

This concept is based on the similarity between spray-drying and the fluid-bed process. Both methods atomise liquid droplets into a chamber of hot air to facilitate drying. By operating with a large air volume, liquid can be sprayed in the chamber at an inlet air temperature much lower than that used for traditional drying systems. This equipment allows to obtain larger quantities of dry product with better dispersibility and flowability and a narrow particle-size distribution (Srivastava & Mishra, 2010). Furthermore, comparing to conventional fluidised-bed systems, this technology does not need previous extrusion and pelletisation of the sample, thus offering lower operating costs and shorter process times (Santivarangkna *et al.*, 2007; Strasser *et al.*, 2009). The suitability of the fluid-bed spray-drying makes this technology an attractive alternative to freeze-drying, spray drying or traditional fluidised-bed drying systems.

Figure 5. Laboratory scale equipment commonly used in formulating BCAs. The aspect of the final product obtained with each system is shown in the corresponding picture's corner.

Formulation not only has to be focused on the drying process, but it also must consider several problems and challenges. Most of them are summarised in the table 2.

Table 2. Problems faced in the formulation process (Jones & Burges, 1998).

Stage	Function
Harvest	Reduction of material bulk Division into particles able to pass spray nozzles
Stabilisation	Prevent growth of agent and contaminant microorganisms Prevent proteases denaturing the active ingredient
Storage	Avoid powders caking due to moisture uptake Control viscosity of liquids to keep particles in suspension without aggregation so that they flow Retain viability
Application	Ensure good performance in applicators for dusts, powders or granules Maintain appropriate viscosity of liquids to form spray droplets Ensure good performance of sprayers without making foam
Post-application	Ensure good coverage of target and good product retention Reduce physical loss by rain or other means Protect agents from inactivating factors, e.g. sun Ensure deposit is palatable and preferably attractive to target pest

It is essential that a formulated product retains their genetic stability, cell viability, attributes as colonisers on fruit surfaces, as well as other aspects of their mechanism of action. Moreover, one of the most important issues involved in the commercial development of biocontrol products is the shelf-life, which should preferably be extended up to two years (Droby *et al.*, 2016; Teixidó *et al.*, 2011; Usall *et al.*, 2016b). Studies regarding the use of different types of packaging (different materials, atmosphere conditions, and storage temperatures) should be carried out to enhance the preservation of the microorganism' viability along this time (Torres *et al.*, 2014).

Organisms are required to remain viable during storage, with minimum loss of potency/activity and without loss of breakdown of the desired formulation properties. Improved stability can, however, be achieved by treatment before formulation. For example, the growth conditions during production could affect in the spore-forming ability of many microorganisms, which allow to obtain products with long-term storability (Collins & Jacobsen, 2003; Nguyen Thi Minh *et al.*, 2011).

In addition or alternatively, additives are included to improve stability. Storage of powders is improved by low moisture content. Dry products have to be kept dried during storage, needing a moisture-proof packaging. Even with dry storage of spores, the microorganism do not become totally inert as respiration does not completely shut down. However, it influences the physiological state of the organism and hence their durability (Burges & Jones, 1998b). Moreover, such additives should ensure that the product is easy to handle and apply. Application equipment is designed to maximise the efficacy of a product through accurate and safe delivery. Regardless of the formulation system, including liquid and dried forms, it is critical to ensure that the correct amount of active ingredient reaches the target. Additives also influence features such as viscosity and the wetting ability of the suspension. Therefore, the choice of thickeners, humectants and wetting agents should be made in the context of the formulation as a whole (Cañamás *et al.*, 2008a; Sui *et al.*, 2015).

3.5. Range of activity

The narrow range of activity (hosts and pathogens) of many BCAs is a serious limitation to their commercial success (Droby *et al.*, 2016; Usall *et al.*, 2016b). Pilot trials are regularly performed under conditions as close as possible to the real conditions under which biological control will be developed in practice. However, it should be convenient to perform those trials comprising several pathogens or crop systems in order to demonstrate the spectrum of action and environmental conditions diverse enough to guarantee a wide range of applicability, which is the most attractive property for the industry (Glare *et al.*, 2012).

3.6 Performance and consistence under field conditions

Many microorganisms with good potential fail to be developed for practical use due to inconsistent performance associated with adverse environmental conditions. Moreover, the concentration of the antagonist used, timing and method of application are also crucial to determine the success of an antagonist in competing with other microorganisms (Guijarro *et al.*, 2007). Therefore, application and optimisation of biocontrol agents before harvest requires considerable understanding of the crop

system, pathogen epidemiology and the biology and ecology of the BCA (Andrews, 1992; Guijarro *et al.*, 2007).

An organism may remain active for a time after application, ideally through the period that a pest is likely to attack the crop or in soil through the crop cycle. Microbes are inactivated by several environmental factors including sun, high temperature, low humidity or dehydration, leaf surface exudates, and competitors. Also they may be lost from the target location by the action of wind, rain or leaching (Jones & Burges, 1998). For this reason, formulations normally contain additives to protect agents from ravages of the environment. Losses in the BCA's viability vary with the type and properties of the formulation, the effects of crop-size and liquid properties on spray retention. Similarly, particle size in dry solid formulations also influences retention: fine dust readily adhere to leaf surfaces and larger and heavy solid formulations will not be easily washed away with the moving water (Jones & Burges, 1998).

As it was mentioned while describing the formulation process (section 3.4), the adequate use of adjuvants can improve the overall coverture of the target and thereby ensuring better biological efficacy of the applied products (Cañamás *et al.*, 2008a; Hunsche *et al.*, 2011). For example, wetters would reduce spray retention if the volume applied results in run off, acting as detergents during rainfall, and decreasing wash-off of the organism.

The approach to biocontrol research has evolved toward being more ecologically holistic and more oriented toward both production strategies and industry's concerns. However, more research is needed in integrating BCAs into cropping systems such as in rotating biocontrol with chemical pesticides and in considering these into forecast models to choose whether to apply a chemical pesticide or biocontrol (Fravel, 2005).

3.7. Commercialisation and costs

The earliest planning on the investigation of an organism must anticipate the ultimate objective, meeting as much as possible both, the scientific and commercial requirements (Burges & Jones, 1998b). The main reasons for dropouts at the prototype and early marketing stages have been: market too small, deterioration in storage, growth of contaminants, poor or inconsistent field results, difficulty of application,

and lack of user education. Moreover, another important reason for products becoming uncompetitive is high cost, which largely depends on the competitive manufacturing of the fully scaled-up (Burges & Jones, 1998b; Droby *et al*., 2016).

A major expense is related to registration procedures, which must be conducted on the final formulated product. In Europe, the cost of registration could be around 1-1.5 million euros, which involves different measures, depending on the country (Usall *et al*., 2016b). Apart from a complete characterisation of the organism under study, the European Commission (EC) regulation 1107/2009 establishes issues that need to be addressed, including the estimation of the fate and distribution of the microorganism in the environment and its impact on non-target species. This information is crucially important to understand its interaction(s) within the environment and hence design meticulous protocols for ecological and safety assessments (Soto-Muñoz *et al*., 2014).

4. THE BCA *Bacillus amyloliquefaciens* CPA-8

The strain CPA-8 used in this study was obtained from the Postharvest Pathology Group Collection of IRTA Centre (Lleida, Catalonia, Spain). It was isolated from the surface of a nectarine fruit in an experimental orchard in Lleida (Catalonia, Spain) and selected for its preliminary efficacy in reducing brown rot caused by *Monilinia* spp. in stone fruit (Casals *et al*., 2010b; Yánez-Mendizábal *et al*., 2011). CPA- 8 was firstly identified by 16S rDNA partial analysis by the Netherland Culture Collection of Bacteria as a member of the *B. subtilis* species complex. The CPA-8's morphologies grown in NYDA (Nutrient Yeast Dextrose Agar) and observed by microscopy are shown in the figure 6 below.

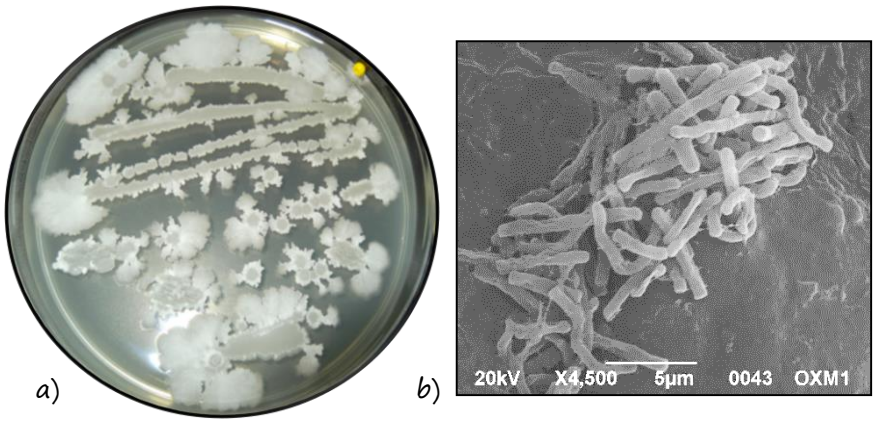

Figure 6. *B. amyloliquefaciens* CPA-8 (before *B. subtilis*) cultured on NYDA (Nutrient Yeast Dextrose Agar) medium at 30 ºC for 24 h (a) and observed by Field Emission Microscopy (FEM) on a nectarine's surface (b).

Bacillus is a diverse bacterial genus characterised by Gram-positive, aerobic or facultative anaerobic bacteria forming dormant ellipsoidal endospores (Fritze, 2004). They are mesophilic and neutrophilic bacteria, regarding temperature and pH, respectively (Mora, 2013). This genus was described for the first time in 1872 by F. Cohn and belongs to the phylum Firmicute, class Bacilli, order Bacillales, family Bacillaceae.

From an ecological point of view, it is a wide distributed genus due to its ubiquity in plants, animals, soil, air, and water (Maughan & Van der Auwera, 2011). *Bacillus* spp. are involved in many chemical processes, which makes it one of the most diverse and commercially useful groups of bacteria (Harwood, 1989; Mora, 2013). The industrial importance of this genus, which deal with the ability to secrete quantities of industrially relevant substances, has promoted the research interest in sequencing the genome of many *Bacillus* strains.

With the development of more precise methods for bacterial characterisation, the main studied *Bacillus* species are included in two groups: *B. subtilis sensu lato* group (which refers to closely species such as *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. atropheaeus*, *B. mojavensis*, and *B. amyloliquefacines*) and *B. cereus sensu lato* group (which includes *B. cereus*, *B. thuringiensis*, *B. mycoides*, and *B. anthracis*) (Fritze, 2004; Hossain *et al*., 2015; Maughan & Van der Auwera, 2011). Recent studies have revealed

that many strains belonging to the first group are phenotypically and genetically very similar and can be easily confused (Fritze, 2004; Reva *et al.*, 2004). The figure 7 illustrates the continuous taxonomic classification within the *B. subtilis* group (Fritze, 2004). The taxonomic development of this group over time reflects the difficulties occurred regarding the identification of these microorganisms. It is noteworthy that *B. amyloliquefaciens* was not classified as a separated specie until the late 1980s, although Welker & Campbell (1967) already reported the unrelatedness between *B. subtilis* and *B. amyloliquefaciens* early in 1967.

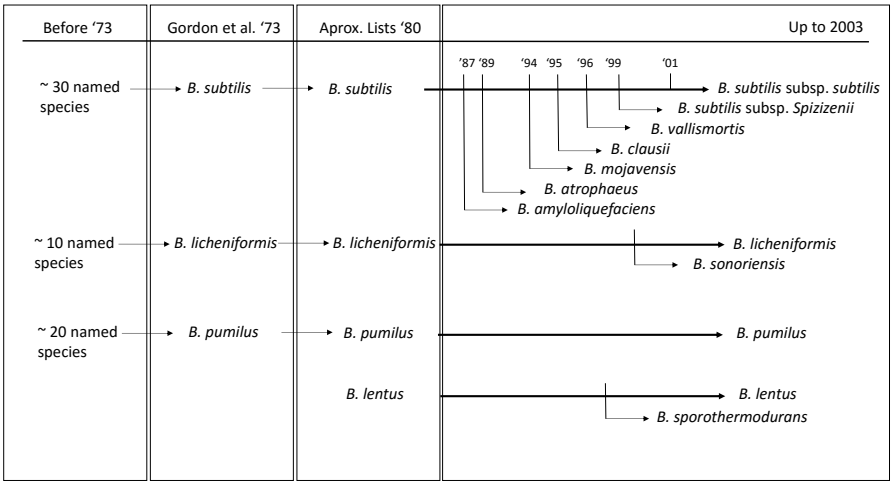

Figure 7. Taxonomic development in the *B. subtilis* group. Adapted from Fritze (2004).

In the last ten years, several *Bacillus* strains of different species have been sequenced, including *B. amyloliquefaciens* FZB42, type strain of *B. amyloliquefaciens* subsp. *plantarum* (Chen *et al.*, 2007; Chen *et al.*, 2009). This feat offers the opportunity to deeply investigate into the characterisation and identification of many bacterial processes such as the sporulation system and the ability of the bacterium to synthesise and secrete industrially important enzymes and antibiotics.

Theoretical Framework

4.1. Production of endospores

The spore-forming ability of many species belonging to the genus *Bacillus*, has received growing attention because of the industrial potential in the development of BCAs. The spore formation is thought to be a strategy for survival when the bacteria are subjected to nutrient depletion or stressing factors (Fritze, 2004). They form intracellular endospores, although they are eventually released as free spores. These structures confer exceptional ecological advantages and allow long-term storage and relatively easy development of *Bacillus*-based products (Collins & Jacobsen, 2003). As they provide tolerance against adverse environmental conditions including heat, desiccation and ionic strength (Nguyen Thi Minh *et al*., 2011), the spores are considered very amenable to drying methods. Thereby, the endospore production is a crucial factor that needs to be considered during the production process of the *Bacillus*-based BCAs.

Bacillus spp. use a system of quorum-sensing to initiate the sporulation process, that occurs in high cell density populations (during the stationary growth phase when nutrients are exhausted) (Posada-Uribe *et al*., 2015). Each particular strain presents its own nutritional requirements, so the optimum culture media and culture conditions to an appropriate endospore production need to be specifically optimised.

Yánez-Mendizábal *et al*. (2011) and Yánez-Mendizábal *et al*. (2012a) demonstrated the capability of CPA-8 to produce endospores as a means of surviving temperatures higher than 80 ^{o}C (Baril *et al*., 2012; Nguyen Thi Minh *et al*., 2011). This capability largely depends on the culture age and is particularly relevant for the BCA formulation process (especially in drying systems that use high temperatures) and also to achieve effective control under field applications.

4.2. Mode of action

Bacillus strains exhibit various cytological traits to compete for niches (Nihorimbere *et al*., 2011). Several mechanisms of action under investigation include the production of hydrolytic enzymes, competition for nutrients and space (such as production of siderophores), emission of volatile organic compounds (VOCs) and the

induction of reactive oxygen species (ROS) in the host (Asari *et al.*, 2016; Elshakh *et al.*, 2016; Zheng *et al.*, 2013).

However, members of the *Bacilllus* genus are often considered microbial factories for the production of a vast array of biologically active molecules potentially inhibitory for the phytopathogen growth (Ongena & Jacques, 2008). The three families of lipopeptides –surfactins, iturins and fengycins- play a key role not only as antagonist of plant pathogens (including bacteria, fungi and oomycetes), but also by stimulating host defence mechanisms (Ongena & Jacques, 2008). These non-ribosomal peptides differ in structural traits and physicochemical properties and mainly act as surfactants (involved in biofilm formation and bacterial motility) and permeabilisers or disruptors of the pathogen's membranes (Cawoy *et al.*, 2015).

By comparison with the reference *B. subtilis* strains UMAF6614 and UMAF6639, fengycin, iturin and surfactin lipopeptides were identified by thin layer chromatography in butanolic extracts from cell-free CPA-8 supernatants (Yánez-Mendizábal *et al.*, 2012d). These results were definitely supported by mutagenesis analysis targeted to supress fengycin biosynthesis by disruption of the *fenB* gene. Fruit trials confirmed that fengycin-defective mutants and their cell-free supernatants lost their ability to control peach brown rot disease in comparison with CPA-8 wild type (Yánez-Mendizábal *et al.*, 2012d). Therefore, these results strongly suggested that the production of such antifungal substances could be a major factor involved in the biocontrol activity of CPA-8.

4.3. Production and formulation

Economical production of microorganisms in a culture medium requires intensive investigation to determine the optimum conditions that ensures a large, stable, and effective microbial population prior to the formulation process (Hynes & Boyetchko, 2006). The work conducted by Yánez-Mendizábal *et al.* (2012c) showed that the use of commercial products and by-products such as Defatted Soy Flour (DSF) in combination with molasses and mineral trace supplements provided high CPA-8 growth while maintaining its biocontrol efficacy.

Regarding CPA-8 formulation, shelf-stable and effective formulates were obtained by spray-drying using different combinations of skimmed milk and $MgSO_4$ as protectants (Yánez-Mendizábal *et al.*, 2012b). These compounds were added before drying to thus obtaining CPA-8 cells by forming a crystalline powder with low water content. Moreover, the spore-forming ability of CPA-8, which provides heat tolerance to the cells, results a crucial factor on CPA-8 survival after spray-drying, technology that usually involves temperatures higher than 100 ºC (Yánez-Mendizábal *et al.*, 2012a).

Although Yánez-Mendizábal *et al.* (2012a) recently reported spray-dried CPA-8 products with final concentrations between 1.6 and 3.3 10^9 CFU g^{-1} and 28-38 % of powder recovery using a pilot spray-dryer without preconditioning, other aspects of CPA-8 formulation could be assessed and implemented to meet the requirements of a high quality product. The study of alternative formulation techniques, liquid or solid, amended or not with protecting agents can contribute to recover higher rates of product with final product concentrations considered acceptable for practical applications.

4.4. Biocontrol efficacy against *Monilinia* spp.

Although CPA-8 formulations provide good quality requirements, it is noteworthy that biocontrol efficacy needs to be retained as one of the most important requisites for commercial purposes.

Many detailed studies have demonstrated the efficacy of the BCA CPA-8 in controlling brown rot disease caused by *Monilinia* spp., either used alone (Yánez-Mendizábal *et al.*, 2011) or in combination with environment-friendly techniques, such as hot water and curing (Casals *et al*. 2012, 2010b). Moreover, the biocontrol ability of this bacterium has been reported not only gainst *Monilinia* spp. in stone fruit ('Baby Gold 9' peaches, 'Andros' peaches and 'Big Top' nectarines) but also against *Botrytis cinerea* in 'Golden Delicious' apples, thus demonstrating the potential antagonistic activity of CPA-8 against different postharvest pathogens (Yánez-Mendizábal *et al.*, 2011).

However, further commercial tests with optimised CPA-8 formulations under packinghouse and field conditions should be the next research step. Frequently, small-

scale experiments may change under a natural environment. An antagonist applied in the field is frequently subjected to severe environmental conditions that may drastically limit BCAs establishment on a host target site (Cañamás *et al.*, 2008b). The step wise screening of microorganisms for commercial use in biological control needs full field testing (including disease control in crops with complete common plant protection schedules) and a well-designed disease program that enables the integration of BCAs into cropping systems (Köhl *et al.*, 2011). Disease control could be achieved by combining BCAs with non-chemical control or prevention methods already existing or under development (Köhl *et al.*, 2011). Therefore, continued research in biocontrol is needed to contribute to the movement toward sustainable agriculture and to ensure available alternatives.

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OBJECTIVES

Objectives

The **main objective** of this thesis is to complete de development of the biocontrol agent *Bacillus amyloliquefaciens* CPA-8 to obtain microorganism-based products that provide an effective strategy to control brown rot in stone fruit.

Specifically, this thesis aimed:

❖ **To characterise the BCA *B. amyloliquefaciens* CPA-8 in order to meet European Commission (EC) regulation 1107/2009 requirements and to develop products suitable to be registered and commercialised**.

- To develop molecular markers to detect and identify CPA-8
- To complete the knowledge on biological and physical properties of CPA-8: to generate new data on growth temperature, water availability (water activity, a_w) and pH, susceptibility to antibiotics, and production of enterotoxic substances
- To investigate the antifungal effect of the volatile organic compounds (VOCs) produced by CPA-8 against postharvest fruit pathogens as alternative mechanism of action

❖ **To improve the production and to optimise the formulation of the BCA *B. amyloliquefaciens* CPA-8 to obtain commercially feasible scaling-up and downstream processing protocols.**

- To improve the conditions to produce CPA-8 under laboratory-scale bioreactors: growth medium, yield process, and evaluation of the optimum harvesting conditions
- To determinate the best formulation system for CPA-8
- To determine the role of some additives (protectants and carriers) in order to improve the viability, shelf-life, and efficacy of CPA-8

❖ **To define and develop new control strategies based on the BCA *B. amyloliquefaciens* CPA-8.**

- To broaden the range of action of CPA-8 to different hosts susceptible to brown rot
- To determine the technical application thresholds of the CPA-8 products developed to survive under practical conditions: tolerance to temperature, desiccation, and rain wash
- To define the best strategy to control brown rot in stone fruit under commercial conditions: determining doses, application times and efficacy

Structure & Methodology

In order to accomplish the objectives proposed, this thesis has been splitted into seven chapters, each one presented as an original research paper. The figure 1 represents the structure of the whole Ph.D. research, briefly detailing the contents of each part (numbered in black). Such numbers, however, do not always correspond with the number of the chapter. According to this distribution, most of the methodologies conducted in the trials have been highlighted below. Moreover, the most representative equipment and data regarding each part, are also represented in the figures 2-8.

1. **Molecular markers' design** (Figure 2)**.**

   To monitor CPA-8 under field conditions, two molecular markers have been developed which identify CPA-8 at the strain level: (i) a SCAR marker using the RAPD methodology and (ii) a strain-specific genomic marker related to ecological adaptations.

2. **Biology & physiology** (Figure 3)**.**

   In order to complete registration requirements for developing a microorganism-based commercial product, better understanding of the CPA-8's biology and physiology has been achieved. Specifically, (i) the effect of the key ecological parameters of temperature, water activity (a_w) and pH on growth rate and lag phase of CPA-8, (ii) the susceptibility/resistance of CPA-8 to different antibiotics at different concentrations under *in vitro* conditions and the presence of *Bacillus cereus* enterotoxic genes in CPA-8 have been evaluated.

3. **Mode of action** (Figure 4)**.**

   The antifungal effect of the volatile organic compounds (VOCs) produced by CPA-8 against three postharvest fruit pathogens on cherries has been investigated. Specifically, (i) an *in vitro* approach was used to evaluate the antifungal effect of CPA-8 on colony and mycelial growth of *Monilinia laxa*, *Monilinia fructicola* and *Botrytis cinerea*; (ii) compounds emitted by CPA-8 were identified by using the SPME-gas chromatographic technique, (iii) the effect of pure compounds on target pathogens was tested *in vitro* and (iv) the antifungal activity of CPA-8 VOCs and pure thiophene was assayed on cherries artificially inoculated with *M. laxa*, *M. fructicola* and *B. cinerea*.

4. **Production process** (Figure 5)**.**

   In order to meet the quality requirements of a CPA-8-based product, three different nitrogen sources were evaluated to improve the low cost medium for CPA-8 production: the boiled extract from Defatted Soy Flour and protein PROSTAR 510A at 10 and 20 g L^{-1}. Once the best medium was selected, the endospore production of CPA-8 under different culture ages was assessed.

5. **Formulation process** (Figure 6)**.**

   Different formulation systems to formulate CPA-8 have been assessed: liquid, freeze-drying and fluid-bed spray-drying. Once the best drying process was defined for CPA-8 formulation, different processing parameters to obtain high performance under commercial conditions were optimised, focusing on the effect of protectants and carrier materials. Moreover, the residual moisture content, the shelf-life, and the effect of the storage temperature were evaluated.

6. **Biocontrol activity in diverse hosts and tolerance to abiotic factors** (Figure 7)**.**

   The biocontrol activity of the CPA-8 formulations against *M. laxa* and *M. fructicola* was tested on different stone fruit including peaches, nectarines, flat peaches, cherries, apricots, and plums. Moreover, the population dynamics of CPA-8 on the surface of nectarines and peaches after being exposed to unfavourable environmental conditions (temperature, relative humidity and simulated rainfall) was described.

7. **Commercial trials** (Figure 8)**.**

   The potential of different formulations of CPA-8 for brown rot control in commercial orchards was assessed as part of a disease program design that enables the integration of BCAs into cropping systems. Specifically, (i) the CPA-8 dose of treatment, (ii) the CPA-8 population dynamics once applied in the orchard and (iii) the CPA-8 efficacy in controlling *Monilinia* spp. incidence at harvest and postharvest time, were evaluated.

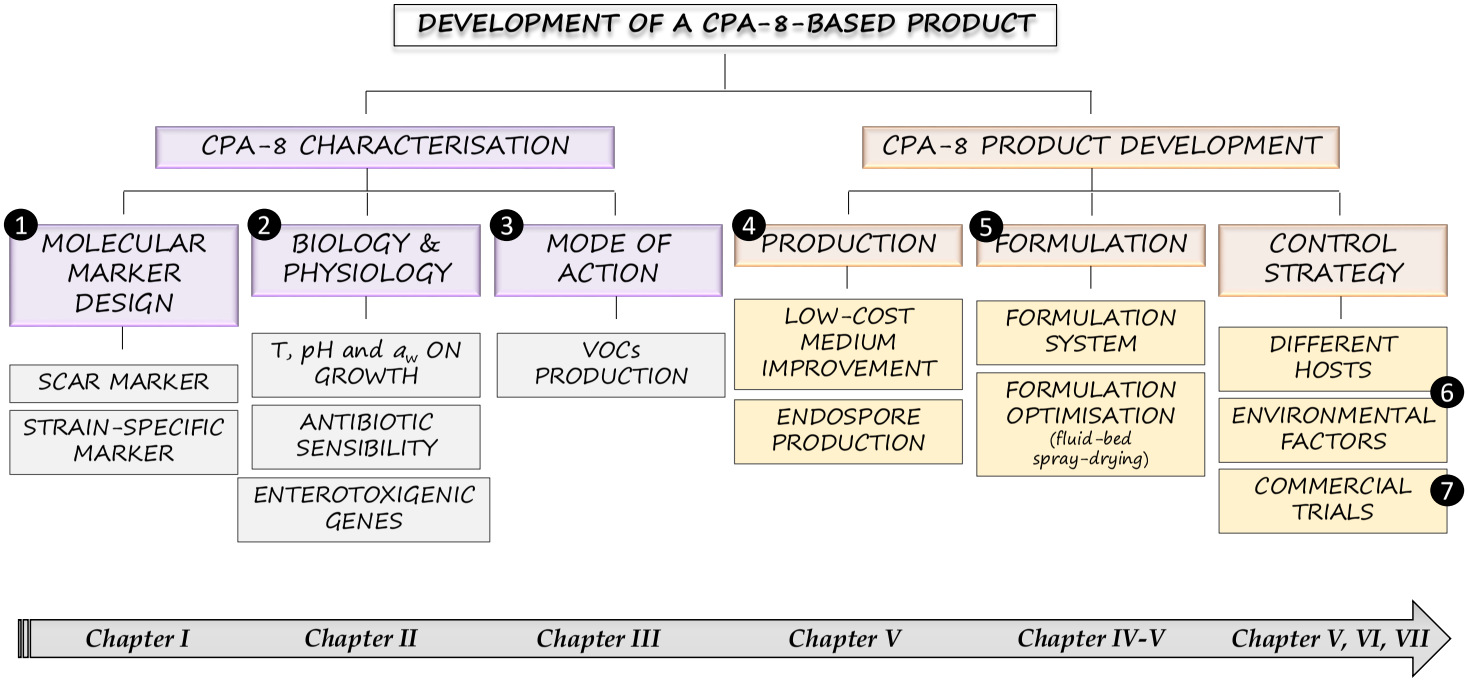

Figure 1. Distribution of the contents of the thesis.

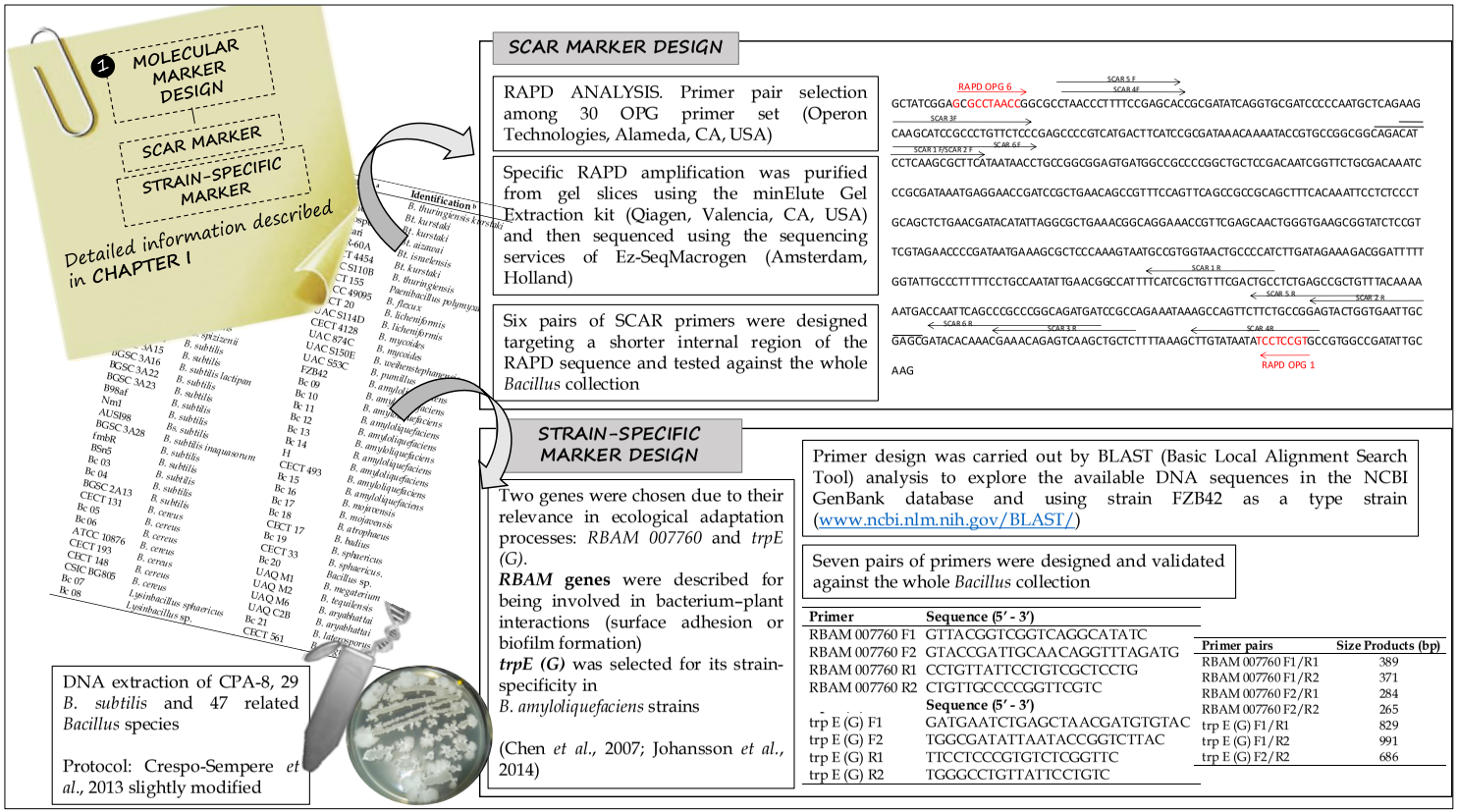

Figure 2. Molecular markers' design.

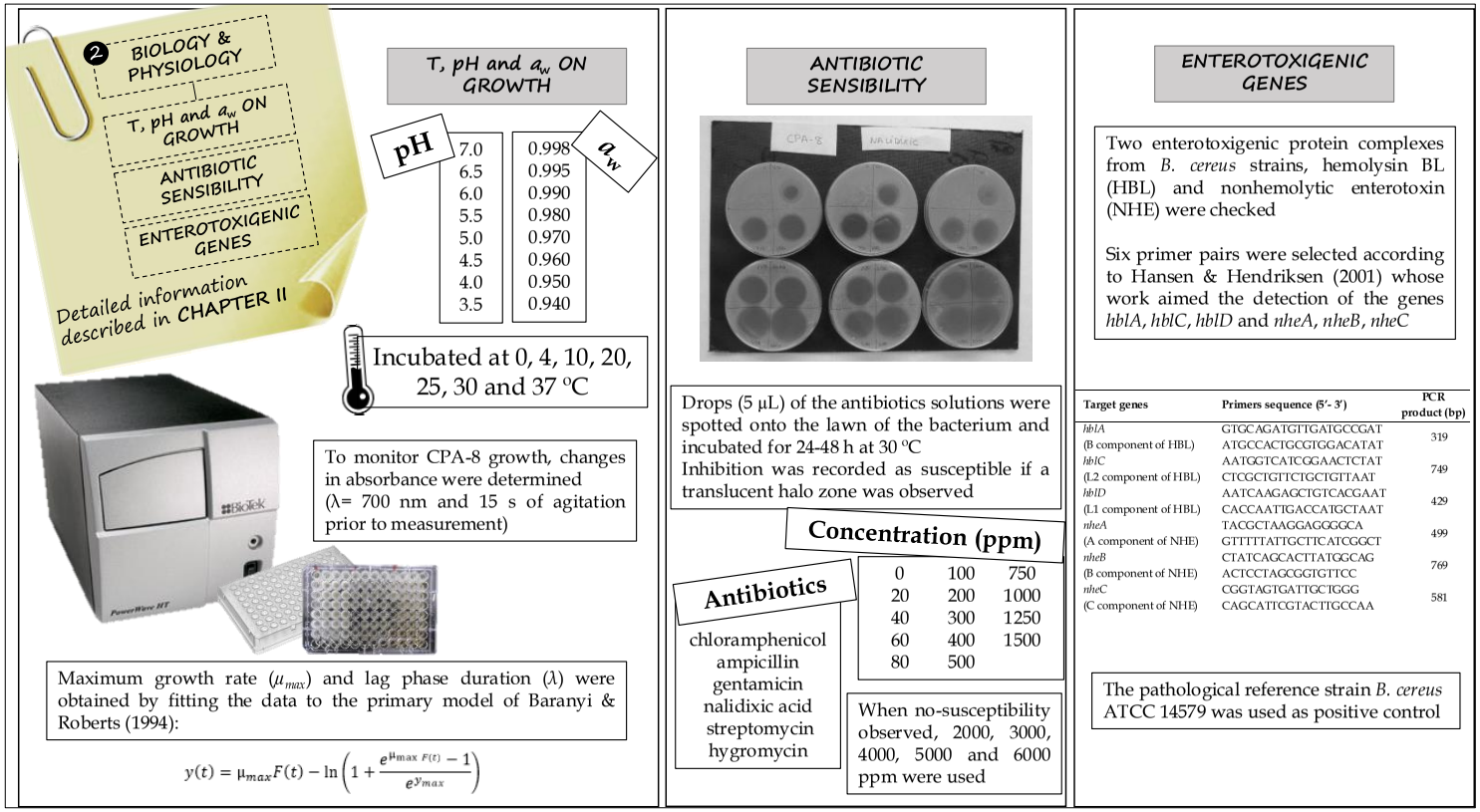

Figure 3. Biology and physiology.

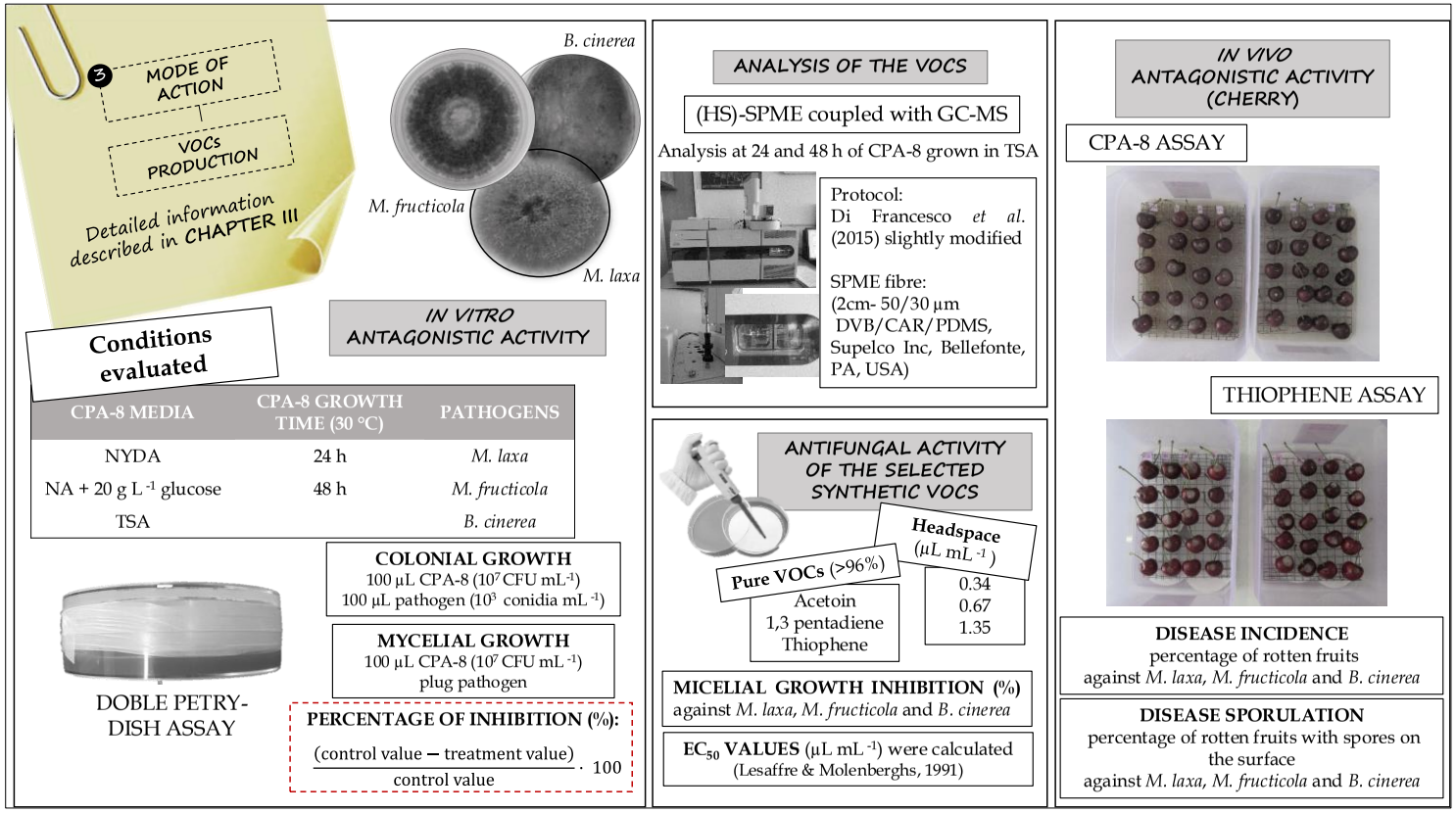

Figure 4. Mode of action.

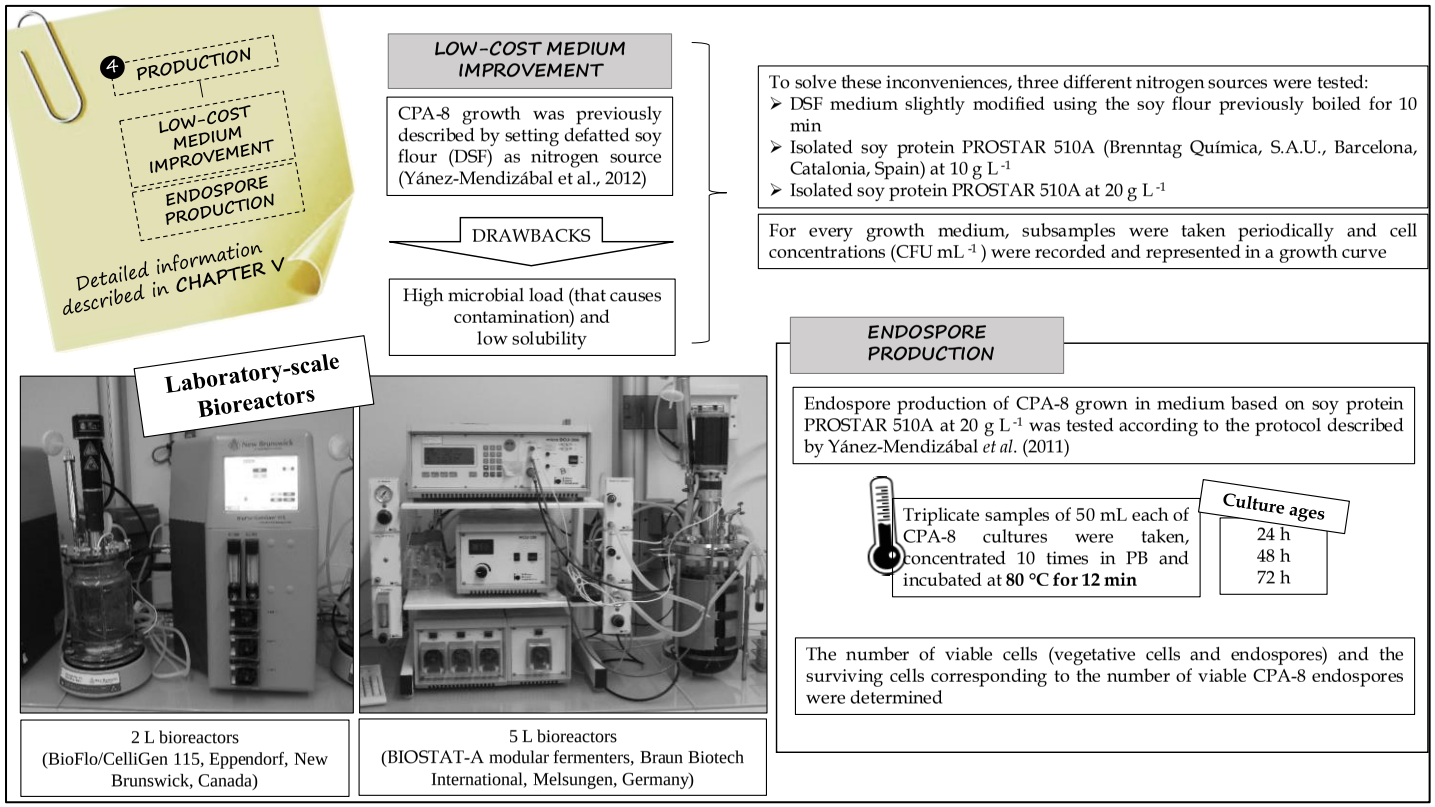

Figure 5. Production process.

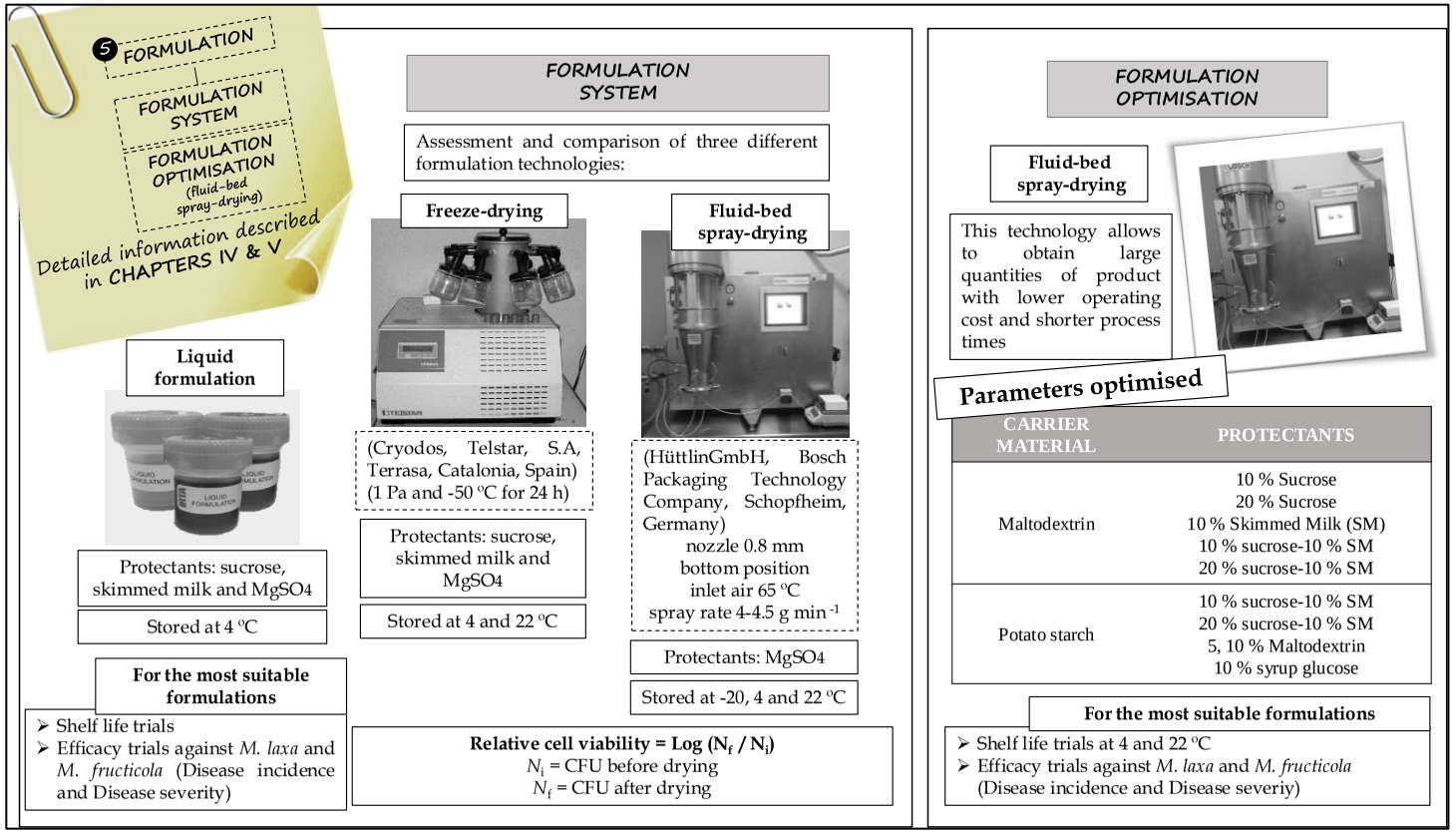

Figure 6. Formulation process.

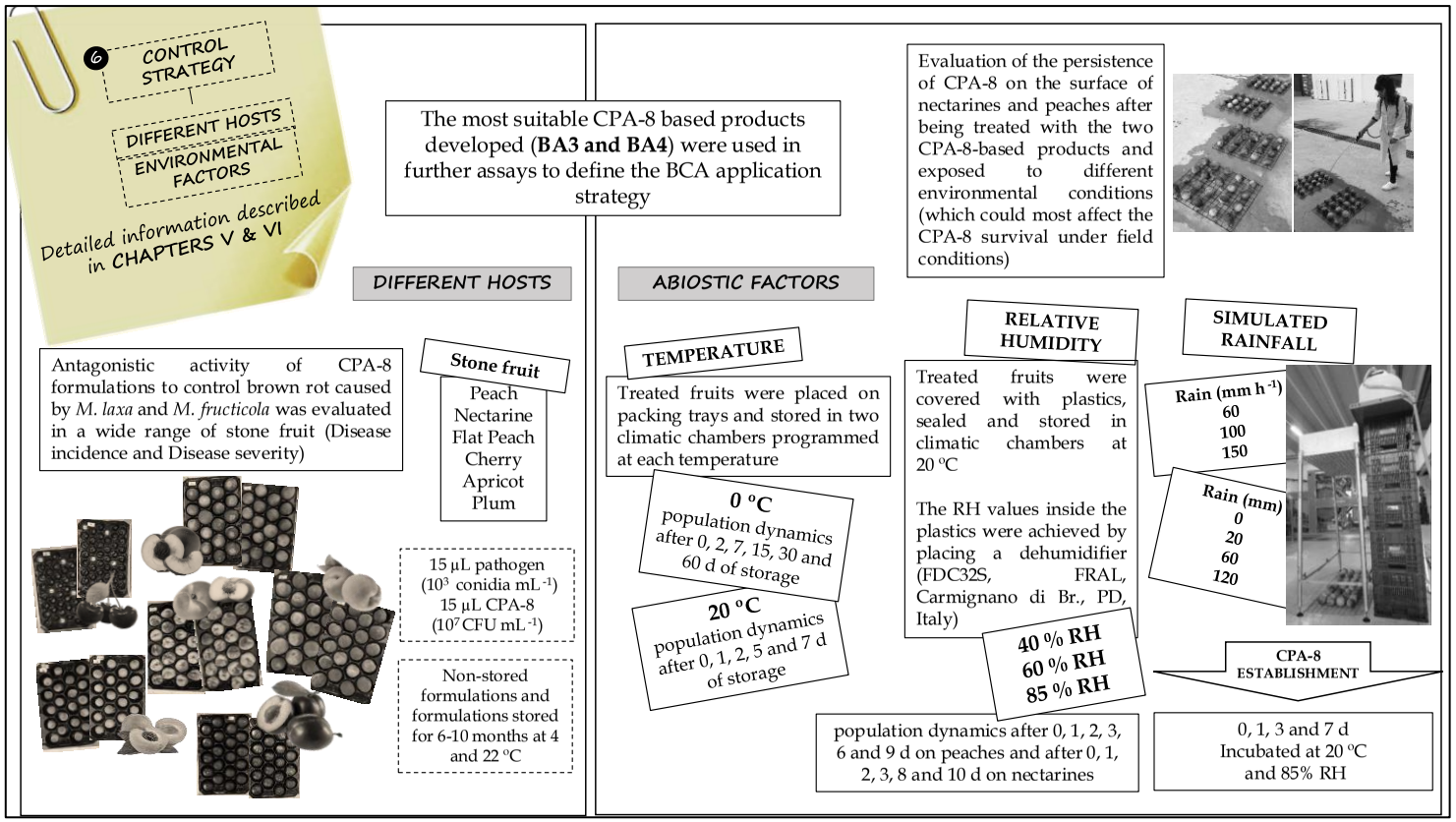

Figure 7. Biocontrol activity in diverse hosts and tolerance to abiotic factors.

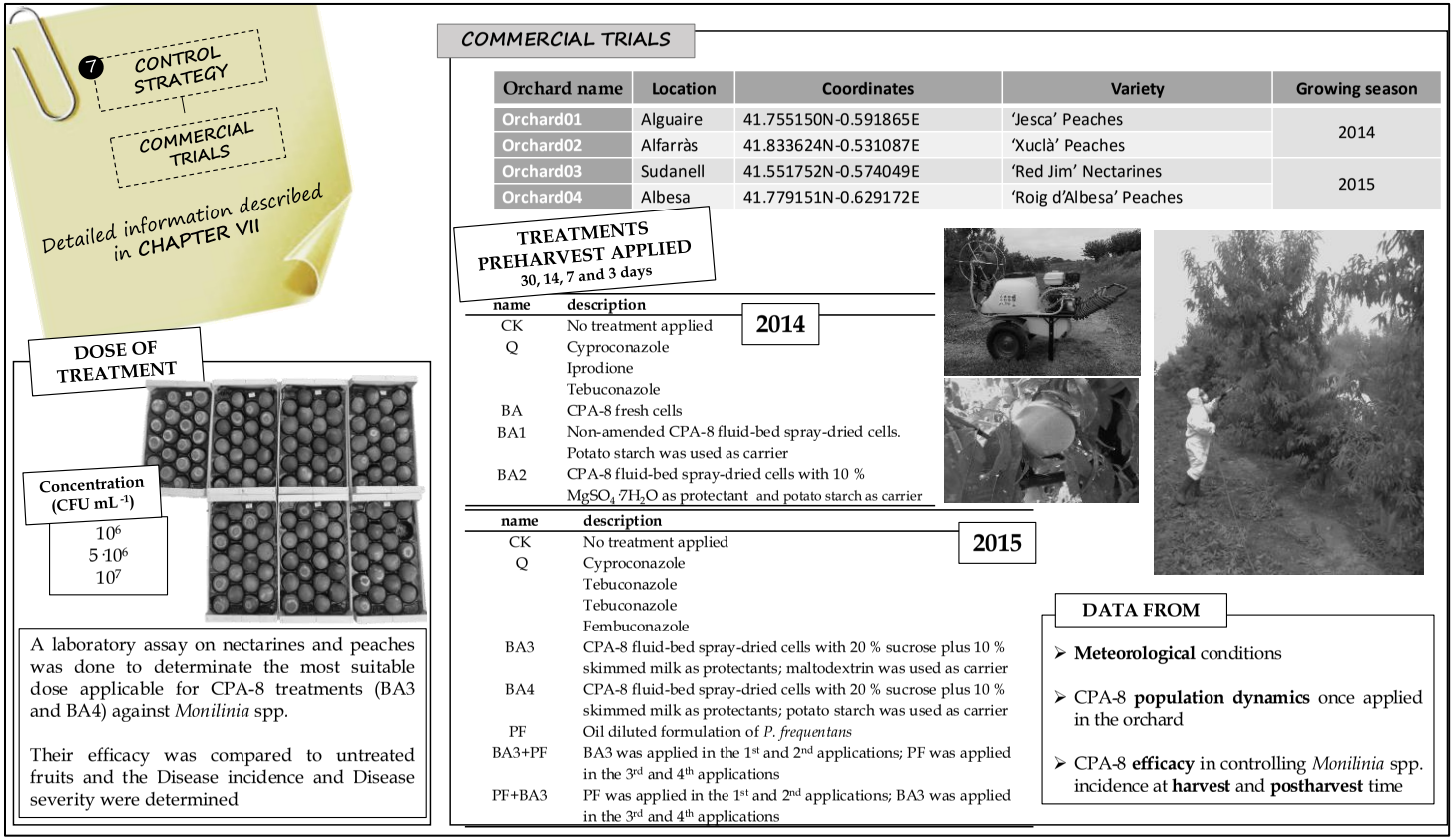

Figure 8. Commercial trials.

CHAPTERS

Chapter 1

Development of a SCAR marker and a strain-specific genomic marker for the detection of the biocontrol agent strain CPA-8 *Bacillus amyloliquefaciens* (formerly *B. subtilis*)

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ABSTRACT

In this work, reliable tools were developed to detect and identify the biocontrol strain CPA-8 using DNA amplification techniques. As a first approach, the RAPD (random amplified polymorphic DNA) technique was applied to a collection of 77 related *Bacillus* species. Among the primers tested, the primer pair OPG1/OPG6 amplified a 668 bp specific product to the strain CPA-8 that was sequenced and used to design SCAR (sequence-characterised amplified regions) primer pairs. The SCAR-4 marker amplified a semi-specific fragment of 665 bp not only for the strain CPA-8 but also for other 12 strains whose morphology was completely different from CPA-8. Another approach was developed to obtain a strain-specific genomic marker related to ecological adaptations of *Bacillus amyloliquefaciens* species. The primer pair F2/R2 obtained from *RBAM 007760*, a gene involved in surface adhesion, amplified a 265 bp fragment unique for strain CPA-8. Our results revealed that these two molecular markers, SCAR-4 and *RBAM 007760* F2/R2 provide suitable monitoring tools to specifically identify the biocontrol agent CPA-8 when applied against brown rot caused by *Monilinia* spp. in stone fruit. Moreover, our findings demonstrate that the strain CPA-8 is affiliated with *B. amyloliquefaciens* species that was formerly designated as *Bacillus subtilis*.

Keywords: *Bacillus* spp.; biological control; molecular marker; monitoring; SCAR.

INTRODUCTION

Fungal diseases are one of the major factors causing considerable economical losses in harvested fruit. The proliferation of fungicide-resistant strains and public concerns about health risk and environmental contamination has promoted the search for alternative methods to control postharvest diseases, such as the use of biological control agents (BCAs) (Janisiewicz & Korsten, 2002; Spadaro & Gullino, 2004; Sharma *et al.*, 2009). During the last few decades, the effectiveness of many antagonist microorganisms against fungal pathogens on fruit has been widely demonstrated (Ippolito *et al.*, 2005; Nunes, 2012).

Bacillus spp. and in particular *Bacillus subtilis sensu lato* group have been reported as effective BCAs based on the capability of production of powerful antifungal compounds and environmentally resistant endospores (Yánez-Mendizábal *et al.*, 2012*a*,*b*; Liu *et al.*, 2014; Zeriouh *et al.*, 2014). In this context, *Bacillus* strain CPA-8 isolated from the surface of a nectarine fruit in Lleida (Catalonia, Spain) and classified initially as *B. subtilis* by 16S rDNA partial analysis is effective for controlling peach brown rot caused by *Monilinia* spp. during postharvest storage, whether used alone (Yánez-Mendizábal *et al.*, 2011) or in combination with other environment-friendly techniques, such as hot water and curing (Casals *et al.*, 2010, 2012).

Before BCAs can be commercialised, the registration procedure is required. As part of this procedure, the microorganisms need to be accurately identified at the species and strain levels (Alabouvette & Cordier, 2011). Monitoring methods can be grouped into cultivation-based and DNA-based techniques. The first technique consists of counting colony forming units on Petri dishes on a selective or semi-selective medium and has the advantage of detecting only viable microorganisms (Teixidó *et al.*, 1999; De Cal *et al.*, 2009). However, this technique lacks specificity, hence non-targeted microorganisms with similar morphology and growth in the selective media, could be confused with the target organism (Nunes *et al.*, 2008). Availability of more specific DNA-based methods has greatly facilitated the surveying and identification of candidate organisms, providing valuable data for registration purposes (Droby *et al.*, 2009). SCAR markers have been commonly used for developing monitoring methods for BCAs because they are natural sequences

present in the genome which allow a simple specific detection by PCR procedure. The most important and significant advantage of SCAR markers is that they do not require any prior knowledge of the strain genome (Pujol *et al.*, 2005). Carrying out RAPD and then designing more repeatable SCAR markers is suggested as a necessary approach that will enable the BCA to be traced and distinguished from other strains of the same species (Schena *et al.*, 2000; Nunes *et al.*, 2008; Alabouvette & Cordier, 2011).

However, recent studies have revealed that many strains belonging to *B. subtilis* and *B. amyloliquefaciens* species are phenotypically and genetically very similar and can be easily confused (Fritze, 2004; Reva *et al.*, 2004). Consequently, the use of SCAR markers as a single tool may not be specific enough to identify CPA-8. In this case, sequencing housekeeping genes has proven to be useful for taxonomic classification and therefore, useful for molecular marker design. These genes are essential and therefore are not lost from genome, but evolve more quickly than 16S rDNA. Moreover, the analysis of the 16S ribosomal sequence is often insufficient or unsatisfactory within *Bacillus* genus (Fritze, 2004; Maughan & Van der Auwera, 2011).

The main objective of the present study was to obtain molecular markers for the BCA CPA-8 at the strain level. In order to do this, two approaches: (a) a SCAR marker using the RAPD method and (b) a strain-specific genomic marker related to the ecological adaptations were developed.

MATERIAL AND METHODS

Antagonist and reference strains

The strain CPA-8 used in this study was obtained from IRTA Centre in Lleida (Catalonia, Spain) and firstly identified by 16S rDNA partial analysis by the Netherlands Culture Collection of Bacteria as a member of the *B. subtilis* species complex. It was isolated from the surface of a nectarine fruit in an experimental orchard from Lleida and selected for its preliminary efficacy in reducing brown rot caused by *Monilinia* spp. (Casals *et al.*, 2010; Yánez-Mendizábal *et al.*, 2011). Reference strains used in this study are listed in Table 1 including 29 *B. subtilis* strains and 47 related *Bacillus* species.

Stock cultures were stored long-term at −80 ºC in CRIOBILLES AEB 400100 (AES Laboratory, Combourg, France) and subcultured on nutrient yeast dextrose agar (NYDA: 8 g L^{-1} nutrient broth, 5 g L^{-1} yeast extract, 10 g L^{-1} dextrose and 20 g L^{-1} agar). The activated culture was maintained on NYDA at 30 ºC for 24 h.

Table 1. Species and strains used in this study.

Strains [a]	**Identification** [b]	**Strains** [a]	**Identification** [b]
CECT 35	*B. subtilis*	Dipel	*B. thuringiensis kurstaki*
CECT 38	*B. subtilis*	Thuricide	*Bt. kurstaki*
CECT 39	*B. subtilis*	Bactospeine	*Bt. kurstaki*
Bc 01	*B. subtilis*	Xentari	*Bt. aizawai*
Bc 02	*B. subtilis*	ONR-60A	*Bt. israelensis*
Serenade	*B. subtilis*	CECT 4454	*Bt. kurstaki*
BG214	*B. subtilis*	UAC S110B	*B. thuringiensis*
168	*B. subtilis subtilis*	CECT 155	*Paenibacillus polymyxa*
BGSC 27E3	*B. subtilis* "natto"	ATCC 49095	*B. flexus*
BGSC 2A11	*B. subtilis spizizenii*	CECT 20	*B. licheniformis*
BGSC 2A12	*Bs. spizizenii*	UAC S114D	*B. licheniformis*
BGSC 2A8	*Bs. spizizenii*	CECT 4128	*B. mycoides*
BGSC 2A9	*Bs. spizizenii*	UAC 874C	*B. mycoides*
BGSC 3A1	*Bs. subtilis*	UAC S150E	*B. weihenstephanensis*
BGSC 3A13	*Bs. spizizenii*	UAC S53C	*B. pumillus*
BGSC 3A14	*B. subtilis*	FZB42	*B. amyloliquefaciens*
BGSC 3A15	*B. subtilis*	Bc 09	*B. amyloliquefaciens*
BGSC 3A16	*B. subtilis lactipan*	Bc 10	*B. amyloliquefaciens*
BGSC 3A22	*B. subtilis*	Bc 11	*B. amyloliquefaciens*
BGSC 3A23	*B. subtilis*	Bc 12	*B. amyloliquefaciens*
B98af	*B. subtilis*	Bc 13	*B. amyloliquefaciens*
Nm1	*B. subtilis*	Bc 14	*B. amyloliquefaciens*
AUSI98	*Bs. subtilis*	H	*B. amyloliquefaciens*
BGSC 3A28	*B. subtilis inaquasorum*	CECT 493	*B. amyloliquefaciens*
fmbR	*B. subtilis*	Bc 15	*B. amyloliquefaciens*
BSn5	*B. subtilis*	Bc 16	*B. mojavensis*
Bc 03	*B. subtilis*	Bc 17	*B. mojavensis*
Bc 04	*B. subtilis*	Bc 18	*B. atrophaeus*
BGSC 2A13	*B. subtilis*	CECT 17	*B. badius*
CECT 131	*B. cereus*	Bc 19	*B. sphaericus*
Bc 05	*B. cereus*	CECT 33	*B. sphaericus.*
Bc 06	*B. cereus*	Bc 20	*Bacillus* sp.
ATCC 10876	*B. cereus*	UAQ M1	*B. megaterium*
CECT 193	*B. cereus*	UAQ M2	*B. tequilensis*
CECT 148	*B. cereus*	UAQ M6	*B. aryabhattai*
CSIC BG805	*B. cereus*	UAQ C2B	*B. aryabhattai*
Bc 07	*Lysinbacillus sphaericus*	Bc 21	*B. laterosporus*
Bc 08	*Lysinbacillus* sp.	CECT 561	*B. coagulans*

[a] Strains labelled CECT were obtained from the Spanish Type Culture Collection; strains labelled ATCC were from the American Type Culture Collection; strains labelled BGSC were from the Bacillus Genetic Stock Center; strains labelled CSIC were from the Spanish Council of Scientific Research; strains labelled UAC were from the University of Azores (Portugal); strains labelled UAQ were from the University of Querétaro (México). All other strains were obtained from commercial products or from our own collection.
[b] Tentative identifications for isolates described in this study.

DNA extraction

Bacterial strains were cultured overnight at 30 ºC in NYDB (NYDA medium without agar) and DNA was extracted according to the method described by Crespo-Sempere *et al.* (2013) with modifications. The protocol was as follows: the bacterial extract was recovered after 10 min of centrifugation at 19 060 *g* and 300 μL of DNA extraction buffer (200 mmol L^{-1} Tris–HCl pH 8.5, 250 mmol L^{-1} NaCl, 25 mmol L^{-1} EDTA, 0.5 % w/v SDS) were added. This cell suspension was vortexed and vigorously shaken in a Fast Prep machine (FP120 Bio101, Thermo Savant, Carlsbad, CA, USA) at speed position 6.5 for 20 s three times in the presence of acid-washed glass beads (425–600 µm diameter). The supernatant was recovered after centrifugation at 19 060 *g* for 10 min and 150 μL of 3 mol L^{-1} sodium acetate (pH 5.2) were added. The tube was gently inverted several times to precipitate the DNA. The supernatant was stored at −20 ºC for 30 min and then centrifuged (19 060 *g*, 10 min). The DNA-containing supernatant was transferred to a new tube and nucleic acids were precipitated by addition of one volume of isopropanol. After a 5-min incubation time at room temperature, the DNA suspension was centrifuged twice (19 060 *g*, 10 min) and the supernatant discarded. The DNA pellet was washed with 70 % ethanol to remove residual salts and vigorously vortexed for 5 min. The suspension was centrifuged (19 060 *g*, 10 min). Finally, the pellet was air dried and the DNA resuspended in 25 μL of TE buffer (10 mmol L^{-1} Tris–HCl pH 8, 1 mmol L^{-1} EDTA). The solution was stored at −20 ºC until its use. The amount and purity of DNA samples were determined spectrophotometrically (NanoDrop ND-1000: NanoDrop Technologies, Wilmington, DE, USA) and DNA integrity was analysed by electrophoresis on 1 % agarose gels run at 100 V for 100 min with TBE buffer (10.8 g Tris base, 5.5 g boric acid, 4 mL EDTA 0.5 mol L^{-1}), stained with gel red (GelRed™ Nucleic Acid Stain, 10 000X in water) and visualised with UV light.

RAPD analysis

Amplification reactions were done in a total volume of 25 μL containing 2.5 μL 10X Complete NH_4Taq buffer (160 mmol L^{-1} $(NH_4)_2SO_4$, 670 mmol L^{-1} Tris HCl pH 8.8, 0.1 % Tween 20, 25 mmol L^{-1} $MgCl_2$), 800 µmol L^{-1} dNTP mix, 0.4 µmol L^{-1} primer (OPG primer set, Operon Technologies Alameda, CA, USA), 1.25 units DFS-Taq DNA polymerase (Bioron GmbH, Ludwigshafen am Rheim, Germany) and 50 ng genomic

DNA. PCR was carried out in the Peltier thermal cycler (GeneAmp PCR System 2700, Applied Biosystems, Foster City, CA, USA). Each PCR program was conducted using a denaturation step of 5 min at 94 ºC, followed by 35 cycles of 94 ºC for 30 s, 35 ºC for 45 s and 72 ºC for 1 min 40 s, with an extension step at 72 ºC for 7 min. Negative controls (no template DNA) were used in every experiment to test the presence of contamination in reagents. PCR products were separated by 1 % agarose gel electrophoresis with TBE buffer and stained with gel red. The sizes of DNA fragments were estimated using a 1 Kb Plus DNA Ladder (Invitrogen, Life Technologies, Carlsbad, CA, USA). Specific RAPD amplification was purified from gel slices using the minElute Gel Extraction kit (Qiagen, Valencia, CA, USA) and then sequenced using the sequencing services of Ez-SeqMacrogen (Amsterdam, Holland). The nucleotide sequence was aligned and analysed and SCAR primer sequences of 19–25 bases were designed (Table 2, Fig. 1).

Table 2. RAPD primers and SCAR primers designed for the detection of strain CPA-8.

RAPD primers	**Sequence (5′ - 3′)**	**Size Product (bp)**
OPG 1	CTACGGAGGA	668
OPG 6	GTGCCTAACC	
SCAR primers	**Sequence (5′ - 3′)**	**Size Product (bp)**
SCAR 1 F	CAGACATCCTCAAGCGCTTC	370
SCAR 1R	GCAGTCGAAACAGCGATGAA	
SCAR 2 F	CAGACATCCTCAAGCGCTTC	480
SCAR 2 R	GCTCGCAATTCACCAGTACTC	
SCAR 3 F	CAAGCATCCGCCCTGTTCTC	584
SCAR 3 R	AGAGCAGCTTGACTCTGTTTCG	
SCAR 4 F	CTAACCCTTTTCCGAGCAC	665
SCAR 4 R	CACGGAGGATATTATACAAGC	
SCAR 5 F	CCTAACCCTTTTCCGAGCA	596
SCAR 5 R	ACCAGTACTCCGGCAGAAGA	
SCAR 6 F	ACATCCTCAAGCGCTTCATAATAAC	504
SCAR 6 R	CTTGACTCTGTTTCGTTTGTGTATC	

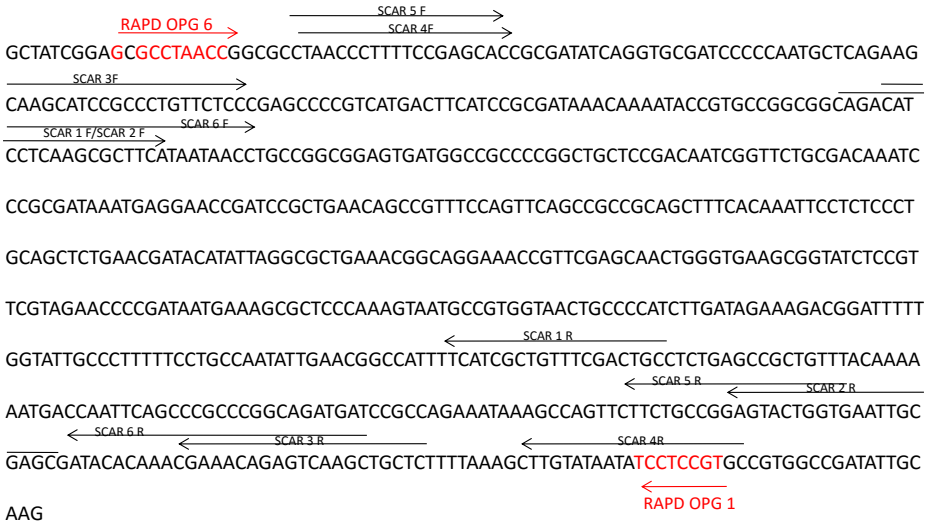

Figure 1. Sequence of the 668 bp DNA fragment which was amplified specifically for the strain CPA- 8 using OPG1 and OPG6 primers. Lines correspond to sequences of OPG1/OPG6 (red arrows) and the primer pairs of the six SCAR designed (black arrows) (SCAR-1 F/R, SCAR-2 F/R, SCAR-3 F/R, SCAR-4 F/R, SCAR-5 F/R and SCAR-6 F/R).

Development of a SCAR marker

Six pairs of SCAR primers were designed targeting a shorter internal region of the RAPD sequence and tested in the Peltier thermal cycler (GeneAmp PCR System 2700, Applied Biosystems). The amplification reaction was prepared in 25 μL using 1.25 units DFS-*Taq* DNA polymerase in 2.5 μL 10X Complete NH_4*Taq*buffer (Bioron GmbH), 200 µmol L^{-1} each dNTP, 50 ng DNA and 0.4 µmol L^{-1} of each SCAR primer. A initial denaturation at 94 ºC for 5 min was followed by 30 cycles of 94 ºC for 30 s, 65 ºC for 45 s, 72 ºC for 30 s and by a final extension step of 72 ºC for 7 min. Negative controls were used. Reaction products were analysed by electrophoresis on 1 % TBE buffer agarose gels stained with gel red and 1 Kb DNA Ladder RTU (NIPPON Genetics Europe GmbH, Düren, Germany) was used as molecular size marker. The PCR products amplified of each strain were also analysed using the sequencing services of Ez-SeqMacrogen, then a sequences alignment was carried out to explore the percentage of sequence identity in all SCAR products.

Development of a strain-specific genomic marker

Two genes were chosen due to their relevance in ecological adaptation processes: *RBAM 007760* and *trpE (G)*. *RBAM* genes were previously described for being involved in bacterium–plant interactions (surface adhesion or biofilm formation) and *trpE (G)* was selected for its strain-specificity in *B. amyloliquefaciens* strains (Chen *et al.*, 2007; Johansson *et al.*, 2014). Using strain FZB42 as a type strain, the assessment of primer design was carried out by BLAST (Basic Local Alignment Search Tool) analysis to explore the available DNA sequences in the NCBI GenBank database (www.ncbi.nlm.nih.gov/BLAST/) and exclude the presence of matching sequences within related microorganisms. Seven pairs of primers were designed: four pairs of primers for *RBAM 007760* gene and three pairs for *trpE (G)* gene (Table 3). The primers were validated against genomic DNA from the strain CPA-8, 29 strains of *B. subtilis* and 47 related *Bacillus* species (Table 1). Bacteria were grown on NYDA medium at 30 ºC for 24 h. For DNA extraction from pure cultures, bacterial colonies were cultured overnight in 500 µL of NYDB and the cell suspensions were processed according to the method of DNA extraction described earlier. Finally, 50 ng of DNA per PCR reaction were analysed by conventional PCR under conditions mentioned in SCAR-marker section. A negative control without DNA and a positive control of CPA-8 were included. The PCR products were separated by electrophoresis on 1.2 % agarose gels and 100 bp DNA Ladder H3 RTU (NIPPON Genetics Europe GmbH) was used as molecular size marker.

Table 3. Strain-specific genomic-marker primers designed for the detection of strain CPA-8.

RBAM 007760	**Sequence (5′ - 3′)**
RBAM 007760 F1	GTTACGGTCGGTCAGGCATATC
RBAM 007760 F2	GTACCGATTGCAACAGGTTTAGATG
RBAM 007760 R1	CCTGTTATTCCTGTCGCTCCTG
RBAM 007760 R2	CTGTTGCCCCGGTTCGTC
trp E (G)	**Sequence (5′ - 3′)**
trp E (G) F1	GATGAATCTGAGCTAACGATGTGTAC
trp E (G) F2	TGGCGATATTAATACCGGTCTTAC
trp E (G) R1	TTCCTCCCGTGTCTCGGTTC
trp E (G) R2	TGGGCCTGTTATTCCTGTC
Primer pairs	**Size Products (bp)**
RBAM 007760 F1/R1	389
RBAM 007760 F1/R2	371
RBAM 007760 F2/R1	284
RBAM 007760 F2/R2	265
trp E (G) F1/R1	829
trp E (G) F1/R2	991
trp E (G) F2/R2	686

RESULTS

RAPD analysis and development of a SCAR marker

In a first approach, we performed a RAPD analysis of the BCA strain CPA-8 and other related species to screen potential DNA markers (Fig. 2). Among the 30 OPG primer pairs used in this first screening, one pair (called OPG1 and OPG6) provided a specific amplification product for CPA-8. The screening of the complete collection of *Bacillus* species (Table 1) using these two RAPD primers allowed selecting the primer pair OPG1: CTACGGAGGA and OPG6: GTGCCTAACC which had a specific DNA fragment for CPA-8 (data not shown). This fragment was purified from gel slices and partially sequenced. Six SCAR primers derived from RAPD primers elongation were designed (Table 2, Fig. 1) and were subjected to specificity tests including the total collection of strains. The SCAR named SCAR-4 of 665 bp was specific for strain CPA-8 except for other 12 strains of the collection but all of them were phenotypically different to CPA-8 (Fig. 3). Most of these non-target SCAR-4 positive strains were classified as *B. amyloliquefaciens* strains (Table 1). These results and an inconclusive classification of the CPA-8 by 16S rDNA partial analysis, suggest that CPA-8 is closely related to *B. amyloliquefaciens* species instead of *B. subtilis*, which is currently its name in the literature. Moreover, after sequencing the SCA-4 PCR products of each positive strain, the sequences were aligned and high sequence

identity (100 %) with *B. amyloliquefaciens* subsp. *plantarum* was obtained for the strain CPA-8 (data not shown).

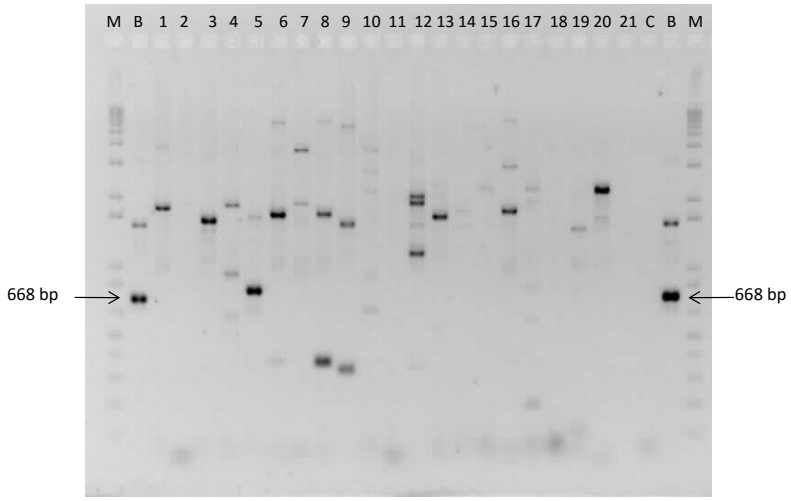

Figure 2. RAPD patterns obtained using *Bacillus* spp. strains and OPG1/OPG6 primers. Lanes: (M) 1Kb Plus DNA Ladder (Invitrogen, Life Technologies, Carlsbad, CA, USA); (C) negative control (without DNA); (B) CPA-8; (1) Bc 10; (2) Bc 11; (3) Bc 12; (4) Bc 13; Bc 14; (6) H; (7) CECT 493; (8) Bc 15; (9) Bc 16; (10) Bc 17; (11) Bc 18; (12) CECT 17; (13) Bc 19; (14) CECT 33; (15) Bc 20; (16) UAQ M1; 17) UAQ M2; (18) UAQ M6; (19) UAQ C2B; (20) Bc 21; (21) CECT 561.

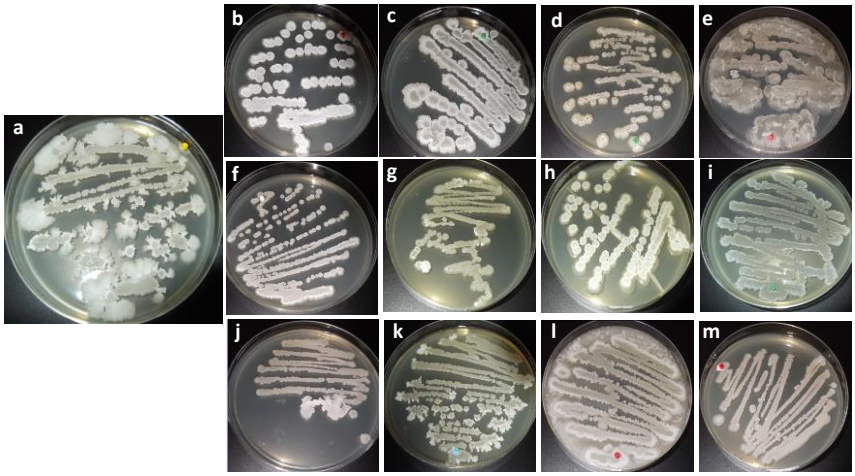

Figure 3. Morphologies of the strain CPA-8 and other non-target SCAR-4 positive strains. Please, note that CPA-8 has white and not bright starred shape. Images: (a) CPA-8; (b) CECT 561; (c) BGSC 3A23; (d) Bc 15; (e) H; (f) Bc 10; (g) Bc 14; (h) Serenade; (i) BGSC 3A14; (j) Bc 11; (k) Bc 12; (l) Bc 13; (m) FZB42.

Identification of the strain-specific genomic marker

Previous work done in sequencing and annotating *Bacillus* genomes allowed us to choose adaptative gene sequences potentially unique for different strains to design specific PCR reactions. Fragments of the sequence of the genes *RBAM 007760* and *trpE (G)* were selected using BLAST analysis for being different among *B. amyloliquefaciens* strains belonging to *B. amyloliquefaciens plantarum* group. In a first step, a non-optimised PCR was performed using the primers designed on the reference strain CPA-8 and the strain FZB42, used as a type strain. The primer pair F2/R2 from *RBAM 007760* gene amplified a product of 265 bp specific for strain CPA- 8. Figure 4 shows the specificity of *RBAM 007760* PCR product on a subset of the 77 strains listed in Table 1. The F2/R2 fragment of *RBAM 007760* gene amplified a specific product of 265 bp in CPA-8 and PCR analysis with heterologous strains of the collection did not give rise to any similar DNA product (Fig. 4b).

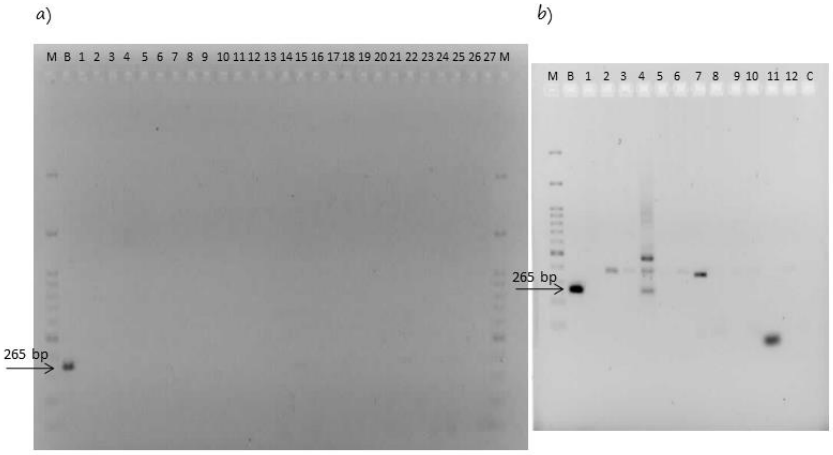

Figure 4. Strain-specific marker obtained with *RBAM 007760* gene F2/R2. (a) The 265 bp fragment was specific for CPA- 8. Lanes: (M) 100 bp DNA Ladder H3 RTU (NIPPON Genetics Europe GmbH, Germany); (C) negative control (without DNA); (B) CPA-8; (1) CECT 131; (2) Bc 05; (3) Bc 06; (4) ATCC 10876; (5) CECT 193; (6) CECT 148; (7) CSIC BG805; (8) Bc 07; (9) Bc 08; (10) Dipel; (11) Thuricide; (12) Bactospeine; (13) Xentari; (14) ONR-60A; (15) CECT 4454; (16) UAC S110B; (17) CECT 155; (18) ATCC 49095; (19) CECT 20; (20) UAC S114D; (21) CECT 4128; (22) UAC 874C; (23) UAC S150E; (24) UAC S53C; (25) Bc 09; (26) CECT 143; (27) Bc 16. (b) The pattern obtained from *RBAM 007760* gene allows distinguish between CPA-8 and non-target SCAR-4 positive strains. Lanes: (M) 100 bp DNA Ladder H3 RTU (NIPPON Genetics Europe GmbH, Germany); (C) negative control (without DNA); (B) CPA- 8; (1) Serenade; (2) BGSC 3A14; (3) BGSC 2A23; (4) FZB42; (5) Bc 10; (6) Bc 11; (7) Bc 12; (8) Bc 13; (9) Bc 14; (10) H; (11) Bc 15; (12) CECT 561.

DISCUSSION

Bacillus amyloliquefaciens CPA-8 has been shown to be reliably effective in controlling brown rot on stone fruit and other works related to the mode of action and to its production and viability have also been published (Yánez-Mendizábal *et al.*, 2012*a*; Yánez-Mendizábal *et al.*, 2012*b*). This indicates that CPA-8 strain could serve as the basis of a new biocontrol product. Ongoing studies to develop this strain as a biopesticide have created the need to develop a monitoring method for tracking it and knowing its environmental fate after its application. In order to overcome the lack of specificity that cultivation-based techniques have in identifying strains with similar morphology, different molecular technologies for monitoring BCAs have been developed.

In this work, a RAPD-PCR method followed by a SCAR marker design was developed to discriminate the BCA CPA-8 from other bacteria. The specificity of the 665 bp fragment amplified with SCAR-4 F/R primers was confirmed by the absence of non-specific amplification signals in almost all *Bacillus* strains. Twelve CPA-8 related isolates of the whole collection were also positive for SCAR-4 marker. However, all of these isolates were phenotypically and morphologically different from strain CPA-8 and could be totally distinguished by plating methods previous DNA amplification. Other authors have succeeded in identifying BCAs by SCAR markers, but frequently no more than 20 strains were compared (Pujol *et al.*, 2005; Nunes *et al.*, 2008) even when BCAs come from phylogenetically complex genus. Results obtained in this study showed that this molecular technique is a reliable method for characterising CPA-8. In addition, a better understanding of the CPA-8 genetics and ecology could be very useful for strain differentiation. Therefore, the development of other molecular marker with higher degree of specificity was carried out through the study of genes related to bacterium–plant interactions and strain-specificity.

To design strain-specific PCR reactions, the genes *RBAM 007760* (collagen like triple helix with GXT repeats proposed to be involved in surface adhesion and biofilm formation) and *trpE (G)*, which is an anthranilate synthase/glutamine amidotransferase for biosynthesis of tryptophan (Chen *et al.*, 2007; Johansson *et al.*,

2014), were analysed. The results showed a specific fragment of 265 bp from the gene *RBAM 007760* F2/R2 primer pair. No amplified bands were present in other strains of the collection except in *B. amyloliquefaciens* FZB42 which product was completely different from strain CPA-8 and both strains could be differentiated. This shows that, sequencing housekeeping genes, which are essential genes but capable of evolving and adapting to different environmental situations, is useful for taxonomic classification. Few authors have reported strain-specific markers to be useful for identifying BCAs (Felici *et al.*, 2008) and others have focused on the development of chemotaxis processes to resolve the complexity between related *B. subtilis* group species (Chen *et al.*, 2009; Borriss *et al.*, 2011; Yssel *et al.*, 2011). However, this work shows the first time that gene *RBAM 007760* has been used for identifying a BCA at a strain level within a large number of *Bacillus* strains. Our results demonstrate that the SCAR-4 was not able to distinguish all strains and should be complemented with plating methods and that the F2/R2 *RBAM 007760* fragment was a strain-specific genomic marker for the strain CPA-8. Owing to an indecisive classification of the strain CPA-8 by 16S rDNA partial analysis, primers from 5′ and 3′ were designed to elongate the 16S rDNA region analysed but no more precise results were obtained (data not shown). However, the homology observed during the alignments realised in this work within CPA-8 sequences and other *Bacillus* related strains sequences have suggest that BCA CPA-8 belongs to *B. amyloliquefaciens* strains and highly probably should be classified as a *B. amyloliquefaciens* subspecies *plantarum*. Since *B. amyloliquefaciens* was recognised as a distinct species from *B. subtilis*, it is known that isolates from plants and soil, which formed a cluster with *B. amyloliquefaciens* type strain FZB42, are generally better adapted to colonisation of the rhizosphere than other members of the *B. subtilis* group and were considered as a distinct ecotype of *B. amyloliquefaciens* (*B. amyloliquefaciens plantarum*) (Fritze, 2004; Reva *et al.*, 2004). Besides, analysis of the whole genome of the strain FZB42 revealed an unexpected potential to produce secondary metabolites such as antibiotics and siderophores by pathways not involved in ribosomes (Chen *et al.*, 2007, 2009). These data agree with the work previously published about CPA-8 mode of action (Yánez-Mendizábal *et al.*, 2012*b*) and suggest that further studies for the better understanding of CPA-8 ecophysiology should be considered.

This work describes the development of two approaches to identify *B. amyloliquefaciens* CPA-8 on a large number of *Bacillus* spp. strains. The study of a semi-specific SCAR marker and a strain-specific genomic marker related to adaptative DNA sequences may have great practical importance. Both markers provide new possibilities for insights into ecophysiology constraints within closely related *Bacillus* strains and can be used to generate valuable data for registration purposes. The molecular markers designed could now be applied in the development of DNA quantification techniques (qPCR) and also be used in monitoring.

Acknowledgments

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Chapter II

Biological characterisation of the biocontrol agent *Bacillus amyloliquefaciens* CPA-8: the effect of temperature, pH and water activity on growth, susceptibility to antibiotics and detection of enterotoxic genes

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ABSTRACT

This work focuses on the biological understanding of the biocontrol agent *Bacillus amyloliquefaciens* CPA-8 in order to accomplish the characterisation required in the registration process for the development of a microorganism-based product. The tolerance of CPA-8 to grow under different pH-temperature and water activity (a_w)-temperature conditions was widely demonstrated. Regarding the pH results, optimum growth at the evaluated conditions was observed at 37 ºC and pH between 7 and 5. On the contrary, the slowest growth was recorded at 20 ºC and pH 4.5. Moreover, the type of solute used to reduce a_w had a great influence on the minimum a_w at which the bacterium was able to grow. The lowest a_w for CPA-8 growth in media modified with glycerol and glucose was 0.950 and 0.960, respectively. Besides, the lowest a_w for CPA-8 growth increased when the temperature decreased to 20 ºC, at which CPA-8 was not able to grow at less than 0.990 a_w, regardless of the type of solute. Antibiotic susceptibility tests were carried out to determine which antibiotic could affect the behaviour of the bacterium and revealed that CPA-8 was clearly resistant to hygromycin. Finally, a PCR amplification assay to detect the presence of enterotoxic genes from *Bacillus cereus* in CPA-8 was also performed. CPA-8 gave negative results for all the genes tested except for *nheA* gene, which is not enough for the toxicity expression, suggesting that fruit treated with this antagonist will not be a potential vehicle for foodborne illnesses.

Keywords: *Bacillus* spp.; biocontrol; ecophysiology; enterotoxicity; sensibility to antibiotics.

INTRODUCTION

Brown rot in stone fruit essentially caused by *Monilinia* spp. is one of the major factors that affect quality of fruit during storage, causing important losses that could reach even higher than 50 % of the production in European Mediterranean countries (Nunes, 2012; Sisquella *et al.*, 2013; Usall *et al.*, 2015). Infection occurs through injuries made during picking or handling and results in decay during storage or marketing. The use of fungicides is still the most effective way for preventing yield losses caused by most fungal diseases on different crops (Mari *et al.*, 2007; Teixidó *et al.*, 2011). However, the use of chemical applications in stone fruit after harvesting is hardly authorized in Europe. In addition, an increase in public concern on the sustainability of agricultural practices and the prevention of acquired fungicide resistance in target pathogens have promoted the development of biological control agents (BCAs) as an effective alternative to chemicals in controlling postharvest diseases of fruits and vegetables (Droby *et al.*, 2016; Sharma *et al.*, 2009; Teixidó *et al.*, 2011). Recently, detailed studies have shown that the BCA *Bacillus amyloliquefaciens* CPA-8 (formerly *B. subtilis*), which is a common constituent of the resident microbiota on peaches, is an effective antagonist to control brown rot in peach caused by *Monilinia* spp., either used alone (Yánez-Mendizábal *et al.*, 2011) or in combination with other environment-friendly techniques, such as hot water and curing (Casals *et al.*, 2010).

In general, the BCAs' efficacy requires specific and suitable environmental conditions. If appropriate temperature and moisture are not consistently available, BCA populations may fail to reduce disease incidence and severity, and may not recover as rapidly as pathogen populations when conducive conditions occur (Garrett *et al.*, 2006). Effective colonisation, high population and viability of BCAs on plant surfaces have been considered important aspects in the successful control of plant diseases. The dynamics of individual populations within the epiphytic community are determined by the most important environmental factors which include water availability (water activity, a_w), prevailing temperatures and pH of the plant tissue (Costa *et al.*, 2002; Mossel *et al.*, 1995). These three factors interact and directly influence the capability for growth and establishment of the BCAs in the host. Thus, it is important to identify the environmental niche in which an individual BCA

can actively grow as this enables abiotic threshold criteria for obtaining efficacy (Teixidó *et al.*, 1998).

Furthermore, in order to develop a commercial product, a deep characterisation of the BCA is required in the registration procedure. Recently, two molecular markers have been described for monitoring CPA-8 under field conditions and during the postharvest storage period (Gotor-Vila *et al.*, 2016). Moreover, the key mode of action of CPA-8 based on fengycin-like lipopeptides production and the emission of volatile organic compounds has also been described (Gotor-Vila *et al.*, 2017; Yánez-Mendizábal *et al.*, 2012b). However, no work is done about the antibiotics sensibility pattern of CPA-8. Isolates of the genus *Bacillus* displayed resistance to an extended spectrum of antibiotics; hence, it is important to know the antibiotic susceptibility. Besides, considering CPA-8 as part of a microorganism-based product, the chance of toxic substances production should be taken into account. Relatively few researches have reported the presence of foodborne illnesses associated with *Bacillus* spp. other than *Bacillus cereus*. The main studied *Bacillus* species are included in two groups: *B. subtilis sensu lato* group (which refers to closely species such as *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. atropheaeus*, *B. mojavensis*, and *B. amyloliquefacines*) and *B. cereus sensu lato* group (which includes *B. cereus*, *B. thuringiensis*, *B. mycoides*, and *B. anthracis*). A priori, CPA-8 should not present enterotoxic genes no lost from *B. cereus* genome. However, due to the high degree of phylogenetic relatedness among members of this genus, a variety of species (including *B. amyloliquefaciens*) should be considered potentially enterotoxigenic (Phelps & McKillip, 2002).

The main aim of this work was to characterise the BCA CPA-8 to complete registration requirements. In order to do this, the specific aims were: (i) to evaluate the effect of the key ecological parameters of temperature, a_w and pH on growth rate and lag phase of CPA-8, (ii) to study the susceptibility/resistance of CPA-8 to different antibiotics tested at different concentrations under *in vitro* conditions and (iii) to detect enterotoxic *B. cereus* genes in CPA-8 by PCR-based technique.

MATERIAL AND METHODS

B. amyloliquefaciens CPA-8

The strain CPA-8 used in this study was obtained from the Postharvest Pathology Group Collection of the IRTA Centre (Lleida, Catalonia, Spain). It was isolated from the surface of a nectarine fruit in an experimental orchard in Lleida (Catalonia, Spain) and selected for its preliminary efficacy in reducing brown rot caused by *Monilinia* spp. in stone fruit (Casals *et al.*, 2010; Yánez-Mendizábal *et al.*, 2011). CPA-8 was firstly identified by 16S rRNA partial analysis by the Netherland Culture Collection of Bacteria as a member of the *B. subtilis* species complex and it has been recently classified as *B. amyloliquefaciens* (Gotor-Vila *et al.*, 2016). Cultures were maintained on nutrient yeast dextrose agar (NYDA: 8 g L^{-1} nutrient broth, 5 g L^{-1} yeast extract, 10 g L^{-1} dextrose and 20 g L^{-1} agar) at 30 ^{o}C for 24 h.

Assays to determine the effect of environmental factors on CPA-8 growth

Growth media

CPA-8 was grown in 250 mL conical flasks containing 50 mL of 863 basic medium (10 g L^{-1} peptone, 10 g L^{-1} yeast extract, and 20 g L^{-1} glucose) and incubated at 30 ^{o}C for 24 h (Yánez-Mendizábal *et al.*, 2011). Fresh inoculum was prepared by transferring a 24 h culture on NYDA to 5 mL of potassium phosphate buffer solution (PB) (70 mL KH_2PO_4 0.2 mol L^{-1}, 30 mL K_2HPO_4 0.2 mol L^{-1} and 300 mL deionized water, v/v/v, pH 6.5) and then added to each flask.

The combined effect of pH-temperature and a_w-temperature on CPA-8 growth

The pH of the 863 medium was adjusted to pH levels from 7.0 (control) to 3.5 using 5 mol L^{-1} malic acid with the pH meter GLP 21 (Crison Instruments S.A., Barcelona, Spain). In this study, seven pH levels were evaluated: 6.5, 6.0, 5.5, 5.0, 4.5, 4.0 and 3.5. For each a_w studies, 863 medium was osmotically modified by adding the non-ionic solutes glycerol or glucose in a broad a_w range from 0.998 (control) to 0.940 following the methodology described by Dallyn & Fox (1980) and Scott (1957), respectively. Specifically, the a_w levels were adjusted at 0.995, 0.990, 0.980, 0.970, 0.960,

0.950 and 0.940 and checked with an Aqualab (Decagon Devices, Pullman, Wash., USA) a_w meter to an accuracy of ± 0.003. For each pH and a_w conditions described, 200 µL of 863 media (modified or non-modified) were contained in 96-well microplates and inoculated by a quantity of a bacterial suspension of CPA-8 to obtain a final concentration adjusted at 10^5 CFU mL^{-1}. The plates were then incubated at 0, 4, 10, 20, 25, 30 and 37 °C. To monitor CPA-8 growth, changes in absorbance of all cultures were determined with a PowerWave HT Microplate Spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA) set at 700 nm and with 15 s of agitation prior to measurement. An uninoculated solution of the same composition for each pH or a_w condition served as negative control. Each condition was replicated three times and the experiment was conducted twice.

CPA-8 susceptibility to antibiotics

Growth medium and antibiotics

CPA-8 was cultured on nutrient yeast dextrose broth (NYDB, NYDA without agar) at 30 °C for 24 h and CPA-8 susceptibility to the following antibiotics was tested: chloramphenicol water soluble (CHL), ampicillin sodium salt (AMP), gentamicin (GEN), nalidixic acid sodium salt (NAL), streptomycin sodium salt (STR) and hygromycin (HYG). All antibiotics were evaluated at different concentrations: 0, 20, 40, 60, 80, 100, 200, 300, 400, 500, 750, 1000, 1250, and 1500 ppm. In the case of no-susceptibility observed, 2000, 3000, 4000, 5000, and 6000 ppm were also evaluated.

In vitro tests: CPA-8 susceptibility

All-purpose agar plates, including meat extract 20 g L^{-1}, glucose 20 g L^{-1} and agar 15 g L^{-1}, were overlaid with 5-6 mL of NYDA soft agar (7.5 g L^{-1}) and inoculated with 50 µL of the 24 h-old CPA-8 culture. Drops (5 µL) of the antibiotics solutions were spotted onto the lawn of the bacterium and incubated for 24-48 h at 30 °C. At each plate, four concentrations of each antibiotic were tested. Inhibition was recorded as positive/susceptible if a translucent halo zone was observed around the spot, or negative/resistant if a no translucent halo was observed. In the case of susceptibility, the diameter of the halo was measured. Three plates were performed for each evaluation and all of the experiments were repeated twice.

Detection of enterotoxic *B. cereus* genes in CPA-8 by PCR-based techniques

DNA extraction

Bacterial strains were cultured overnight at 30 ºC in NYDB and DNA was extracted according to the method described by Crespo-Sempere *et al.* (2013) and modified by Gotor-Vila *et al.* (2016). The amount and purity of DNA samples were determined spectrophotometrically (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE, USA) and DNA integrity was analysed by electrophoresis on 1 % agarose gels run at 100 V for 100 min with TBE (10.8 g Tris base, 5.5 g boric acid, 4 mL EDTA 0.5 mol L^{-1}), stained with gel red (GelRed™ Nucleic Acid Stain, 10.000X in water) and visualised with UV light.

Analysis of *B. cereus* genes of virulence on CPA-8

The presence of two enterotoxigenic protein complexes from *B. cereus* strains, hemolysin BL (HBL) and nonhemolytic enterotoxin (NHE) was checked in CPA-8. Different primer pairs were selected according to Hansen & Hendriksen (2001), Kumar *et al.* (2010), and Ngamwongsatit *et al.* (2008), whose work aimed the detection of the genes *hblA*, *hblC*, *hblD* and *nheA*, *nheB*, *nheC* (genes from HBL and NHE complexes, respectively) (Table 1). PCR was performed by using a peltier thermal cycler (GeneAmp PCR System 2700, Applied Biosystems, Foster City, CA, USA). The amplification reactions were prepared in 25 µL using 1.25 units DFS-Taq DNA polymerase in 2.5 µL 10X Complete NH4 Taq Buffer (Bioron GmbH, Ludwigs am Rhein, Germany), 200 µmol L^{-1} each dNTP, 50 ng DNA and 0.4 µmol L^{-1} each primer. A initial denaturation at 94 ºC for 5 min was followed by 30 cycles of 94 ºC for 30 s, x ºC for 45 s (Table 1), 72 ºC for 30 s and by a final extension step of 72 ºC for 7 min. The pathological reference strain *B. cereus* ATCC 14579, which exhibits detectable signal for the enterotoxic genes used in this study, was used as a positive control and negative controls (without DNA) were also included. Reaction products were analysed by electrophoresis on 1 % TBE buffer agarose gels stained with gel red and visualised with UV light. DNA standard 100 bp DNA Ladder H3 RTU (NIPPON Genetics Europe GmbH, Düren, Germany) was used as molecular size marker.

Table 1. Sequences of the primers, temperature of annealing, and size of the PCR products of the enterotoxic *B. cereus* genes tested in CPA-8: (1) Hansen and Hedriksen, 2001; (2) Ngamwongsatit et al. (2008), and (3) Kumar et al. 2010.

Target genes	Primers sequence (5′- 3′)	T annealing (°C)	Product (bp)
hblA (B component of HBL)	GTGCAGATGTTGATGCCGAT ATGCCACTGCGTGGACATAT	65	319
hblC (L2 component of HBL)	AATGGTCATCGGAACTCTAT CTCGCTGTTCTGCTGTTAAT	65	749
hblD (L1 component of HBL)	AATCAAGAGCTGTCACGAAT CACCAATTGACCATGCTAAT	65	429
nheA$_1$ (A component of NHE)	TACGCTAAGGAGGGGCA GTTTTTATTGCTTCATCGGCT	65	499
nheB$_1$ (B component of NHE)	CTATCAGCACTTATGGCAG ACTCCTAGCGGTGTTCC	65	769
nheC$_1$ (C component of NHE)	CGGTAGTGATTGCTGGG CAGCATTCGTACTTGCCAA	65	581
nheA$_2$	TAAGGAGGGGCAAACAGAAG TGAATGCGAAGAGCTGCTTC	58	759
nheB$_2$	CAAGCTCCAGTTCATGCGG GATCCCATTGTGTACCATTG	58	935
nheC$_2$	ACATCCTTTTGCAGCAGAAC CCACCAGCAATGACCATATC	58	618
nheA$_3$	AAGGCGAATGTACGAGAGTGG CTTCTCTCGTTTGACTATCTGCAG	58	553

Statistical analysis

To determine the effect of temperature, a_w, and pH on CPA-8 growth responses, the absorbance data were ln-transformed and plotted against time. For each pH-temperature or a_w-temperature combination, the maximum growth rate (μ_{max}) and lag phase duration (λ) were obtained by fitting the data to the primary model of Baranyi & Roberts (1994):

$$\text{Eq. (1)} \qquad y(t) = \mu_{max} F(t) - \ln\left(1 + \frac{e^{\mu_{max} F(t)} - 1}{e^{y_{max}}}\right)$$

and

$$\text{Eq. (2)} \qquad F(t) = t + \frac{1}{\mu_{max}} \ln(e^{-\mu_{max} t} + e^{-\mu_{max} \lambda} - e^{-\mu_{max} t - \mu_{max} \lambda})$$

Where $y(t)$ is the natural logarithm of absorbance values at time t (h), y_{max} is the natural logarithm of maximum absorbance value, μ_{max} is the maximum growth rate (h^{-1}) and λ is the lag phase duration (h).

Growth curves were fitted to Eq. (1) using the package JMP® 8.0 (SAS institute, Cary, NC, USA). Subsequently, analysis of variance (ANOVA) was performed to test the influence of each combination of CPA-8 growth condition on maximum growth rate and lag phase duration. Statistical significance was judged at the level of $P<0.05$. When the analysis was statistically significant, the Tukey's HSD test was used for the separation of means. Root-square transformation of the maximum growth rate was performed prior to analysis to normalise the data. Non-transformed means are presented.

RESULTS

Effect of the environmental factors on CPA-8 growth

CPA-8 was not able to grow on liquid 863 medium after being incubated at 0, 4 and 10 ºC for more than one month regardless of the pH and a_w. However, CPA-8 was maintained alive at the conditions mentioned (data not shown). The absorbance curves fitted by Baranyi model at the ranges of pH-temperature and a_w-temperature at which CPA could grow are shown in Figures 1-3.

The minimum pH at which CPA-8 was able to grow was 4.5 regardless of the temperature. In the conditions evaluated, the optimum growth was observed at 37 ºC and pH between 7 and 5 (Fig. 1). Maximum growth rates of 0.43-0.47 h^{-1} (Table 2a) and lag phase durations of 3.74-5.23 h (Table 2b) were recorded. On the contrary, the slowest growth was recorded at 20 ºC and pH 4.5 (Fig. 1) with a maximum growth rate of 0.12 h^{-1} (Table 2a) and lag phase duration of 34.41 h (Table 2b). Generally, for the same pH, the reduction of the incubation temperature from 37 to 30, 25 and 20 ºC reduced the maximum growth rate and increased the lag phase duration (Table 2a- b). Regarding the influence of pH on growth of CPA-8 at 37 and 30 ºC, the maximum growth rate was not significantly affected by pH (Table 2a). However, the lag phase duration significantly increased when pH was reduced to 5 or 4.5 (Table 2b). In contrast, when the temperature of incubation was lower (25 ºC), both growth parameters estimated were significantly affected by pH, since maximum growth rate decreased and lag phase duration increased when pH of the medium was modified from 7-5.5 to 5 and 4.5 (Table 2a-b). At 20 ºC, no influence of pH was observed

regarding the maximum growth rate. Nevertheless, the lag phase duration was also significantly increased when the pH of the medium was reduced to 5 and 4.5 (Table 2b).

In terms of the combined parameters a_w-temperature, regardless of the type of solute used to reduce a_w, the greatest growth was generally observed at 37 ºC and 0.998-0.990 a_w (Fig. 2-3) with maximum growth rates of 0.36-0.50 h^{-1} (Table 3a and Table 4a) and 3.48-4.49 h lag phase durations (Table 3b and Table 4b). On the contrary, the slowest growth was recorded at 20 ºC and 0.990 a_w (Fig 2-3), which was the lowest a_w at which CPA-8 was able to growth at this temperature (maximum growth rate of 0.09 h^{-1} and lag phase duration of 24.62 h when glucose was used as solute) (Table 4). The type of solute used to reduce a_w had a great influence in the minimum a_w at which the bacterium was able to grow, indicating that the a_w-temperature range with the solute glucose was more limited than with the solute glycerol. The lowest a_w for growth at 37 and 30 ºC was 0.950 in media modified with glycerol and at 25 ºC, no growth was observed at this a_w (Fig. 2). However, for the media modified with glucose, the lowest a_w for growth at 37 ºC and 30-25 ºC was 0.960 and 0.970, respectively (Fig.3). Although there is not a clear tendency in the maximum growth values when the a_w decreased for the same temperature, a significant increase in the lag phase duration is observed for both solutes. However, significant lower growth rate values and longer lag phase durations were generally detected when the temperature decreased for the same a_w (Table 3-4).

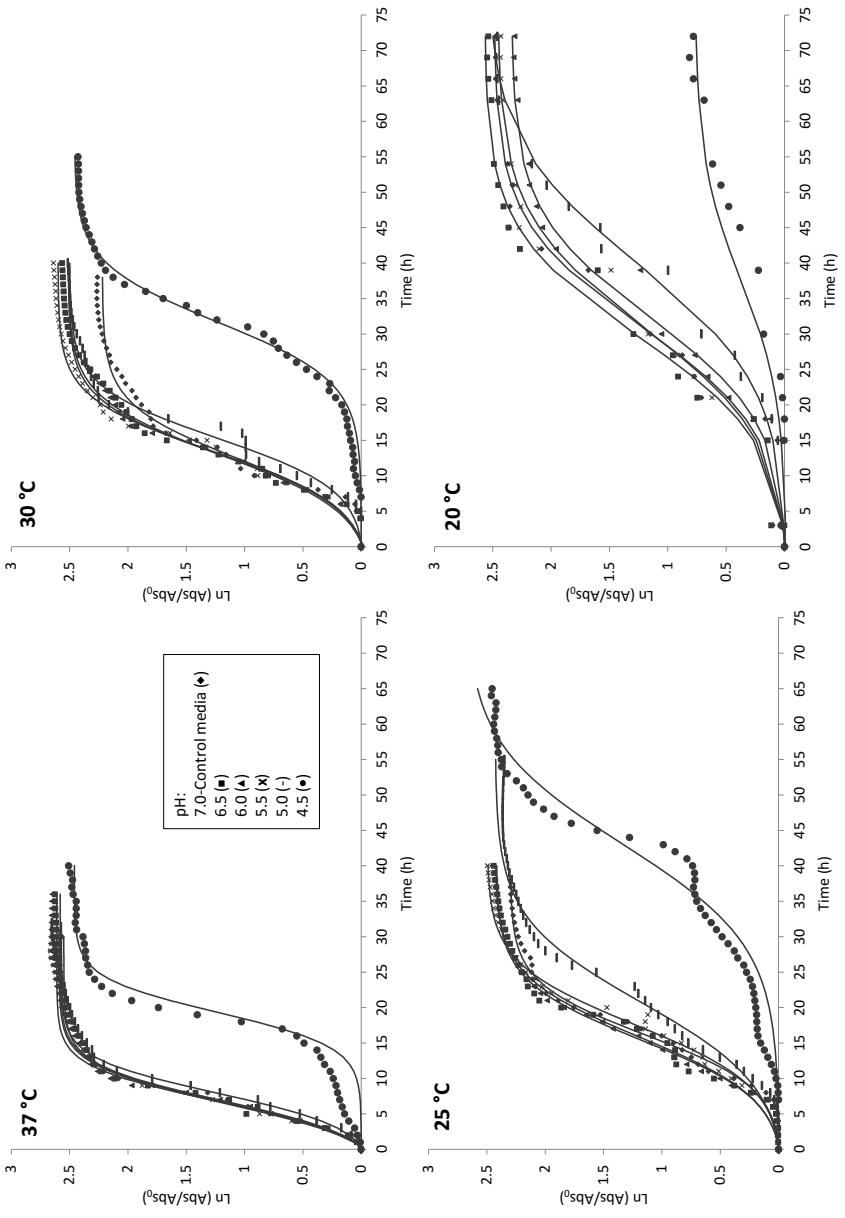

Figure 1. Absorbance growth curves of strain CPA-8 at different temperatures and pH. Symbols refer to experimental data and solid lines to curves fitted by Baranyi model.

Table 2a. Maximum specific growth rate (h^{-1}) of CPA-8 at different temperatures and pH estimated by Baranyi model. Root square transformation of the data was performed before statistical analysis. Non-transformed means are shown. Means with the same uppercase letter for each temperature or with the same lowercase letter for each pH are not significantly different ($P<0.05$) according to Tukey's HSD test.

	Temperature (°C)			
pH	37	30	25	20
7.0	0.45 Aa	0.29 Ab	0.30 Ab	0.15 Ac
6.5	0.43 Aa	0.28 Ab	0.26 ABb	0.15 Ac
6.0	0.47 Aa	0.30 Ab	0.27 Ab	0.15 Ac
5.5	0.43 Aa	0.30 Ab	0.26 ABb	0.14 Ac
5.0	0.46 Aa	0.31 Aa	0.19 BCa	0.13 Aa
4.5	0.46 Aa	0.27 Aab	0.16 Cab	0.12 Ab

Table 2b. Lag phase duration (h^{-1}) of CPA-8 at different temperatures and pH estimated by Baranyi model. Means with the same uppercase letter for each temperature or with the same lowercase letter for each pH are not significantly different ($P<0.05$) according to Tukey's HSD test.

	Temperature (°C)			
pH	37	30	25	20
7.0	4.18 Aa	8.77 Ab	12.31 ABb	22.81 ABc
6.5	3.88 Aa	8.61 Ab	11.21 Ac	21.32 Ad
6.0	4.16 Aa	8.96 Aab	11.04 Ab	24.63 ABc
5.5	3.74 Aa	9.16 Ab	12.83 ABc	22.67 ABd
5.0	5.23 Aa	11.31 Bb	14.52 Bc	30.29 BCd
4.5	15.65 Ba	27.12 Cb	34.71 Cc	34.41 Cc

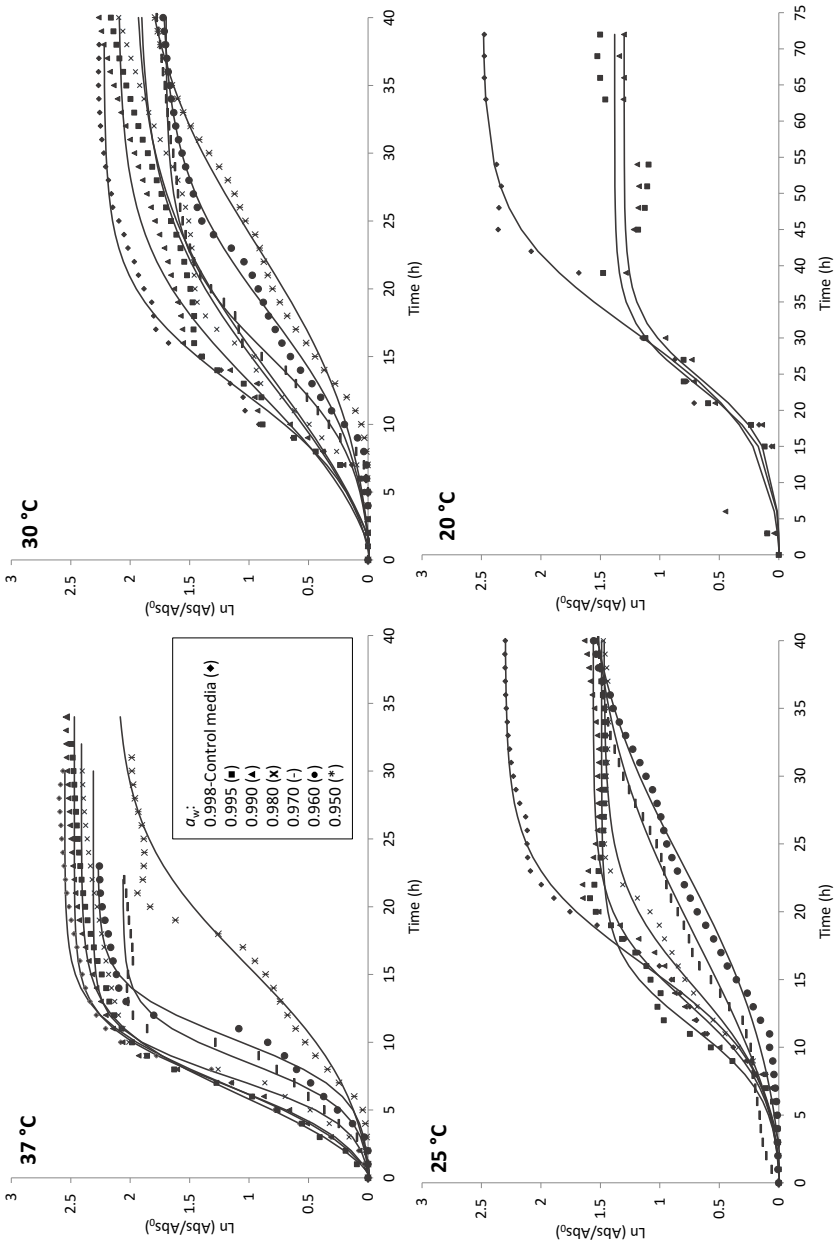

Figure 2. Absorbance growth curves of strain CPA-8 at different temperatures and water activity modified by adding glycerol. Symbols refer to experimental data and solid lines to curves fitted by Baranyi model.

Table 3a. Maximum specific growth rate (h^{-1}) of CPA-8 at different temperatures and a_w modified with glycerol solution estimated by Baranyi model. Root square transformation of the data was performed before statistical analysis. Non-transformed means are shown. Means with the same uppercase letter for each temperature or with the same lowercase letter for each a_w are not significantly different (P<0.05) according to Tukey's HSD test.

	Temperature (ºC)			
aw	37	30	25	20
0.998	0.45 CDa	0.26 Ab	0.30 Bb	0.15 Ac
0.995	0.42 Da	0.17 BCb	0.40 Aa	0.23 Ab
0.990	0.50 BCa	0.21 Bc	0.33 Bb	0.23 Abc
0.980	0.64 Aa	0.16 BCb	0.27 Bc	-
0.970	0.61 Aa	0.28 Ab	0.13 Cc	-
0.960	0.56 ABa	0.20 Bb	0.15 Cc	-
0.950	0.22 Ea	0.15 Cb	-	-

(-): No growth observed

Table 3b. Lag phase duration (h^{-1}) of CPA-8 at different temperatures and a_w modified with glycerol solution estimated by Baranyi model. Means with the same uppercase letter for each temperature or with the same lowercase letter for each a_w are not significantly different (P<0.05) according to Tukey's HSD test.

	Temperature (ºC)			
aw	37	30	25	20
0.998	4.18 Aa	7.83 Ab	12.31 Bb	22.81 Ac
0.995	3.48 Aa	7.96 Ab	9.69 Ab	20.47 Ac
0.990	4.40 Aa	8.01 Ab	10.92 ABc	21.36 Ad
0.980	5.57 Ba	8.19 Ab	11.75 ABc	-
0.970	6.86 Ca	12.08 Bb	12.24 Bb	-
0.960	8.21 Da	14.04 Cb	17.35 Cc	-
0.950	11.33 Ea	16.92 Db	-	-

(-): No growth observed

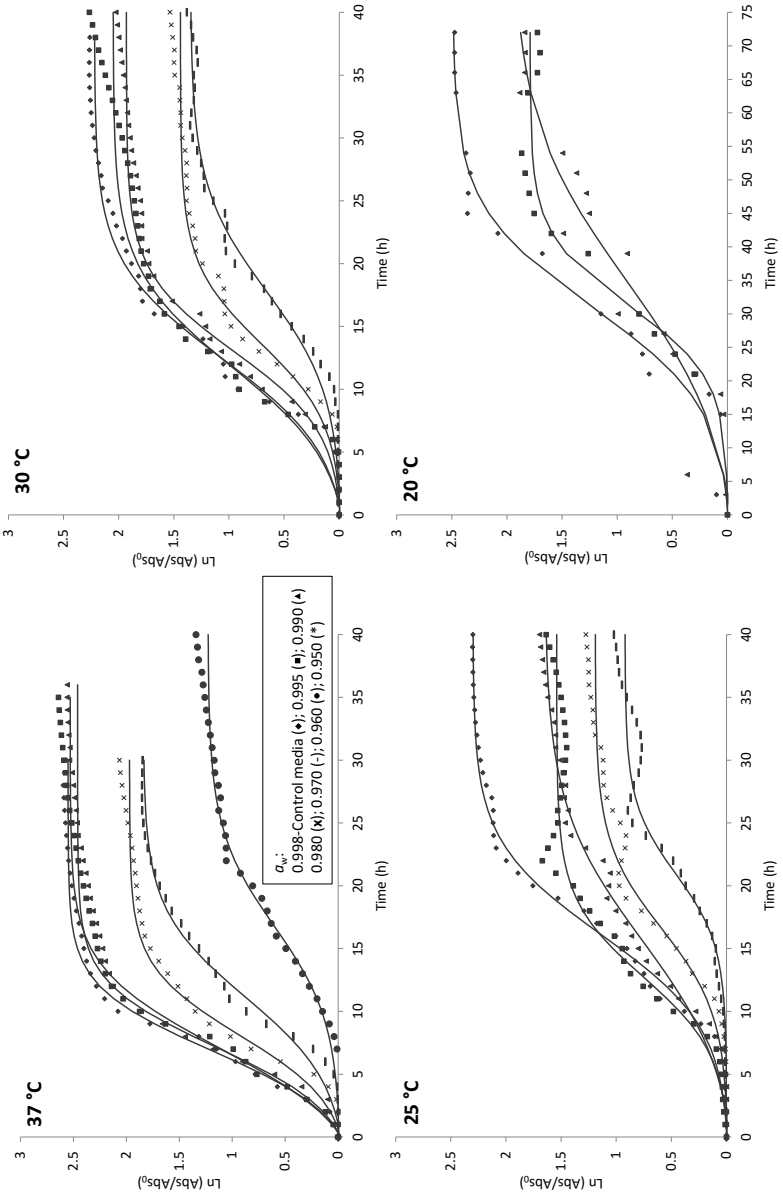

Figure3. Absorbance growth curves of strain CPA-8 at different temperatures and water activity modified by adding glucose. Symbols refer to experimental data and solid lines to curves fitted by Baranyi model.

Table 4a. Maximum specific growth rate (h^{-1}) of CPA-8 at different temperatures and a_w modified with glucose solution estimated by Baranyi model. Root square transformation of the data was performed before statistical analysis. Non-transformed means are shown. Means with the same uppercase letter for each temperature or with the same lowercase letter for each a_w are not significantly different (P<0.05) according to Tukey's HSD test.

	Temperature (ºC)			
aw	37	30	25	20
0.998	0.45 Aa	0.29 Ab	0.30 ABb	0.15 Ac
0.995	0.36 Aa	0.28 Aa	0.34 ABa	0.20 Aa
0.990	0.44 Aa	0.34 Aab	0.20 Bbc	0.09 Bc
0.980	0.40 Aa	0.33 Aa	0.30 ABa	-
0.970	0.31 Aa	0.25 Aa	0.36 Aa	-
0.960	0.27 aa	-	-	-
0.950	-	-	-	-

(-): No growth observed

Table 4b. Lag phase duration (h^{-1}) of CPA-8 at different temperatures and a_w modified with glucose solution estimated by Baranyi model. Means with the same uppercase letter for each temperature or with the same lowercase letter for each a_w are not significantly different (P<0.05) according to Tukey's HSD test.

	Temperature (ºC)			
aw	37	30	25	20
0.998	4.18 ABa	8.77 Bb	12.31 ABb	22.81 Ac
0.995	3.89 Aa	8.23 Ab	10.50 Ac	26.67 Ad
0.990	4.49 ABa	10.16 BCb	11.75 Ab	24.62 Ac
0.980	5.72 Ba	11.46 Cb	14.61 Bc	-
0.970	8.26 Ca	15.15 Db	19.67 Cc	-
0.960	14.52 Da	-	-	-
0.950	-	-	-	-

(-): No growth observed

CPA-8 susceptibility to antibiotics

The susceptibility or resistance of CPA-8 to different antibiotic substances was determined (Table 5). Our results showed that CPA-8 was clearly resistant to hygromycin at all concentrations tested (from 20 to 6000 ppm). Moreover, when CPA- 8 grew in the presence of chloramphenicol and streptomycin no halo was observed when they were used at concentrations from 20 to 80 ppm and from 20 to 200 ppm, respectively. In contrast, CPA-8 was susceptible to gentamicin, ampicillin and nalidixic acid at all concentrations evaluated except when nalidixic acid was used

at 20 ppm, in which the growth of CPA-8 could be distinguished. As it was expected, larger halo diameters were detected as the antibiotic concentrations increased.

Table 5. CPA-8 susceptibility and resistance to different antibiotic substances by detecting zone of inhibition (halo) around bacterial growth

	Antibiotics					
Concentration (ppm)	**CHL**	**AMP**	**GEN**	**NAL**	**STR**	**HYG**
0	+	+	+	+	+	+
20	+	- (8.7±0.6)	- (7.0±0.0)	+	+	+
40	+	- (8.2±0.3)	- (10.7±0.3)	- (9.3±0.6)	+	+
60	+	- (9.7±0.6)	- (10.7±0.6)	- (11.7±0.6)	+	+
80	+	- (9.7±0.6)	- (11.0±1.0)	- (13.3±1.5)	+	+
100	- (9.3±1.2)	- (10.8±1.9)	- (12.0±0.5)	- (13.3±2.6)	+	+
200	·	- (12.0±0.0)	- (14.0±1.0)	- (18.3±0.6)	+	+
300	·	- (11.3±1.5)	- (13.3±0.6)	- (19.3±1.2)	- (4.0±0.0)	+
400	·	- (11.7±2.5)	- (14.3±1.2)	- (22.0±0.0)	- (4.7±0.6)	+
500	- (18.2±1.9)	- (14.5±1.5)	- (14.3±0.6)	- (21.4±1.1)	- (8.5±2.9)	+
750	- (19.0±2.5)	- (19.7±0.6)	·	- (24.5±1.9)	- (14.0±0.0)	+
1000	- (20.2±2.7)	- (19.7±0.6)	·	- (26.3±1.0)	- (15.0±0.0)	+
1250	- (24.0±1.0)	- (19.7±0.6)	·	- (26.0±1.0)	- (16.0±0.0)	+
1500	- (23.8±1.0)	- (19.7±1.2)	·	- (27.7±1.2)	- (16.7±0.6)	+
2000	·	·	·	·	·	+
3000	·	·	·	·	·	+
4000	·	·	·	·	·	+
5000	·	·	·	·	·	+
6000	·	·	·	·	·	+

CHL: chloramphenicol, AMP: ampicillin, GEN: gentamicin, NAL: nalidixic acid, STR: streptomycin and HYG: hygromycin.
+: growth of CPA-8 (resistance)
- : no growth of CPA-8 (susceptibility). In brackets, diameter of the halo ± standard deviation (mm)
·: no data available

Detection of enterotoxic *B. cereus* genes

A molecular study to detect the presence of *B. cereus* enterotoxic genes in the strain CPA-8 was performed using PCR. The results showed that the genes *hblA*, *hblC*, *hblD*, *nheB* and *nhC* were not detected. However, *nheA* gene amplified when the primers described by Hansen & Hendriksen (2001) were used. Otherwise, this gene did not give rise to any DNA product when using the primer pairs described by either, Kumar *et al.* (2010) or Ngamwongsatit *et al.* (2008) (Fig. 4). It should be pointed out that NHE is a three-component enterotoxin which is composed by three genes (*nheA*, *nheB* and *nheC*) that constitute one operon, being all three components necessary for enterotoxigenic activity. Regarding these results, we suggest that the genes encoding the protein complexes (HBL and NHE), responsible for enterotoxigenicity in *B. cereus* foodborne pathogen, are not present in CPA-8.

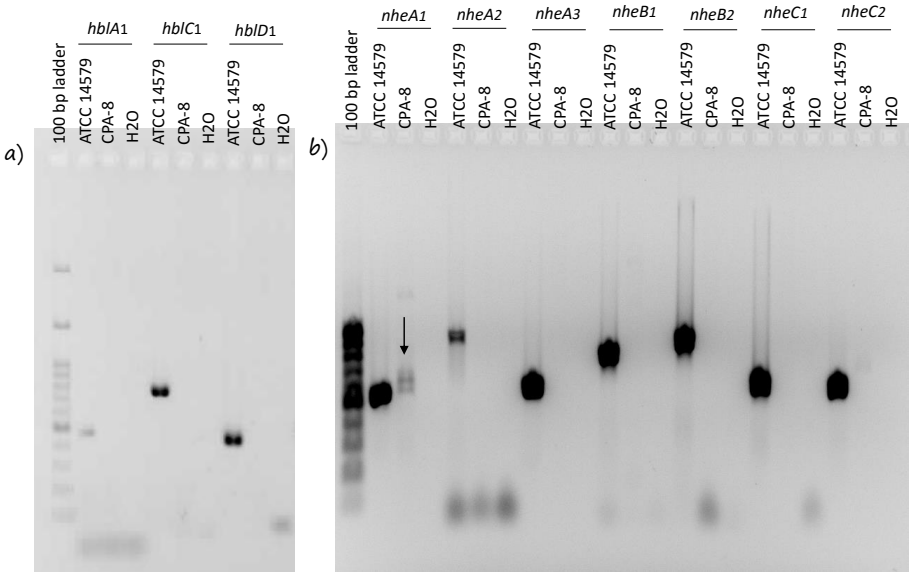

Figure 4. PCR products from enterotoxic *B. cereus* genes amplification. a) HBL complex and b) NHE complex. Target genes are indicated in the figure and subindex indicates the primer pairs used in the PCR: ($_1$) Hansen and Hedriksen, 2001; ($_2$) Ngamwongsatit et al. (2008), and ($_3$) Kumar et al. 2010, and. The CPA-8 *nheA*$_1$ gene amplification is indicated by an arrow ↓

DISCUSSION

This study is the first detailed investigation into the a_w, temperature, and pH relation on growth of the BCA CPA-8. In addition, a better biological characterisation of the microorganism has been accomplished.

These findings report the most favourable environmental conditions in which CPA-8 is able to grow, which is particularly relevant for predicting biocontrol response. On the whole, CPA-8 could grow in a wide range of stress conditions. Specifically, the results have confirmed that CPA-8 grew faster in warmer temperatures. Minimum temperature is an intrinsic property of the organisms when growth conditions other than temperature are non-limiting (Ratkowsky *et al.*, 1982) and for CPA-8 growth seems to be higher than 10 ºC. Maximum growth was obtained in a no modified media and neutral pH when incubation temperatures were 37 and 30 ºC, suggesting that CPA-8 is a mesophilic bacterium. This strain is a really adapted microorganism to high temperatures, providing an advantage when a BCA has to be applied at field conditions (specifically in summer time) and needs to be formulated by drying technologies. However, although the results obtained in this work indicate that CPA-8 could not grow at cold temperatures, we could observe that CPA-8 survived at temperatures lower than 10 ºC. Moreover, the maintenance of CPA-8 cells on peaches after storage at 0 ºC has already been demonstrated (Yánez-Mendizábal *et al.*, 2012a). These data indicate that CPA-8 is not restricted to warm temperatures for storage conditions.

According to Padan *et al.* (2005), who described that neutral or even basic pH are required for better bacterial development, acidic pH under *in vitro* conditions seemed an important restriction for the growth of CPA-8. Nevertheless, the work conducted by Yánez-mendizábal *et al.* (2012a) also proved that CPA-8 cells remained over the surface of artificially wounded peaches and nectarines with pH lower than 4.5.

Regarding water availability, the minimum a_w at which this BCA was able to grow at 37 ºC was 0.960 when glucose was used to modify the medium and 0.950 at 30 and 37 ºC when glycerol was used. However, the minimum a_w was 0.990 at 20 ºC,

which was the lowest temperature at which CPA-8 grew among the temperatures tested. These results are in agreement with those obtained by Mossel *et al.* (1995), who observed that a large proportion of bacilli are not able to grow when the a_w is lower than 0.950.

A similar study with the BCA *Pantoea agglomerans* CPA-2 (Costa *et al.*, 2002) described that the minimum a_w for growing was 0.960 in media modified with glycerol and 0.950 in media modified with NaCl or glucose. As we could observe, the solute used to reduce a_w has a great influence on bacterial growth, especially at unfavourable conditions (low temperature). It has been reported that some bacteria, such as *P. agglomerans* and *Bacillus* spp., generally respond to elevated ionic-strength media by synthesising or accumulating any of a variety of osmolytes (e.g., glycine-betaine, proline) (Loshon *et al.*, 2006; Teixidó *et al.*, 2005). These compounds enable the microorganism to retain water in the cytoplasm and thus maintaining turgor pressure and providing tolerance to desiccation conditions (Loshon *et al.*, 2006; Teixidó *et al.*, 2005). Therefore, additional studies should be done to define the compatible solutes accumulated by CPA-8.

Few works have already reported how important is to provide a detailed description of the ecophysiology of the BCAs and the pathogens. Teixidó *et al.* (1998) described the variety of responses of the yeast *Candida sake* to water, temperature, and pH stress, suggesting that changes in nutrients and a_w may be useful for improving environmental competence of the microorganism in the environment. Moreover, the comparison of a_w and temperature impacts on growth of *Fusarium langsethiae* strains from northern Europe indicated which environmental profiles could be beneficial for improving the ecological knowledge of these fungui (Medina & Magan, 2010).

In order to accomplish a better understanding of the biology of CPA-8, two different analysis have also been conducted: antibiotic sensibility and production of enterotoxins. A panel of six common antibiotic substances belonging to different antibiotic groups such as penicillin, quinolones and aminoglycosides was tested against CPA-8 growth. If the growth of the organism is inhibited by the action of the substance, it has been reported as susceptible to that antibiotic. A clearly resistance was observed when hygromycin was applied at all concentrations tested, even at 6000 ppm. The results obtained suggest that CPA-8 can grow in the presence of

hygromycin as a tool for avoiding other microbial contamination during different stages of the CPA-8-based products development such as, formulation process or shelf-life storage. To the best of our knowledge, little is known about the antibiotic sensibility patterns of the BCAs. In the work reported by Kadaikunnan *et al.* (2015), *B. amyloliquefaciens* VJ-1 was classified as sensitive or resistant to an antibiotic according to the diameter of inhibition zone given in a standard antibiotic disc chart. However, whereas some results could be comparable, hygromycin was not evaluated.

Regarding the CPA-8 capability for encoding genes responsible for the production of enterotoxins, a PCR test was carried out. *B. cereus* is traditionally considered the most problematic member of the genus *Bacillus* in the food industry due to the ability of many strains to produce enterotoxins (Phelps & McKillip, 2002). Many *B. cereus* isolates express at least two distinct multiple-component enterotoxins: a tripartite hemolytic heat-labile enterotoxin designated HBL and the nonhemolitic enterotoxin NHE (Hansen & Hendriksen, 2001; Phelps & McKillip, 2002). Toxicological activity has also been found in other *Bacillus* species including *B. circulans*, *B. lentus*, *B. mycoides* and, *B. subtilis* (Beattie & Williams, 1999). Conversely, the results obtained in this work suggest that CPA-8 do not encode either HBL or NHE proteins. Although the subunit NheA amplified by using the primer pair described by Hansen & Hendriksen (2001), specific studies exist which demonstrated that all three components (NheA, NheB, and NheC) are necessary for cytotoxic activity (Lindbäck *et al.*, 2004; López & Alippi, 2010).

In summary, this study has shown that from an ecological point of view, CPA-8 has a wide tolerance to different pH-temperature and a_w-temperature profiles which should enable this strain to grow actively under a wide range of environmental conditions. This work also focused on the better understanding of the CPA-8's biology. Results from antibiotic tests have been obtained and the absence of *B. cereus* HBL and NHE enterotoxins has been demonstrated. Thus, the findings here reported meaningfully contribute to the characterisation of *B. amyloliquefaciens* CPA-8 in order to complete registration requirements for developing a microorganism-based commercial product.

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Chapter III

Antifungal effect of volatile organic compounds produced by *Bacillus amyloliquefaciens* CPA-8 against fruit pathogen decays of cherry

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ABSTRACT

The present work focuses on the antifungal effect of volatile organic compounds (VOCs) produced by *Bacillus amyloliquefaciens* CPA-8 against *Monilinia laxa*, *Monilinia fructicola* and *Botrytis cinerea*, three postharvest fruit pathogens of sweet cherry fruit. VOCs were evaluated with a double petri dish assay against mycelial and colony growth of target pathogens. For this purpose, CPA-8 was grown on different media and cultured for 24 and 48 h at 30 ^{o}C before assays. Data showed that mycelial growth inhibition was higher when CPA-8 was grown on Tryptone Soya Agar (TSA) while no differences were generally observed when CPA-8 was cultured for either, 24 or 48 h. Moreover, no effects were observed on colony growth. The main volatile compounds emitted by CPA-8 were identified by solid-phase microextraction (SPME)-gas chromatography as 1,3 pentadiene, acetoin (3-hydroxy-2-butanone) and thiophene. Pure compounds were also tested *in vitro* on mycelial growth inhibition and their EC_{50} values against the three pathogens were estimated. Thiophene was the most effective VOC, showing more than 82 % suppression of mycelial growth at the highest concentration (1.35 μL mL^{-1} headspace) and EC_{50} values ranging from 0.06 to 6.67 μL mL^{-1} headspace. Finally, the effectiveness of thiophene and CPA-8 VOCs was evaluated against artificially inoculated cherry fruit. Among the target pathogens, *M. fructicola* was clearly controlled by CPA-8 with less than 25 % of rotten fruits compared to the control (65 % disease incidence) and for all pathogens, less than 37.5 % of CPA-8 treated decayed fruit produced spores (disease sporulation). Otherwise, pure thiophene showed no effect against any pathogen on disease incidence and disease sporulation. The results indicated that VOCs produced by *B. amyloliquefaciens* CPA-8 could develop an additive antifungal effect against postharvest fruit pathogens in stone fruit.

Keywords: Biocontrol; *Bacillus* spp.; *Monilinia* spp.; *Botrytis* spp.; VOCs; cherry.

INTRODUCTION

Postharvest decay of fruit presents a major factor causing postharvest losses and limits the duration of storage and shelf-life of produce. Numerous fungal pathogens infect stone fruit after harvest, including the wound-invading fungi *Monilinia* spp. and *Botrytis* spp., and cause economically important diseases of stone fruit worldwide (Mari *et al.*, 2016; Usall *et al.*, 2015). *Monilinia* rot is specially responsible for substantial postharvest losses, reaching even as high as 80 % in years when the climate conditions are favorable for the development of the disease, especially in late-ripening varieties (Usall *et al.*, 2015).

Traditionally, synthetic fungicides have been used to control postharvest decays; however, the appearance of fungicide-resistant population of pathogens and the concerns of the consumers about the possible toxicological risks of the residues have resulted in the need of developing other methods that involve a reduction in the number of field chemical applications (Droby *et al.*, 2016; Sharma *et al.*, 2009; Usall *et al.*, 2016). The biological control of postharvest pathogens using microbial antagonists has been the focus of considerable research over the last 30 years by many scientists and several commercial companies worldwide (Droby *et al.*, 2016) although it is not already routinely applied in fruit industry.

Antagonists can display a wide range of modes of action, at different stages of their activity, relating to different hosts and pathogens. Sometimes, different modes act simultaneously and it is therefore difficult to establish which individual mechanism has contributed to a specific antifungal function (Di Francesco *et al.*, 2016). To clarify the mechanism of action, as well as the understanding of biocontrol systems, it is crucial to know the interactions among environment, pathogen, and biocontrol agent (BCA) and, therefore, the expected biocontrol efficacy (Parafati *et al.*, 2015).

Bacillus strains exhibit various cytological traits, such as stress-resistant endospore formation and the synthesis of extracellular enzymes, to compete for niches (Nihorimbere *et al.*, 2011). Other mechanisms of action, such as volatile organic compounds (VOCs), siderophore production and the induction of reactive oxygen species (ROS) in the host, are under investigation (Asari *et al.*, 2016; Elshakh *et al.*, 2016; Zheng *et al.*, 2013).

Chapter III

Among the substances produced by BCAs, VOCs are frequently involved in the biological control of several fungal diseases of fruit. These compounds typically constitute a complex mixture of low-molecular weight lipophilic compounds derived from different biosynthetic pathways by many microorganisms as part of their metabolism. Some of these secondary products could be potentially employed with success as gaseous treatments in a process defined by the term biofumigation (Di Francesco *et al.*, 2016; Mari *et al.*, 2016). The microbial activity of VOCs produced by *Bacillus* strains has been widely studied. In the nineties, Fiddaman & Rossall (1994) reported the importance of substrate on the production of antifungal volatiles from *Bacillus subtilis* and the response of fruit pathogens to the volatile organic compounds produced by *Bacillus amyloliquefaciens* strains has been described during recent years (Asari *et al.*, 2016; Raza *et al.*, 2016; Yuan *et al.*, 2012). Integration of VOCs as a different strategy to achieve higher levels of disease control will contribute to a successful handling of postharvest diseases (Mari *et al.*, 2016).

B. amyloliquefaciens CPA-8 -formerly *B. subtilis*-, has been previously reported as BCA due to its effectiveness against postharvest diseases caused by *Monilinia* spp. and *Botrytis* spp. (Casals *et al.*, 2012; Yánez-Mendizábal *et al.*, 2011). Regarding its mode of action, the work conducted by Yánez-Mendizábal *et al.*, (2012b) provided experimental evidence about the strong antifungal effect against *Monilinia* species, mainly based on fengycin-like lipopeptides production. However, the synthesis of this product could not be considered as the only mechanism of action.

The objective of the present work was to investigate the antifungal effect of the VOCs produced by CPA-8 against three postharvest fruit pathogens on cherries. In order to do this, (i) an *in vitro* approach was used to evaluate the antifungal effect of CPA-8 on colony and mycelial growth against *Monilinia laxa*, *Monilinia fructicola* and *Botrytis cinerea*; (ii) compounds emitted by CPA-8 were identified by using the SPME-gas chromatographic technique, (iii) the effect of pure compounds on target pathogens was tested *in vitro* and (iv) the antifungal activity of CPA-8 VOCs and pure thiophene was assayed on cherries artificially inoculated with *M. laxa*, *M. fructicola* and *B. cinerea*.

MATERIALS AND METHODS

Microorganisms and culture media

The antagonist CPA-8 was isolated from a nectarine surface by the Postharvest Pathology Group of IRTA (Lleida, Catalonia, Spain) and has been recently reclassified as member of *B. amyloliquefaciens* species (Gotor-Vila *et al.*, 2016). Stock cultures were stored at 4 °C and subcultured on Nutrient Yeast Dextrose Agar (NYDA: 8 g L^{-1} nutrient broth, 5 g L^{-1} yeast extract, 10 g L^{-1} dextrose and 20 g L^{-1} agar) at 30 °C for 24 h when required. Fresh bacteria were suspended in potassium phosphate buffer (PB, 70 mL KH_2PO_4 0.2 mol L^{-1}; 30 mL K_2HPO_4 0.2 mol L^{-1} and 300 mL deionized water v/v/v pH 6·5) and adjusted by hemocytometer to a final concentration of 10^7 CFU mL^{-1}.

As pathogens, *M. laxa* (co33), *M. fructicola* (McL4), isolated from decayed stone fruit, and *B. cinerea*, isolated from decayed kiwi fruit, were obtained in Bologna (Italy) and identified by the Department of Agricultural Sciences CRIOF-DipSA (Bologna, Italy). They were maintained on Potato Dextrose Agar (PDA, Sigma-Aldrich, St. Louis, MO, USA, 39 g L^{-1}) plates at 25 °C for a maximum of 15 days. Conidia from the strains were collected and suspended in sterile distilled water containing 0.05 % (v/v) Tween 80. The suspension was adjusted by hemocytometer to a final concentration of 10^3 conidia mL^{-1}.

In vitro antagonistic activity of VOCs produced by CPA-8

The efficacy of the VOCs produced by CPA-8 on the mycelium and colony growth of the target pathogens was tested by the double petri dish assay. For this purpose, 100 µL of CPA-8 cell suspension (10^7 CFU mL^{-1}) were sprayed in petri dishes containing three different growth media, parafilmed, and incubated for 24 and 48 h at 30 °C. The culture media used in this study were NYDA, Nutrient Agar (NA, Oxoid, Cambridge, UK, 31 g L^{-1}) supplemented with glucose 20 g L^{-1} (NAglu20) and Triptone Soya Agar (TSA, Oxoid, Cambridge, UK, 40 g L^{-1}). After CPA-8 incubation time, two different trials were done. (i) For colony growth trials, the lid of the plate was removed and replaced by a base plate inoculated with 100 µL of a conidia suspension of each pathogen (10^3 conidia mL^{-1}). For *M. laxa* and *M. fructicola* conidia suspension, PDA base plates where used. In case of *B. cinerea*, the suspension was sprayed on Malt Extra Agar (MEA, Oxoid, Cambridge, UK,

33.6 g L^{-1}) base plates. The two base plates were sealed immediately with double layer of parafilm and incubated at 25 °C. After two days, the number of colonies were counted. (ii) For mycelial growth trials, mycelial agar discs (5-mm square plug) of every fungus were placed in the centre of the PDA base plate when either *M. laxa* or *M. fructicola* was used or MEA base plates for *B.cinerea*. Every double petri dish was double sealed as previously described and incubated at 25 °C for 3 days. The diameter of the colony was measured and expressed in millimetres. The percentage of inhibition of the colony and mycelial growth was calculated on the basis of the difference between treatment and control according to the formula: ((C-T)/C) x 100, where C is the control value and T is the measurement of the fungus in each antagonist-fungal set-up. The sample unit was represented by nine plates (replicates) for each pathogen and antagonist interaction in every condition mentioned. Plates without CPA-8 served as control.

Analysis of the VOCs produced by CPA-8

A qualitative evaluation of CPA-8 VOCs composition was done using HeadSpace Solid Phase MicroExtraction (HS)-SPME coupled with gas chromatography tandem mass spectrometry analysis (GC-MS) according to the method previously described by Di Francesco *et al.*, (2015) with modifications. SPME fibre (2cm- 50/30 µm DVB/CAR/PDMS, Supelco Inc, Bellefonte, PA, USA) was preconditioned according to manufacturer's recommendations and exposed to the headspace of CPA-8 petri dishes for 5 min at 30 °C. Analysis were performed in CPA-8 plates grown in the optimum media tested above for 24 and 48 h.

Trapped compounds were then thermally desorbed from the fibre for 2 min in the GC injection port at 250 °C in the split-less injection mode. For peak separation and detection, a Bruker GC 451 gas chromatograph equipped with a HP-5 fused silica capillary column (30 m by 0.25 mm inside diameter; 0.25 µm film thickness, J&W Scientific Inc, Folsom, CA, USA) connected to a quadrupole mass detector Bruker Scion SQ Premium (Bruker Daltonics, Macerata, Italy) was used. The transfer line was heated at 250 °C, the ion source at 220 °C, and carrier gas (He) flow rate was 1 mL min^{-1}. The mass spectrometer was operated in electron impact mode at 70 eV, scanning the range of 35/500 m/z in a full scan acquisition mode. The GC oven temperature was set at 40 °C for 4 min and then programed to rise from 40 to 90 °C at 10 °C min^{-1}, from 90 to 160 °C at 5 °C min^{-1}, and from 160 to 280 °C at 40 °C min^{-1}. The tentative identification of VOCs was

done by comparing the mass spectra and the retention times with the data system library (NIST 11 MS Library) and GC peak data were used to estimate the relative abundance (relative peak area, RA) of each volatile compound. Blank sample analysis (growth medium not inoculated with CPA-8) was performed under the same conditions in order to exclude interfering substances. All measurements were made with three replicates, each replicate representing the analysis of a different petri dish.

Antifungal activity of selected synthetic VOCs

Pure standards of the most representative VOCs produced by CPA-8 and identified by GC-MS analysis (Table 1) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and individually tested for suppressing mycelial growth of target pathogens. For this purpose, mycelial agar discs (5-mm square plug) were placed in the centre of petri dishes containing PDA or MEA, depending on the pathogen. Then, a paper filter (90 mm diameter) with different aliquots of pure compounds: 15, 30 and 60 µL were positioned inside the cover of the dishes. The aliquots of pure compounds introduced in the petri dishes corresponded to 0.34, 0.67 and 1.35 µL mL^{-1} headspace, respectively. The dishes were immediately doubled sealed with parafilm and incubated at 25 ^{o}C for three days. The sample unit was represented by three replicates for each dose and pathogen and plates with a non-spread paper filter were used as control. The percentage of inhibition of mycelial growth was calculated according to the formula described above and EC_{50} values (expressed as µL mL^{-1}) were calculated as the effective headspace concentration that inhibit fungal mycelial growth by 50 % in comparison to the control.

***In vivo* test of disease control by CPA-8 and pure thiophene**

Two different trials were conducted with 'Skeena' cherries to evaluate the antagonistic activity of both, the volatiles produced by CPA-8 and the pure compound thiophene, to control brown rot caused by *M. laxa* and *M. fructicola* and grey mold caused by *B. cinerea*. Fruit selected without visible injuries and rots and homogeneous in maturity and size was wounded in the equator with a sterile nail (3 mm wide and 3 mm deep) and inoculated with 15 µL of *M. laxa, M. fructicola* or *B. cinerea* conidial suspension (10^3 conidia mL^{-1}).

In the CPA-8 trials, sterile plastic boxes (30 x 19 x 12 cm. LxWxH) containing in the bottom a thin layer of TSA (400 mL) were inoculated 24 h before with 2 mL of a CPA-8 suspension adjusted at 10^7 CFU mL^{-1} and incubated at 30 ºC. For thiophene evaluation, six paper filters (90 mm diameter) were spread with 60 µL of the pure volatile compound each and placed in the bottom of sterile plastic boxes. Then, the inoculated cherries were placed inside the box. To avoid the contact between fruit and substrate, a sterile grid was used. Boxes non-inoculated with the antagonist or non-spread with thiophene were used as control. The boxes were closed with plastic lids and double sealed with parafilm. For each pathogen and trial (natural VOCs or pure thiophene), five cherries constituted a single replicate and each treatment was replicated four times. The percentage of rotten fruit (disease incidence) and the percentage of rotten fruit with spores on the surface (disease sporulation) were determined after four days of storage at 20 ºC and 85 % relative humidity, RH.

Data analysis

Data on the percentage of colony and mycelial growth inhibition was calculated according to the formula described before. Disease incidence and sporulation were also analysed and expressed as percentage. Differences in mycelial growth inhibitions as well as differences in disease incidence and sporulation data were evaluated using analysis of variance (ANOVA) with the JMP®8 statistical software (SAS Institute, Cary, NC, USA). Statistical significance was judged at the level $P<0.05$. When the analysis was statistically significant, the Tukey's HSD Test was used for separation of the means. EC_{50} of each substance was calculated using the probit analysis applied to the percentage of mycelial growth inhibition (Lesaffre & Molenberghs, 1991).

RESULTS

***In vitro* antagonistic activity of VOCs produced by CPA-8**

Data from the double petri dish assays indicated that VOCs produced by CPA-8 inhibited the mycelial growth of all tested pathogens (*M. laxa*, *M. fructicola* and *B. cinerea*) with variable efficacy depending on the growth media of CPA-8 (Fig. 1). On the whole, mycelial growth inhibition was higher when CPA-8 was cultured on TSA medium than when NYDA or NAglu20 were used. Otherwise, the antagonistic activity was the same

(data no significant) when CPA-8 was cultured for either, 24 and 48 h. In detail, higher values of mycelial growth inhibition were observed for *M. laxa* when CPA-8 grew on TSA medium (>78.6 %) compared to NYDA and NAglu20 (no more than 53.4 %). Similar results were obtained for *B. cinerea*, showing the highest mycelial growth inhibition when CPA-8 was grown on TSA medium for 24 h (86.8 %). When *M. fructicola* was tested, CPA- 8 grown on TSA for 24 h also showed the best results (68.6 %) and no differences were observed compared to CPA-8 grown on NAglu20 for 24 h (48.5 %). Regarding the colony growth, no differences were observed between the control and CPA-8 in all conditions tested. However, the size of the colony of each pathogen was meaningfully smaller when CPA-8 was co-incubated (data not shown).

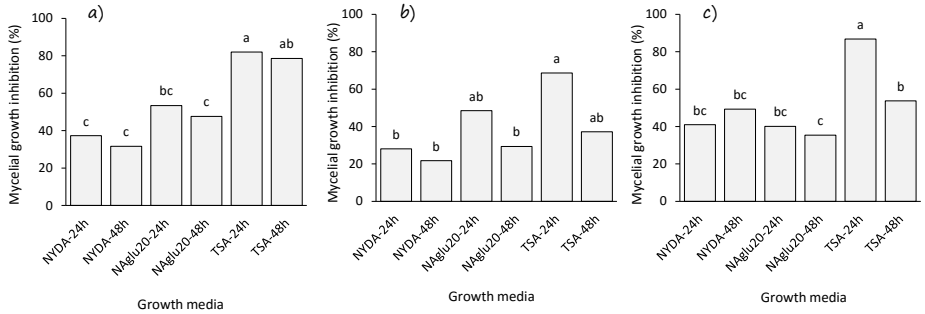

Figure 1. Effects of volatile organic compounds from *B. amyloliquefaciens* CPA-8 grown on NYDA, NAGlu20 and TSA medium for 24 and 48 h at 30 ºC on mycelial growth inhibition of a) *M. laxa*, b) *M. fructicola* and, c) *B. cinerea*, previously incubated at 25 ºC for three days on PDA or MEA medium. Within the same figure, different letters indicate significant differences (P<0.05) according to Tukey's HSD test.

SPME GC-MS analysis of VOCs produced by CPA-8

The headspace analysis indicated that CPA-8 grown on TSA produced a diverse volatile profile including ketones, aromatic compounds, hydrocarbons, and esters. Table 1 shows the tentative identification of the most representative VOCs emitted by CPA-8 plates but not by control plates. No differences were observed between CPA-8 cultured for 24 and 48 h. The compound 1,3 pentadiene was the most abundant VOC produced by CPA-8 (highest RA) followed by acetoin (3-hydroxy-2-butanone), thiophene, and ethylacetate. Compounds such as 2 butanone, 1-butanol 3-methyl and 1-butanol 2-methyl were also found in both tests, CPA-8 and control (data not shown), probably due to the protein source of the growth medium used. For this reason, these compounds have not been considered.

Table 1. Most representative volatile organic compounds detected and identified by SMPE-GC-MS analysis in the headspace of *B. amyloliquefaciens* CPA-8 plates grown in TSA medium for 24 h at 30 ºC. Retention Time (RT), Molecular Weight (MW) and GC peak Relative Area (RA) are shown.

RT (min)	Compound	MW	RA (%)
1.8	1,3 Pentadiene	68.12	61.01
2.3	Ethylacetate	88.11	6.61
3.0	Thiophene	84.14	11.32
6.8	Acetoin	88.11	21.06

Antifungal activity of selected synthetic VOCs

The pure VOCs 1,3 pentadiene, acetoin, and thiophene were tested for antifungal activity against *M. laxa*, *M. fructicola*, and *B. cinerea*. Results showed that thiophene was the most effective one in mycelial growth inhibition, showing over 82 % suppression when the highest concentration was used (1.35 µL mL^{-1} headspace). For this compound, EC_{50} values ranging from 0.06 to 6.67 µL mL^{-1} headspace were obtained for the target pathogens (Table 2). No inhibition was observed when 1,3 pentadiene was used against *M. laxa* and *M. fructicola* at any concentration tested; however, approximately 50 % of mycelial growth inhibition was observed against *B. cinerea* at the two highest concentrations (0.67 and 1.35 µL mL^{-1} headspace) with EC_{50} values over 10^5 µL mL^{-1} headspace (Table 2). Acetoin showed poor antifungal activity against *M. laxa* and *B. cinerea* (<50 %) and no mycelial growth inhibition was observed against *M. fructicola* (Table 2).

Table 2. Antifungal activity of pure volatile organic compounds on the *in vitro* mycelial growth inhibition (%) tests against *M. laxa*, *M. fructicola* and *B. cinerea*. Three different concentrations of each compound were used (0.34, 0.67, and 1.35 µL mL^{-1} headspace). When possible, EC_{50} values were represented (µL mL^{-1} headspace).

Pathogen	Compound	Concentration	Mycelial growth inhibition	EC_{50}
M. laxa	Acetoin	0.34	9.7	-
		0.67	8.7	
		1.35	23.2	
	1,3 Pentadiene	0.34	ni	-
		0.67	ni	
		1.35	ni	
	Thiophene	0.34	44.0	2.62
		0.67	68.9	
		1.35	95.1	
M. fructicola	Acetoin	0.34	ni	-
		0.67	ni	
		1.35	ni	
	1,3 Pentadiene	0.34	ni	-
		0.67	ni	
		1.35	ni	
	Thiophene	0.34	50.7	0.06
		0.67	86.4	
		1.35	100.0	
B. cinerea	Acetoin	0.34	34.3	-
		0.67	41.4	
		1.35	47.1	
	1,3 Pentadiene	0.34	30.0	$2.20 \cdot 10^5$
		0.67	62.9	
		1.35	57.1	
	Thiophene	0.34	27.6	6.67
		0.67	48.6	
		1.35	82.9	

ni: no mycelial growth inhibition observed
-: < 50 % mycelial growth inhibition. Insufficient data to calculate EC_{50} values

In vivo test of disease control by CPA-8 and thiophene

Results obtained from the *in vivo* tests are shown in Figures 2 and 3. Regarding the percentage of disease incidence, the pathogen *M. fructicola* was clearly susceptible to VOCs produced by CPA-8 (Fig. 2). In this case, less than 25 % of rotten fruit (compared to 65 % in the control) was observed. Otherwise, no significant differences (P<0.05) were detected between treatments and control in *M. laxa* and *B. cinerea*. Concerning the presence of spores on the surface of rotten fruit (Fig. 3) it could be observed that for all pathogens, less than 37.5 % of rotten fruit showed spores on the surface when CPA-8 VOCs were used. Furthermore, the quantity of the spores observed was small and

restricted to the wound (Fig. 4). The VOCs produced by CPA-8 against *M. laxa* and *M. fructicola* showed more than 41 % reduction of the percentage of disease sporulation compared with the control but no statistically differences were found between treatments due to the variability of the data (Fig. 3). However, results for *B. cinerea* revealed a complete disease sporulation reduction (100 %) as no spores were observed over the decayed fruit (Fig. 3). Otherwise, when thiophene was tested, no effect was observed in either disease incidence or disease sporulation against any of the target pathogens (Fig. 2 and Fig. 3).

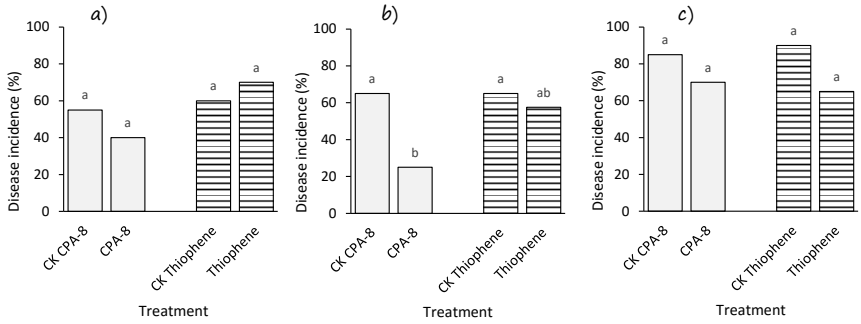

Figure 2. *In vivo* antagonistic activity of volatile organic compounds from *B. amyloliquefaciens* CPA-8 grown on TSA ( ) and the pure compound thiophene ( ). The figure shows the disease incidence (percentage of rotten fruit) of cherry fruit artificially inoculated with a) *M. laxa*, b) *M. fructicola,* and c) *B. cinerea* and incubated for four days at 20 ºC and 85 % RH. CK means control treatment, without CPA-8 or thiophene, respectively. Within the same figure, different letters in the same column pattern indicate significant differences (P<0.05) according to Tukey's HSD test.

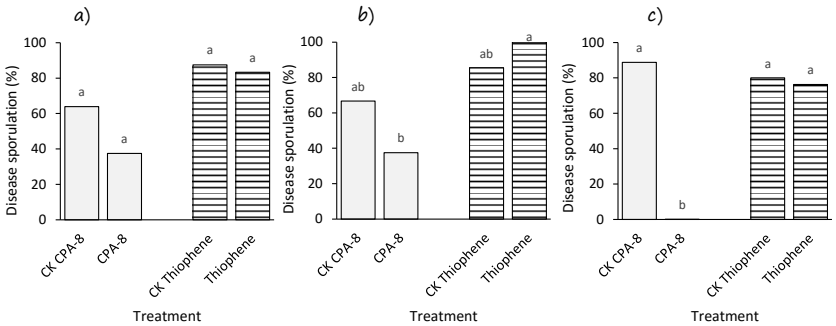

Figure 3. *In vivo* antagonistic activity of volatile organic compounds from *B. amyloliquefaciens* CPA-8 grown on TSA ( ) and the pure compound thiophene ( ). The figure shows the disease sporulation (percentage of rotten fruit with spores on the surface) of cherry fruit artificially inoculated with a) *M. laxa*, b) *M. fructicola* and c) *B. cinerea* and incubated for four days at 20 ºC and 85 % RH. CK means control treatment, without CPA-8 or thiophene, respectively. Within the same figure, different letters in the same column pattern indicate significant differences (P<0.05) according to Tukey's HSD test.

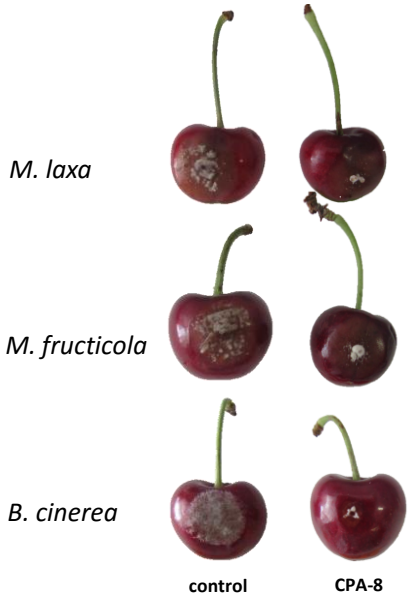

Figure 4. Effect of the volatile organic compounds from TSA cultures of *B. amyloliquefaciens* CPA-8 on sporulated cherry fruit artificially inoculated with *M. laxa*, *M. Fructícola*, and *B.cinerea*.

DISCUSSION

In this study, the antifungal effects of VOCs produced by *B. amyloliquefaciens* CPA-8 against the postharvest fruit pathogens *M. laxa*, *M. fructicola*, and *B. cinera* were determined. The results of the antagonistic activity in the preliminary *in vitro* assays demonstrated that VOCs emitted by CPA-8 were able to supress the mycelial growth of all target pathogens. Regarding the raw data obtained, the diameter of the colonies of all pathogens ranged from 18.7 to 64.7 mm in case of the control plates and from 0 to 7.8 mm when the pathogens were co-incubated with CPA-8. Results also indicated that the antifungal activity of CPA-8 volatiles was variable, depending on the growth media, with TSA probing to be the most effective. Therefore, this results are in agreement with the development of effective formulations for CPA-8, in which the low-cost media used for the production of this bacterium contained defatted soya flour as nitrogen source (Yánez-Mendizábal *et al.*, 2012a). The works previously conducted by Fiddaman and Rossall (1933, 1994) also reported the importance of the substrate on the production of antifungal volatiles; however, they indicated that NA and diluted TSA media were poor substrates for *B. subtilis* volatile production.

Whereas our results demonstrated a clear reduction in the mycelial growth, no differences (except the size of the colony) were observed in the colony growth when comparing the control and CPA-8, indicating that the volatiles from this bacterium have a fungistatic effect rather than a fungicidal action towards the target pathogens. Furthermore, VOCs produced by CPA-8 did not show selective effect against any target pathogen.

The work conducted by Arrebola *et al.* (2010) previously revealed microscopic observations in which VOCs from *Bacillus* strains induced morphological abnormalities on the conidia of *Penicillium crustosum*. Other authors have also reported the ability of volatiles produced by different *Bacillus* species to inhibit not only mycelial growth but also spore germination and tube elongation of *B. cinerea*, *Penicillium* spp. and *Fusarium oxyxporum* (Arrebola *et al.*, 2010; Chen *et al.*, 2008; Yuan *et al.*, 2012). These data suggest that VOCs produced by CPA-8 could represent an effective tool in the biocontrol of postharvest diseases caused by fungi.

In order to understand the nature of VOCs produced by CPA-8, SPME coupled with GC-MS was used. This simple and rapid technology for sampling volatile compounds at low concentrations in headspace analysis has been successfully used to characterise the VOCs profile produced by fungi, bacteria and yeasts (Chaves-Lopez *et al.*, 2015; Di Francesco *et al.*, 2015; Strobel *et al.*, 2001). The fibre used in this analysis was previously optimised by Di Francesco *et al.* (2015). The dual coated fibre selected allowed to extract a great number of compounds and also adsorb volatile molecules at low concentration in a wide molecular weight range due to its physico-chemical characteristics suitable for complex mixtures. CPA-8 VOCs were detected after 24 and 48 h of bacteria incubation and no different peak areas were observed between different culture times. As expected, the relative percentage of compounds detected in the headspace analyses did not vary due to the physiological state of the bacterium after being cultured 24 h. However, these results differ from the work conducted by Di Francesco *et al.* (2015) on yeasts in which different VOCs were observed starting from 48 h of yeast incubation. The main volatile compounds produced by CPA-8 were identified as 1,3 pentadiene, acetoin (3-hydroxy-2-butanone), and thiophene. Although most of the compounds detected have already been reported to be produced by different *Bacillus* strains (Arrebola *et al.*, 2010; Chaves-Lopez *et al.*, 2015), it should be taken into account that the methodologies applied to collect and detect VOCs can strongly influence the results and often confuse the comparison between

different studies. Moreover, RA values should not be extrapolated as quantitative considerations because these data not only depend on compound concentration but also on the fibre affinity and the detector sensing to the different analytes.

Pure synthetic compounds were purchased to determine the EC_{50} values against the fungal pathogens in *in vitro* mycelial growth inhibition tests. The compound thiophene resulted the most effective, showing over 82 % suppression of mycelial growth at the highest concentration (1.35 µL mL^{-1} headspace) and EC_{50} values ranging from 0.06 to 6.67 µL mL^{-1} headspace depending on the pathogen. Fokialakis et al., (2006) reported that constituents from bioactive extracts from different species of the genus *Echinops* resulted in the isolation of eight thiophenes possessing varying degrees of termiticidal activity against the Formosan subterranean termite, whose ability to damage wood and trees is widely known. However, to the best of our knowledge, little is known about thiophene production from BCAs and the ecological significance of sulphur compounds is still poorly understood. Regarding the use of either acetoin or 1,3 pentadiene, poor results were observed, suggesting that higher concentrations were needed. Nevertheless, VOCs produced by microorganisms are commonly found at very low concentrations and their effect is supposed to be due to synergic or additive action and not to a single component activity (Mercier & Jimenez, 2004; Strobel *et al*., 2001). Therefore, more research would be necessary to determine lethal concentrations of the VOCs produced by CPA-8 and whether their combined action against fungal growth is additive or synergistic.

The inhibitory effect of VOCs from TSA cultures of CPA-8 on wounded cherry fruit artificially inoculated with *M. laxa*, *M. fructicola* and *B. cinerea* was also demonstrated, providing the best results for *M. fructicola* disease incidence. Furthermore, CPA-8 reduced and minimised the presence of spores on the surface of decayed fruit and soft rot symptoms were generally observed in all target pathogens. *B. cinerea* reached a complete suppression in the percentage of sporulation disease, indicating that VOCs from CPA- 8 are able to fit in well with the wound environment of artificially inoculated cherry fruit. These data meaningfully differ from the results obtained under *in vitro* conditions, suggesting that the high biocontrol efficacy observed when CPA-8 was applied against fungal mycelial growth *in vitro* is not always enough to explain the accumulative effects of several control mechanisms occurred in *in vivo* assays. It is known, for instance, that

each volatile compound has its own production or release dynamic depending on the available volume/headspace (Mercier & Jimenez, 2004).

Even though some studies reported *in vitro* analysis of VOCs produced by *Bacillus* species, little is known about volatiles produced by *Bacillus* spp. in controlling postharvest disease of fruit. Chen *et al.* (2008) demonstrated that volatiles generated by *B. subtilis* JA had significant effect on *B. cinerea* inhibition. Furthermore, the volatiles produced by *B. subtilis* and *B. amyloliqueaciens* also had a significant reduction in the decay incidence of citrus diseases in *in vitro* and *in vivo* trials in oranges (Arrebola *et al.*, 2010).

This work, investigates for the first time the capability of the volatiles produced by CPA-8 as an effective mechanism of action against postharvest fruit pathogens. In this context, this study provides experimental evidence about the antifungal effect and biological control ability of CPA-8 to reduce cherry rot caused by *M. laxa*, *M. fructicola* and *B. cinerea*. Although Yánez-Mendizábal *et al.* (2012b) have previously described the main mode of action of CPA-8 based on lipopeptide production, this works proves that this mechanism is not the only factor that needs to be considered in a biological control program for fruit disease. Future research would be needed on designing agriculturally acceptable and practical ways for an efficient use of *B. amyloliquefaciens* CPA-8 antifungal volatiles to keep stone fruit quality during postharvest storage, distribution, and marketing period.

Acknowledgments

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Chapter IV

Formulation of the biocontrol agent Bacillus amyloliquefaciens CPA-8 using different approaches: liquid, freeze-drying and fluid- bed spray-drying

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ABSTRACT

The present work focuses on the assessment and comparison of three different formulation technologies and the effect of protectants on cell viability, storage stability, and antagonistic activity of the biocontrol agent *Bacillus amyloliquefaciens* CPA-8. Cultures were concentrated with different protective substances such as $MgSO_4$, sucrose, and skimmed milk (SM) and subjected to liquid formulation, freeze-drying, and fluid-bed spray-drying. Results showed that CPA-8 freeze-dried cells without protectants or amended with SM suffered the highest losses in cell viability (0.41-0.48 log). Moreover, the cell viability of the tested freeze-dried products decreased after four months of storage at both tested temperatures (4 and 22 ^{o}C). Otherwise, liquid and fluid-bed spray-dried products were stable for four months at 4 ^{o}C and for twelve months at 22, 4 and -20 ^{o}C, respectively, and no effect of the protectants was observed. The most suitable CPA-8 products were then tested against *Monilinia laxa* and *Monilinia fructicola* on artificially wounded nectarines and in all cases, the antagonistic activity was maintained similar to fresh cells. The efficacy results revealed that the formulation process did not affect the biocontrol potential of CPA-8. This work led us to conclude that effective formulations with final concentrations ranging from $1.93 \cdot 10^9$ to $2.98 \cdot 10^9$ CFU mL^{-1} and from $4.76 \cdot 10^9$ to $1.03 \cdot 10^{10}$ CFU g^{-1} were obtained for liquid and dried products, respectively. Additionally, the suitability of the fluid-bed spray-drying technology should be taken into account to develop a stable and effective CPA-8 product for practical applications to control brown rot in stone fruit.

Keywords: *Bacillus* spp., protectants, shelf-life, *Monilinia* spp., biocontrol efficacy.

INTRODUCTION

Postharvest decays of fruit have been traditionally controlled by synthetic pesticides. However, the appearance of pathogen resistance to these compounds and the public concerns about environmental contamination and human health have revealed the need to develop other methods that would help reduce field chemical applications (Droby *et al.*, 2009; Janisiewicz & Korsten, 2002; Sharma *et al.*, 2009).

Postharvest losses can reach high values, reaching up to 50 % of the total fruit production (Nunes, 2012). Brown rot caused by the wound-invading fungus *Monilinia* spp. is one of the most important postharvest diseases affecting stone fruit (Larena *et al.*, 2005; Mari *et al.*, 2007). The application of environment-friendly approaches such as those using biological control agents (BCAs), either alone or in combination with physico-chemical treatments, has been strongly considered in recent years (Casals *et al.*, 2012; Casals *et al.*, 2010; Mari *et al.*, 2007; Usall *et al.*, 2015).

The spore-forming ability of *Bacillus* species provides high resistance to extreme environmental conditions, making this genus a good candidate for developing stable and efficient biocontrol products (Mari et al. 2014). The efficacy of the BCA *Bacillus amyloliquefaciens* CPA-8, formerly known as *B. subtilis* (Gotor-Vila *et al.*, 2016), has been previously described against postharvest diseases caused by *Monilinia* spp. based on its ability to produce fengycin-like lipopeptides (Yánez-Mendizábal *et al.*, 2012a; Yánez-Mendizábal *et al.*, 2012d)

One of the major bottle necks in the commercialisation of biocontrol products is the development of shelf-stable formulations (Droby *et al.*, 2009; Navarta *et al.*, 2011). A useful microbial formulation should be economical to produce, easy to distribute to the intended environment, contain enough colony forming units (CFU), and provide a long shelf-life (preferentially stored at room temperatures and maintained for at least six months) (Melin *et al.*, 2007; Teixidó *et al.*, 2011). Finally, biological formulations should maintain the efficacy against plant pathogens similar to fresh cells.

Formulations can be accomplished by different methods including liquid and dry preparations. Compared to liquid forms, dried products obtained by spray-drying,

drying with fluidised-bed dryers or freeze and vacuum-drying are more feasible due to the storage capability, transportation, and their ability to produce large amounts of dried products at low cost. However, dried products frequently show low viability rates because of the thermal and dehydration stress suffered during the drying process (Abadias *et al.*, 2005; Melin *et al.*, 2007). Nevertheless, the microbial cell viability and efficacy resulting after the formulation process can be improved by the addition of certain stabilising substances (e.g., polymers, sugars, albumin, milk, salts, honey, polyols or aminoacids) to the formulation medium. Components of the formulation media have two main functions in preserving the viability of formulated cells: to provide a dry residue and thus acting as a receptor in the rehydration process and also to protect biochemically the cells against damage during the drying process (Abadias *et al.*, 2001; Melin *et al.*, 2011; Sabuquillo *et al.*, 2010).

The genus *Bacillus* is considered very amenable to drying because of its capability of spore production, which provides heat tolerance (Nicholson *et al.*, 2000; Yánez-Mendizábal *et al.*, 2012a). The study of spray-drying effect on *Bacillus* strain CPA-8 has been recently reported and spray-dried CPA-8 formulations have been obtained with final concentrations around 1.6–3.3 $\cdot 10^9$ CFU g^{-1} and 28-38 % of powder recovery using a pilot spray-dryer without preconditioning (Yánez-Mendizábal *et al.*, 2012b). The study of alternative formulation techniques, liquid or solid, amended or not with protecting agents can contribute to recover higher rates of the product with final concentrations considered acceptable for practical applications. Regarding drying methods, a lot of work is done by freeze-drying or fluidised-bed drying microorganisms. However, little is known about the innovative combination of spray-drying and conventional fluidised-bed drying techniques (fluid-bed spray-drying).

This work aimed to evaluate and compare different technologies to formulate CPA- 8: liquid, freeze-drying, and fluid-bed spray-drying. Specifically, we studied the following: (i) the effect of different protectants on cell viability and residual moisture content of CPA-8 formulations; (ii) the shelf-life and the effect of the storage temperature on CPA-8 formulations, and finally (iii) the efficacy of CPA-8 formulations against *Monilinia laxa* and *Monilinia fructicola* on nectarines.

MATERIALS AND METHODS

Antagonist strain and culture conditions

CPA-8 was originally isolated from a nectarine surface, identified by the Netherlands Culture Collection of Bacteria and belongs to the Postharvest Pathology Group Collection of IRTA (Lleida, Catalonia, Spain). Stock cultures were stored at 4 °C and subcultured on nutrient yeast dextrose agar (NYDA: 8 g L^{-1} nutrient broth, 5 g L^{-1} yeast extract, 10 g L^{-1} dextrose and 20 g L^{-1} agar) at 30 °C for 24 h when required. CRIOBILLES AEB 400100 (AES laboratory, Combourg, France) were used for long term storage at -80 °C.

Fresh bacteria suspended in potassium phosphate buffer (PB, 70 mL KH_2PO_4 0.2 mol L^{-1}; 30 mL K_2HPO_4 0.2 mol L^{-1} and 300 mL deionised water v/v/v pH 6.5) were used to inoculate a 5 L (BIOSTAT-A modular fermenters, Braun Biotech International, Melsungen, Germany) or 2 L (BioFlo/CelliGen 115, Eppendorf, New Brunswick, Canada) bioreactors containing growth medium as previously described Yánez-Mendizábal *et al.* (2012c) and slightly modified in the nitrogen source manipulation process: 100 g L^{-1} extracted defatted soy flour, 5 g L^{-1} molasses, 1.9 g L^{-1} KH_2PO_4, 0.001 mg L^{-1} $CuSO_4$, 0.005 mg L^{-1} $FeCl_3 \cdot 6H_2O$, 0.004 mg L^{-1} Na_2MoO_4, 0.002 mg L^{-1} KI, 3.6 mg L^{-1} $MnSO_4 \cdot H_2O$, 0.92 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.14 mg L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.01 mg L^{-1} H_3BO_3 and 10 mg L^{-1} $C_6H_8O_7$. The initial inoculum was adjusted at $2 \cdot 10^5$ CFU mL^{-1} with an spectrophotometer (Yánez-Mendizábal et al., 2012c) and cells were grown for 68-72 h at 30 °C to obtain high endospore concentration (Yánez-Mendizábal *et al.*, 2012a). Agitation was set to 200 rev min^{-1} and the air feeding rate was 0.33 vvm. Antifoam (1 ml per litre) was added if needed (30 % Simethicone emulsion USP, Dow Corning®, USA).

Pathogens

M. laxa (CPML2) and *M. fructicola* (CPMC1) were isolated from decayed stone fruit, identified by the Department of Plant Protection of INIA (Madrid, Spain) and belongs to the Postharvest Pathology Group Collection of IRTA (Lleida, Catalonia, Spain). They were maintained on potato dextrose agar plates (PDA, Biokar Diagnostics, 39 g L^{-1}) at 25 °C for a maximum of 15 days. To induce conidial production, healthy fruit (peaches or

nectarines) were artificially infected with the pathogens. Infected fruit were maintained at 20 ºC and 85 % relative humidity (RH) in the dark for 7 days.

Preparation of CPA-8 formulations

CPA-8 liquid formulation

For liquid formulation (LQF), cells were grown as described above and harvested by centrifugation at 9820 *g* for 12 min at 10 ºC in an Avanti J-20 XP centrifuge (Beckman Coulter, CA, USA). The resulting pellet was resuspended in the same CPA-8 supernatant medium to include the antifungal lipopeptides synthesised by the bacterium during the production process. Samples of 50 mL of CPA-8 prepared approximately at $5 \cdot 10^9$ CFU mL^{-1} were mixed with 10 % sucrose, 10 % powder skimmed milk (SM), 10 % $MgSO_4$, 10 % sucrose plus 10 % SM or 10 % $MgSO_4$ plus 10 % SM and homogenised at 150 rev min^{-1} for 20 min at room temperature. One sample was non-supplemented with protectants and used as control (without protectant, WP). For each LQF product, the initial cell concentration per millilitre (CFU mL^{-1}) was determined by plating ten-fold dilutions on NYDA medium. The experiment was conducted twice. Subsequently, all LQF products were kept at 4 ºC.

CPA-8 freeze-drying formulation

For each suspension prepared in LQF (including WP), samples of 50 mL each were distributed in autoclaved flasks and frozen at -20 ºC overnight. Thereafter, the flasks were connected to a freeze-dryer (Cryodos, Telstar, S.A, Terrasa, Catalonia, Spain) operating at 1 Pa and -50 ºC for 24 h. The experiment was conducted twice and the dried products were stored at 4 and 22 ºC.

To determine the CPA-8 viability after freeze-drying (FD), three replicate samples (0.5 g) of each FD product were rehydrated with 5 mL of PB. Samples were shaken vigorously for 1 min and then allowed to rehydrate for other 9 min in static. Ten-fold dilutions of each suspension were plated on NYDA to determine the cell concentration per gram (CFU g^{-1}). The relative cell viability was calculated for each FD product by the difference between the CPA-8 concentration after drying and the initial concentration. The rate of surviving cells was calculated as follows:

$$\text{Relative cell viability} = \text{Log}\ (N_f / N_i)$$

Where N_i represents the CFU in the suspension of CPA-8 before being formulated: population of the suspension (CFU mL^{-1}) x amount of solution (mL), and N_f is the CFU of CPA-8 obtained after drying: population of the powder (CFU g^{-1}) x amount of powder (g).

To calculate the moisture content of the CPA-8 dried products, duplicate samples of 0.5 g were placed in aluminium-weighing boats and dried in a convection oven at 100 °C for 24 h. The dry matter was calculated based on the weight loss after drying and expressed as % RH.

CPA-8 fluid-bed spray-drying formulation

Fluid-bed spray-drying (FBSD) of CPA-8 was performed with cells cultured for 68-72 h, harvested by centrifugation and resuspended approximately at 10^{10} CFU mL^{-1} as described before. Suspensions of 500 mL each were prepared as control (WP) or supplemented with 10 % $MgSO_4$ as protectant. A fluid-bed spray-dryer (HüttlinGmbH, Bosch Packaging Technology Company, Schopfheim, Germany) was used. Each suspension was sprayed by a 0.8 mm nozzle in bottom-spray position using a peristaltic pump, applying a spraying air pressure of 80 kPa. Each trial was sprayed onto 300 g of powdered carrier material previously loaded into the drying camera of the pilot scale fluid-bed spray-dryer. To facilitate the grain formation, 3.5 g of binder were added to the cells solution. Inlet air temperature was set to 65 ^{o}C which resulted in a maximal product temperature of 42 ^{o}C depending on the spraying rate, which ranged between 4 and 4.5 g min^{-1} to avoid the agglomeration of particles. The experiment was repeated and the FBSD products were stored at 22, 4, and -20 ^{o}C.

To optimise the final powder recovery, potato soluble starch, pregelatinised potato starch and pea protein were tested as carrier materials and two different binders were evaluated: pregelatinised potato starch and hydroxymethylpropylcellulose (HPMC).

The viability of CPA-8 cells after FBSD was calculated for both, the control and the suspension with 10 % $MgSO_4$ by the difference between the CFU after the drying process

(rehydrated with PB) and before drying, as it has been explained for FD products. The moisture content of each formulation was also estimated.

Shelf-life and effect of storage temperature on viability of CPA-8 formulations

Polypropylene tubes containing solid and liquid products were sealed with laboratory film (Parafilm 'M'; Picgenet Plastic Packaging, Chicago, IL, USA) and then kept in an airtight container filled with silica gel in order to avoid product humidification. CPA-8 LQF products were maintained under refrigeration conditions (4 ^{o}C). Solid formulations were stored at 22 and 4 °C (FD products) and at 22, 4 and -20 °C (FBSD products). To determinate the shelf-life of each product, cell viability was determined monthly for four months for LQF and FD products and for a year in the case of FBSD products. Three replicates of each product and temperature were sampled and ten-fold dilutions were plated on NYDA to determine the CFU mL^{-1} or CFU g^{-1}. In case of dried formulations, 0.5 g were rehydrated in 5 mL of PB.

Antagonistic activity of CPA-8 formulations against *M. laxa* and *M. fructicola* on stone fruit

Antagonistic activity of CPA-8 to control brown rot caused by *M. laxa* and *M. fructicola* was tested on nectarines cv. 'Early Sungrand'. Treatments were prepared from those liquid and solid CPA-8 products that showed the best cell viability after formulation and their efficacy was compared to fresh cells and water as the treatment without CPA-8 (control CK).

Fruit selected without visible injuries and rots and homogeneous in maturity and size was stored at 0 °C and 85 % RH until required for experimentation (not exceeding 15 days). Fruit was wounded in the equator with a sterile nail (3 mm wide and 3 mm deep) and inoculated with 15 µL of *M. laxa* or *M. fructicola* conidial suspension. Conidia of each pathogen were scraped from infected fruit using a sterile loop and transferred to 5 mL of sterile distilled water amended with Tween-80 (one drop per litre). Conidia concentrations were adjusted at 10^3 conidia mL^{-1} by hemocytometer.

After 2 h at room temperature, a 15 µL suspension (10^7 CFU mL^{-1}) of each CPA-8 treatment was applied. The treatments were prepared as follows: CPA-8 fresh suspension from 72 h-old culture in NYDB medium (NYDA without agar) and formulated CPA-8

treatments from stored powders or liquid suspensions based on CPA-8 concentrations determined on the storage stability assay. Five nectarines constituted a single replicate and each treatment was replicated four times. The percentage of fruit infected (disease incidence) and the mean lesion diameter of brown rot (disease severity) were determined after five days of storage at 20 ^{o}C and 85 % RH.

Data analysis

Relative cell viability of CPA-8 was estimated on the basis of CFU counted before (fresh cells) and after being formulated (dried cells). Brown rot incidence and severity data were also analysed and expressed as percentage of rotten fruit and rot diameter over the fruit surface (cm), respectively. Incidence percentage was transformed to the arcsine of the square root to normalise the data. Differences in relative cell viability as well as the brown rot incidence and severity data were evaluated using analysis of variance (ANOVA) with the JMP®8 statistical software (SAS Institute, Cary, NC, USA). Statistical significance was judged at the level $P<0.05$. When the analysis was statistically significant, the least significance difference (LSD) test was used for mean separation. Cell viability (CFU mL^{-1} and CFU g^{-1}) from the shelf-life study were log-transformed to achieve a normal distribution of the data and plotted in figures where the error was represented by the standard deviation (±SD) of three replications of each sampling data.

RESULTS

Effect of protectants on shelf-life of CPA-8 after liquid formulation

In order to test whether addition of a specific additive can protect CPA-8, either 10 % sucrose, 10 % SM, 10 % $MgSO_4$, 10 % sucrose plus 10 % SM or 10 % $MgSO_4$ plus 10 % SM was mixed with CPA-8 cells and the cell viability of each product was compared to cells that were resuspended in a formulation medium without protectants. The initial concentrations of CPA-8 LQF products ranged from 2.92 to 4.47 $\cdot 10^9$ CFU mL^{-1}. After four months of storage (Fig. 1), no appreciable alterations in cell viability were observed (1.93-2.98 $\cdot 10^9$ CFU mL^{-1}). Furthermore, no effects were detected in the addition of protectants to the formulation medium. According to these results, all liquid preparations showed

acceptable shelf-life and the CPA-8 LQF product with no protectants (WP) was selected for efficacy evaluation.

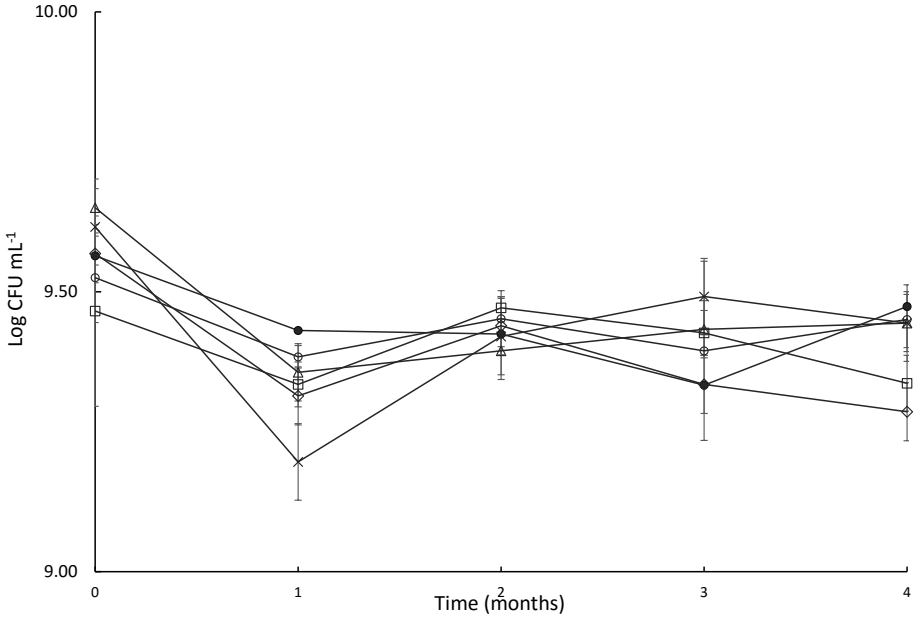

Figure 1. Cell viability of CPA-8 liquid products stored at 4 ºC in the absence "WP" ( ) and in the presence of the protectants 10 % sucrose ( ), 10 % SM ( ), 10 % $MgSO_4$ ( ); 10 % sucrose-10% SM ( ) and 10 % $MgSO_4$-10 % SM ( ). Values are the averages of three determinations and bars indicate the standard deviations.

Effect of protectants on viability of CPA-8 after freeze-drying formulation

The protective ability of sucrose, SM, and $MgSO_4$ on CPA-8 during the FD process was evaluated against non-amended (WP) CPA-8 cells (Fig. 2). The ANOVA analysis revealed significant differences between formulations ($F_{5,29}$=46.33 P<0.0001). After means separation, the results indicated a positive effect in the addition of protectants. The use of 10 % $MgSO_4$ plus 10 % SM showed the best results in CPA-8 viability after the drying process (0.17-log cell viability reduction) but not significant differences were observed when it was compared to 10 % sucrose and 10 % $MgSO_4$. However, when CPA-8 was amended with only SM, the CPA-8 viability was reduced 0.41-log. For FD products without protectants (WP), the cell viability was also decreased almost five times (0.48-

log). For shelf-life and efficacy evaluation, CPA-8 FD products supplemented with $MgSO_4$ alone and combined with SM, were selected.

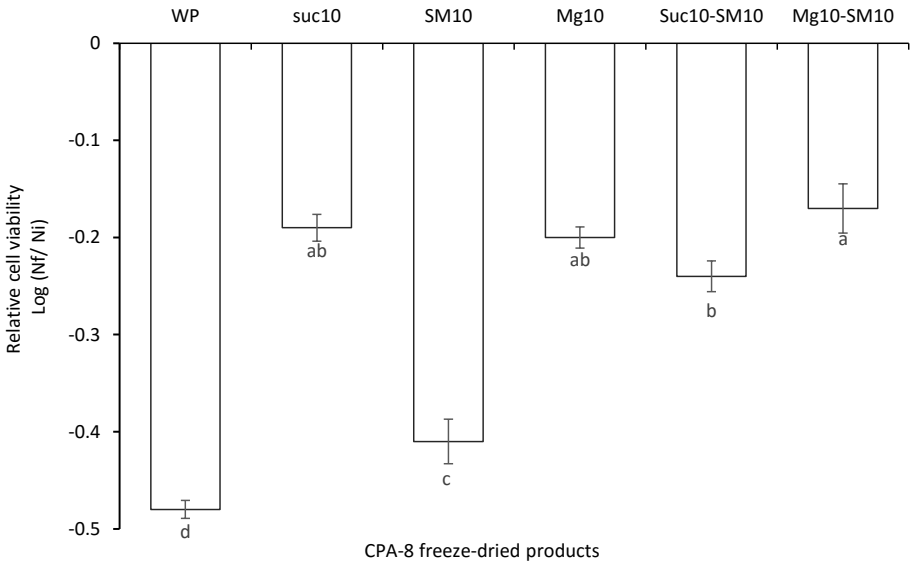

Figure 2. Relative cell viability of CPA-8 after freeze-drying in the absence "WP" and in the presence of the protectants 10 % sucrose (suc10), 10 % SM (SM10), 10 % MgSO4 (Mg10); 10 % sucrose-10 % SM (Suc10-SM10) and 10 % MgSO4-10 % SM (Mg10-SM10). Different letters indicate significant differences (P<0.05) according to LSD test. Values are the averages of six determinations and bars indicate the standard error.

Effect of protectants on viability of CPA-8 after fluid-bed spray-drying formulation

Two CPA-8 solutions were tested, one without protectants (WP) and another one amended with 10 % $MgSO_4$. No significant differences were found when comparing both FBSD products ($F_{1,9}$=3.87 P=0.0806) and minor reductions in cell viability (0.06 and 0.14-log for WP and 10 % $MgSO_4$, respectively) were observed. Consequently, both FBSD products were selected for shelf-life and efficacy trials.

Shelf-life and effect of storage temperature on viability of CPA-8 dried formulations: freeze-drying and fluid-bed spray-drying

The cell viability of each solid CPA-8 product was tested at different shelf-life periods and storage temperatures (Fig. 3). In general, the viability was stable

independently of the temperature. For CPA-8 FD products, a minor reduction in cell viability was detected when the storage temperature was either, 4 or 22 ºC. In every case, logarithm of CFU g^{-1} decreased between 0.24-0.34. CPA-8 cells amended with 10 % $MgSO_4$ and stored at 22 ºC (most pronounced decrease) meant a reduction from $1.79 \cdot 10^{10}$ to $8.09 \cdot 10^{9}$ CFU g^{-1}. The CFU g^{-1} of FBSD formulations were practically unchanged during one year of storage; after this period, viability values between 4.76 and $8.16 \cdot 10^{9}$ CFU g^{-1} were obtained.

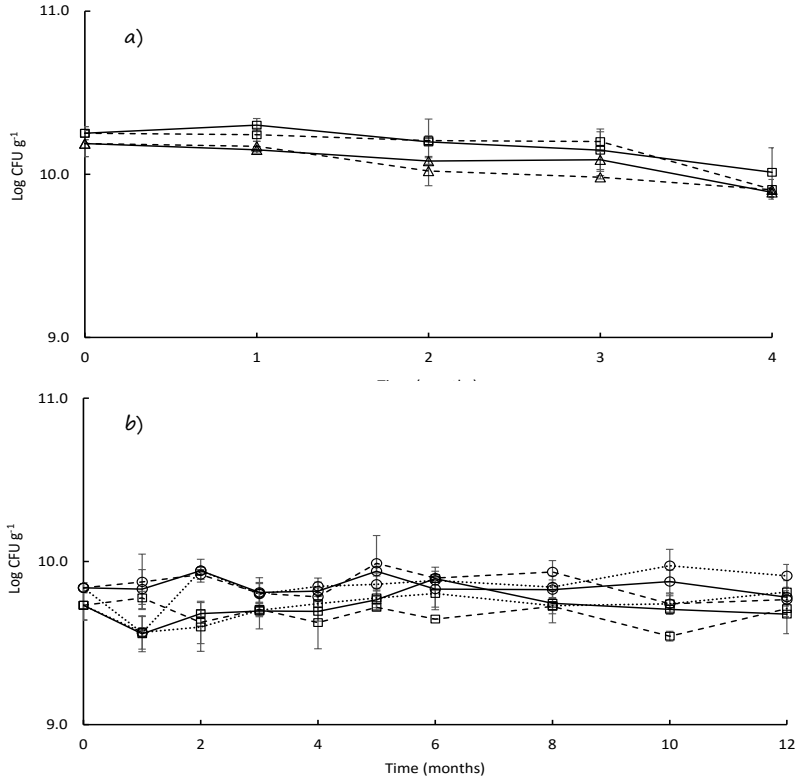

Figure 3. Cell viability of CPA-8 dried products after long-term storage at different temperatures. In the figure, only the most successful products are shown. Values are the averages of three determinations and bars indicate the standard deviations. (a): four months shelf-life of CPA-8 freeze-dried products previously amended with 10 % $MgSO_4$ and stored at 4 ( ) and at 22 ºC ( ); and four months shelf-life of CPA-8 freeze-dried products previously amended with 10 % $MgSO_4$-10 % SM and stored at 4 ( ) and at 22 ºC ( ); (b): one year shelf life of CPA-8 fluid-bed spray-dried products without protecting agents "WP" stored at 4 ( ), at 22 ( ) and at -20 ºC ( ) and one year shelf-life of CPA-8 fluid-bed spray-dried products previously amended with 10% $MgSO_4$ stored at 4 ( ), at 22 ( ) and at -20 ºC ( ).

Moisture contents of CPA-8 resulting powders

Residual moisture contents of FD products laid in the range between 2.3 and 8.8 %. In contrast, FBSD products varied from 7.0 to 11.4 %. The results indicated that FD yielded drier products than BFSD and that the introduction of SM into the formulation medium before drying significantly reduced the moisture content of the final products. When SM was added, the products obtained contained the lowest % RH values (from 2.3 to 4.9 %). For both techniques, acceptable final moistures were obtained.

Antagonistic activity of CPA-8 formulations against *M. laxa* and *M. fructicola* on stone fruit

Figure 4 shows the effect of formulated CPA-8 products on the reduction of brown rot decay caused by *M. laxa* and *M. fructicola* compared to untreated fruit and fruit treated with CPA-8 fresh cells. The most successful formulations stored for 2 months at 4 ºC were tested and taking into account the feasibility of the FBSD technology, FBSD products stored for 9 months at 4 and 22 ºC were also evaluated.

After 5 days at 20 ºC and 85 % RH, all formulated products maintained similar antagonistic activity than fruit treated with fresh cells. The ANOVA analysis revealed that the treatments applied significantly affected brown rot decay (*M. fructicola* incidence: $F_{8,27}$=21.99 P<0.0001; *M. fructicola* severity: $F_{8,27}$=64.55 P<0.0001; *M. laxa* incidence: $F_{8,27}$=8.04 P<0.0001; *M. laxa* severity: $F_{8,27}$=21.51 P<0.0001). The percentage of disease incidence in untreated fruit inoculated with *M. fructicola* was 70 %, with a mean lesion diameter of 3.9 cm. In contrast, fresh cells showed decay incidence of 5 % with 0.1 cm of mean lesion diameter and no statistical differences were observed compared to the formulated products.

In general, there was higher incidence and bigger diameter of rot decay in fruit inoculated with *M. laxa* than with *M. fructicola*. The rot incidence of untreated fruit inoculated with *M. laxa* was 85 % and the mean diameter of lesion was 5.7 cm. No differences were observed between CPA-8 formulated products and fresh cells in disease severity. Regarding the percentage of disease incidence, the results showed 35 % reduction in case of fresh cells and over 62 % in all formulated treatments compared to untreated fruit. No statistical differences were obtained between formulated treatments

and all of them (except nine months-old CPA-8 FBSD product stored at 22 ºC) showed better efficacy against *M. laxa* than fresh cells.

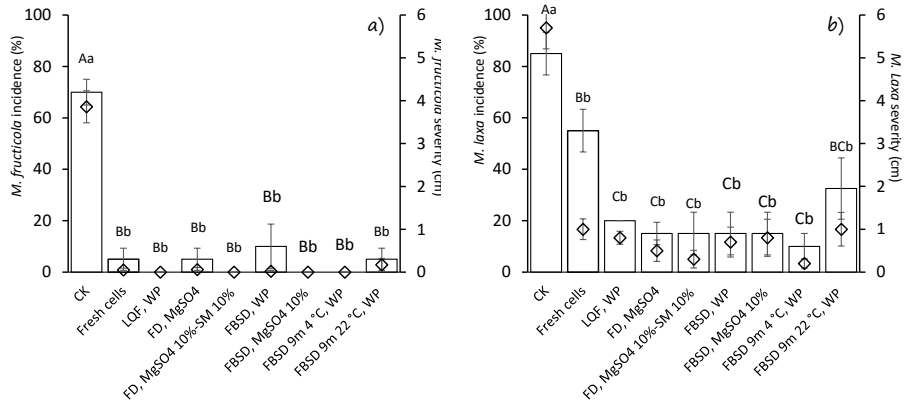

Figure 4 Antagonistic activity of CPA-8 fresh cells and formulations (10^7 CFU mL^{-1}) against artificial infection with *M. fructicola* (a) and *M. laxa* (b) on nectarines after 5 days of incubation at 20 ºC and 85% RH. Within the same figure, different letters indicate significant differences (P<0.05) according to LSD test. Uppercases and bars refer to *Monilinia* incidence (%) and lowercases and diamonds refer to *Monilinia* severity (cm). Values are the averages of four determinations and bars indicate the standard error. The treatments tested were: negative control, without CPA-8 (CK); 72 h-old CPA-8 fresh cells (Fresh cells); two months (stored at 4 ºC) liquid formulation of CPA-8 without protectants (LQF, WP), two months (stored at 4 ºC) CPA-8 freeze-dried cells amended with 10 % $MgSO_4$ (FD, $MgSO_4$ 10 %) and with 10 % $MgSO_4$ plus 10 % SM (FD, $MgSO_4$ 10 %-SM 10 %), two months (stored at 4 ºC) CPA-8 fluid-bed spray-dried cells without protectants (FBSD, WP) and amended with 10 % $MgSO_4$ (FBSD, $MgSO_4$ 10 %), nine months (stored at 4 ºC) CPA-8 fluid-bed spray-dried cells without protectants (FBSD 9m 4 ºC, WP) and nine months (stored at 22 ºC) CPA-8 fluid-bed spray-dried cells without protectants (FBSD 9m 22 ºC, WP).

DISCUSSION

This work focused on a direct comparison of LQF, FD, and the innovative FBSD technology in terms of viability of CPA-8 cells as alternative formulations to spray-drying, which was previously described (Yánez-Mendizábal *et al.*, 2012b). The effect of protective agents (sucrose, $MgSO_4$ and SM, alone or combined) was also investigated because the preconditioning of bacterial cells prior the drying process and during storage has been demonstrated to be of practical importance.

The decisive role of fengycin-like lipopeptides in the antagonism of CPA-8 against *Monilinia* spp. has been previously determined by Yánez-Mendizábal *et al*. (2012d). For

this reason, CPA-8 was not separate from the growth medium and cells were formulated with the supernatant containing synthesised lipopeptides.

In general, storage at 4 ºC has given the highest degree of cell viability for many microorganisms formulated into a liquid form, while depending on species and strains, the shelf-life can be short at ambient or elevated temperatures (Abadias *et al.*, 2003; Melin *et al.*, 2007). According to our results, we have confirmed that CPA-8 supernatant medium with no protectants should be used as medium for preservation of CPA-8 aqueous cells because a suitable number of living cells were obtained after four months of storage. This suggests that the culture medium retains the nutrients needed for the metabolism of the microorganism and as no cells are died during formulation, the addition of protectants does not mean a beneficial effect in the bacteria's viability during shelf-life. It is noteworthy that LQF requires the manipulation of high volumes with no protection from contamination. For this reason and taking into account that the refrigerated conservation system has the disadvantage to involve high costs of storage, this was not the system chosen to formulate CPA-8 and no more than four months of shelf-life were studied.

FD (or lyophilisation), is a common industrial technique. However, it is a cost-sensitive application whose damaging factors in drying the microorganisms must be considered. For avoiding the undesirable effects, such as denaturation of proteins and decrease in cell viability of many cell types, some protecting agents have been added to the medium. In this work, sucrose, $MgSO_4$, and SM were tested. The SM at concentrations of 1-10 % has often been used for cryopreservation but even more frequently in mixtures with other cryoprotectants (Hubalek, 2003; Li & Tian, 2007). As described Navarta *et al.* (2011), the use of SM in the FD process of the BCA *Ranhella aquatilis* had lower protective effect than the SM used in combination with other additives such as sugars or polyols. This concords with the results obtained in this work. The lowest cell viability obtained for FD products, and even obtained when compared to all formulated products, was shown for FD products amended with 10 % SM and for those formulated without additives (relative cell viabilities of 0.41 and 0.48-log, respectively). The poor results in viability were probably caused because during the drying process it was too fast to let the internal water migrate outside the cell, and water frozen inside the cell resulted in lethal damage. In contrast, CPA-8 cells amended with sucrose or sucrose plus SM provided higher degree of cell protection during drying. Other authors have reported the beneficial role of

sugars (Costa *et al.*, 2000; Navarta *et al.*, 2011; Zhan *et al.*, 2011), which replace structural water in membrane after rehydration and prevent unfolding and aggregation of proteins (Champagne *et al.*, 1991). The results obtained when $MgSO_4$ was added to CPA-8 cells prior FD –alone or in mixture with SM- are in agreement with the work conducted by Yánez-Mendizábal *et al.* (2012b). It reported the importance to use $MgSO_4$ salts in spray-dried CPA-8 cells to thus obtaining formulations with acceptable shelf-life even at ambient temperatures.

In this work, satisfactory FD products were generally obtained. Although a minor reduction in cell viability after storage was observed, it was not considered decisive. However, this process is highly time-consuming and involves elevated costs in massive production. For that, the authors considered that FD is not the best alternative to formulate the BCA CPA-8.

FBSD is currently known as an attractive alternative to FD, spray-drying or traditional fluidised-bed drying systems. By operating with a large air volume, liquid can be spray-dried in the chamber at an inlet air temperature much lower than that used for traditional spray-drying. Srivastava and Mishra (2010) have also reported that FBSD products generally involve larger quantities, have better dispersibility and flowability, and exhibit a narrow particle-size distribution. Furthermore, comparing to conventional fluidised-bed drying, FBSD does not need previous extrusion and pelletization of the sample. This combined technology also offers lower operating costs and shorter process times compared to FD (Strasser *et al.*, 2009).

In this work, three different carriers and two binders were tested to obtain a suitable FBSD product. The poor solubility of pea protein and pregelatinised potato starch as carrier materials triggered clogging the spray nozzle and were not appropriated for CPA- 8 formulation. Moreover, the agglutinant power of the polymer HPMC also meant the formation of agglomerates. At the end, the combination of potato soluble starch as carrier and pregelatinised potato starch as binder were chosen for enhancing powder recovery with no agglomerates and also for being low-cost commercial products (data not shown).

In terms of CPA-8 viability, we have experimented with two cell suspensions: one without additives and other one amended with 10 % $MgSO_4$. $MgSO_4$ salt was chosen for

its good results in CPA-8 FD -reported in this work- and also for the results showed in the work conducted by Yánez-Mendizábal *et al.* (2012b) on CPA-8 spray-drying. Both formulations provided high bacterial viability after drying and were found shelf-stable in all storage temperatures tested (22, 4 and -20 ºC) extending the viability of CPA-8 for up to twelve months. This means that practically all the cells survived successfully the process. However, different findings were reported by Strasser *et al*. (2009) when lactic acid bacteria were dried using the same technique. In that case, without the addition of protectants, the viability of the cells was higher after FD than after FBSD. The results we obtained are important for subsequent CPA-8 distribution and application, considering that commercial products must have a long storage life and also should not require special cold conditions that increase their management cost.

Although CPA-8 can be stored and kept viable after long incubation periods, this does not guarantee that the biocontrol potential of the strain would be maintained. The biocontrol efficacy after the formulation process should be retained as one of the most important requisites for commercial purposes. Based on this, the antifungal activity of CPA-8 formulations against *M. laxa* and *M. fructicola* was tested on nectarines. CPA-8 formulated products have successfully controlled the pathogen compared to untreated fruit. These results supported that the formulating process did not have any negative effect on the biocontrol efficacy of CPA-8.

In conclusion, we could confirm that apart from the reported spray-drying method to formulate *B. amyloliquefaciens* CPA-8, this strain could be satisfactorily formulated with all the techniques evaluated in this work: LQF, FD and FBSD. In all cases, CPA-8 formulated products were obtained with satisfactory final concentrations and stable shelf-life. Taking into account the considerations mentioned, FBSD appeared to be the most suitable approach. Although the results presented are promising, CPA-8 FBSD needs to be improved in order to develop a commercially available product. Nevertheless, this work presented and discussed that FBSD technology ensures the efficacy, stability, and low-cost easily application of CPA-8 formulated products.

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Chapter V

Biocontrol products based on *Bacillus amyloliquefaciens* CPA-8 using fluid-bed spray-drying process to control postharvest brown rot in stone fruit

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ABSTRACT

Two products based on the biocontrol agent *Bacillus amyloliquefaciens* CPA-8 have been developed as an effective alternative to chemical applications to control postharvest brown rot in stone fruit. As part of the production and formulation processes, the effects of three different nitrogen sources on growth media and the effects of different carrier materials and protectants on fluid-bed spray-drying were studied. CPA-8 populations achieved 10^9 CFU mL^{-1} after 72 h of culture. However, the protein PROSTAR 510A at 20 g L^{-1} provided better growth curves compared to the boiled extract from Defatted Soy Flour and protein PROSTAR 510A at 10 g L^{-1}. Furthermore, culture ages of 72 h were needed to obtain high endospore production and therefore, suitable heat tolerance of CPA-8. The use of the protectants 20 % sucrose plus 10 % skimmed milk resulted in the best formulations when either carrier material, maltodextrin or potato starch, was used. These two products were then selected for assays of shelf-life and efficacy. CAP-8 viability was unchanged after 15 months of storage at 4 and 22 °C, maintaining concentrations between $7.8 \cdot 10^9$ and $1.2 \cdot 10^{10}$ CFU g^{-1}. Finally, the efficacy of the CPA-8 products against *Monilinia* spp. was confirmed (> 44.4 % disease reduction) on peaches, nectarines, flat peaches, cherries, apricots and plums.

Keywords: *Bacillus* spp.; protectants; shelf-life; *Monilinia* spp.; biocontrol efficacy.

INTRODUCTION

Brown rot caused by *Monilinia* spp. is specially responsible for substantial postharvest losses of stone fruit, reaching even as high as 80 % of the production in years when the conditions of temperature and wetness are favorable for the development of the disease, especially in late-ripening varieties (Mari *et al.*, 2014; Usall *et al.*, 2015). Traditionally, synthetic fungicides have been used to control postharvest decays; however, the appearance of fungicide-resistant population of pathogens and the concerns of the consumers about the possible toxicological risks of the residues have resulted in the need of developing other methods that involve a reduction in the number of field chemical applications (Droby *et al.*, 2016; Usall *et al.*, 2016a). For this reason, the biological control of postharvest pathogens using microbial antagonists has become in the last decades an effective alternative to reduce or replace the chemicals applied for controlling postharvest diseases of fruit and vegetables (Wisniewski *et al.*, 2016). The efficacy of the biocontrol agent (BCA) *Bacillus amyloliquefaciens* CPA-8, formerly identified as *Bacillus subtilis* (Gotor-Vila *et al.*, 2016), has been previously described against postharvest diseases caused by *Monilinia* spp. based on its capability of production of fengycin-like lipopeptides and emission of volatile organic compounds (Casals *et al.*, 2012; Gotor-Vila *et al.*, 2017a; Yánez-Mendizábal *et al.*, 2012d).

While an abundance of reports exists describing beneficial microorganisms with effective antagonistic activity against postharvest diseases, little success rate of postharvest biocontrol products has been realised (Usall *et al.*, 2016b). For the development of a commercial microorganism-based product, two fundamental steps should be considered: the economical production of large quantities of the microorganism and the development of a formulation strategy that ensures reasonable shelf-life (stored preferentially for 12-24 months at room temperature) and maintains efficacy on a wide range of hosts compared to fresh cells (Droby *et al.*, 2016; Teixidó *et al.*, 2011).

CPA-8 formulated products have been successfully obtained in a liquid state requiring refrigeration (Gotor-Vila *et al.*, 2017b) and dried by different dehydration processes such as spray-drying (Yánez-Mendizábal *et al.*, 2012b), freeze-drying and fluid-bed spray-drying (Gotor-Vila *et al.*, 2017b). The feasibility of the fluid-bed spray-

drying, which operates with a large air volume and lower temperatures than spray-drying, makes this technology an attractive alternative to the traditional drying systems (Srivastava & Mishra, 2010).

Although drying methods are generally more suitable due to the storage capability and transportation, desiccation frequently produces cell damage (Fu & Chen, 2011; Morgan *et al.*, 2006). Nevertheless, to provide a good matrix that allows stability and cell rehydration, substances such as polymers, sugars, milk, and polyols have been tested for their protective effect during drying (Navarta *et al.*, 2011; Strasser *et al.*, 2009). In many cases, such additives were found to be effective toward protection of dried bacteria because they replace structural water in cell membrane after rehydration and prevent unfolding and aggregation of proteins (Stephan *et al.*, 2016).

The genus *Bacillus* is considered very amenable to drying methods because of its spore-forming ability that provides tolerance against diverse environmental stresses including heat, desiccation, and ionic strength (Nguyen Thi Minh *et al.*, 2011). Thereby, the endospore production is a crucial factor that needs to be considered during the production process of BCAs. These structures confer exceptional ecological advantages and allow long-term storage and relatively easy development of *Bacillus*-based products (Collins & Jacobsen, 2003).

Although the formulated products could be stored and kept viable after long incubation periods, this does not guarantee that the biocontrol potential of the microorganism would be maintained. The narrow range of activity of many BCAs is a serious limitation to their commercial success. Many postharvest antagonists target a single major pest in a particular host, which generally limits their potential market size. To make development and commercialisation more successful, it would be beneficial to broaden the spectrum of action of these products to different hosts and pathogens (Droby *et al.*, 2016; Glare *et al.*, 2012).

The aim of this study was to develop stable and effective CPA-8-based products. In order to meet the requirements of product quality, we studied the following: (i) different nitrogen sources to improve the low cost medium for CPA-8 production, (ii) the endospore production of CPA-8 under different culture ages, (iii) the effect of

different protectants and carriers on CPA-8 fluid-bed spray-drying and (iv) the efficacy of CPA-8 products against *Monilinia laxa* and *Monilinia fructicola* on a wide range of stone fruit.

MATERIALS AND METHODS

Microorganisms

B. amyloliquefaciens CPA-8 was isolated from a nectarine surface and belongs to the Postharvest Pathology Group Collection of IRTA (Lleida, Catalonia, Spain). Bacteria were subcultured on nutrient yeast dextrose agar (NYDA: 8 g L^{-1} nutrient broth, 5 g L^{-1} yeast extract, 10 g L^{-1} dextrose and 20 g L^{-1} agar) at 30 °C for 24 h when required.

M. laxa (CPML2) and *M. fructicola* (CPMC2) were obtained from decayed stone fruit, identified by the Department of Plant Protection of INIA (Madrid, Spain) and belong to the Postharvest Pathology Group Collection of IRTA (Lleida, Catalonia, Spain). Cultures were maintained on potato dextrose agar plates (39 g L^{-1} PDA, Biokar Diagnostics, France) at 25 ºC for 15 days.

CPA-8 production

Culture media optimisation

Low cost media with a different nitrogen source each were used to evaluate the CPA-8 growth in 2 L bioreactors (BioFlo/CelliGen 115, Eppendorf, New Brunswick, Canada). CPA-8 growth was previously described by setting defatted soy flour (DSF) as nitrogen source (Yánez-Mendizábal *et al*., 2012c). In the present study, the DSF medium was slightly modified using the soy flour extracted by boiling for 10 min: 100 g L^{-1} extracted defatted soy flour, 5 g L^{-1} molasses, 1.9 g L^{-1} KH_2PO_4, 0.001 mg L^{-1} $CuSO_4$, 0.005 mg L^{-1} $FeCl_3 \cdot 6H_2O$, 0.004 mg L^{-1} Na_2MoO_4, 0.002 mg L^{-1} KI, 3.6 mg L^{-1} $MnSO_4 \cdot H_2O$, 0.92 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.14 mg L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.01 mg L^{-1} H_3BO_3 and 10 mg L^{-1} $C_6H_8O_7$. Two more media were prepared replacing the soy flour by PROSTAR 510A (Brenntag Química, S.A.U., Barcelona, Catalonia, Spain), an isolated soy protein (90 % protein content) prepared at two concentrations (10 and 20 g L^{-1}). For every growth medium, subsamples were taken periodically and cell concentrations were recorded and presented

in a growth curve. CFU mL^{-1} were estimated by ten-fold dilutions and plated on NYDA. Each medium was tested in triplicate.

Culture conditions with the optimised medium

Fresh bacteria cultured overnight at 30 ºC in NYDA plates and suspended in potassium phosphate buffer (PB, 70 mL KH_2PO_4 0.2 mol L^{-1}; 30 mL K_2HPO_4 0.2 mol L^{-1} and 300 mL deionized water v/v/v pH 6.5) were used to prepare an appropriate volume of inoculum to inoculate 5 L laboratory scale bioreactors (BIOSTAT-A modular fermenters, Braun Biotech International, Melsungen, Germany) or 2 L bioreactors containing the culture medium. The initial concentration was adjusted at $2 \cdot 10^5$ CFU mL^{-1}. CPA-8 cells were grown for 68-72 h at 30 °C. Agitation was set at 200 rev min^{-1} and the air feeding rate was 0.33 vvm. Antifoam (30 % Simethicone emulsion USP, Dow Corning®, USA) was added as needed. This procedure was further used in sections 2.3, 2.4, and 2.5 for CPA-8 production.

Heat resistance test of CPA-8 for endospore production

As a crucial factor during the production of BCAs, heat resistance and consequently endospore production of 24-, 48- and 72-h-old CPA-8 cultures was tested. Triplicate samples of 50 mL each of CPA-8 cultures were taken from the bioreactor, centrifuged at 9820 *g* for 12 min at 10 ºC in an Avanti J-20 XP centrifuge (Beckman Coulter, CA, USA) and concentrated 10 times in PB. Then, CPA-8 solutions were incubated at 80 °C for 12 min to kill the vegetative cells as reported Yánez-Mendizábal *et al.* (2011). The number of viable cells (vegetative cells and endospores) and the surviving cells corresponding to the number of viable CPA-8 endospores were determined by 10-fold dilutions plated on NYDA and incubated at 30 °C for 24 h (CFU mL^{-1}). The experiment was repeated twice.

CPA-8 formulation

Fluid-bed spray-drying optimisation process

To obtain fluid-bed spray-dried CPA-8 products, a fluid-bed spray-dryer (HüttlinGmbH, Bosch Packaging Technology Company, Schopfheim, Germany) was used. The bacterial solution was atomised by a 0.8 mm nozzle in bottom-spray position using a peristaltic pump, applying a spraying air pressure of 80 kPa. Each trial was sprayed onto 300 g of powdered carrier material previously loaded into the drying

camera of the pilot scale fluid-bed dryer. To facilitate the grain formation, 3.5 g of pregelatinised potato starch were added to the cells solution due to its agglomerating function. Inlet air temperature was set to 65 ºC which resulted in a maximal product temperature of 42 ºC depending on the spraying rate, which ranged between 4 and 4.5 g min^{-1}. The formulation process was performed with cells grown for 68-72 h and harvested by centrifugation at 9820 *g* for 12 min at 10 ºC. The resulting pellet was resuspended approximately at 10^{10} CFU mL^{-1} in the same CPA-8 supernatant medium to include the antifungal lipopeptides synthesised by the bacterium during the production process (Yánez-Mendizábal *et al.*, 2012d). For every trial, 160 g of the suspension were supplemented with different protective substances in order to improve the CPA-8 survival during the drying process and storage. The CPA-8 suspensions with each protective substances were homogenised in a rotary shaker at 150 rev min^{-1} for 60 min at room temperature before drying. CPA-8 suspensions without additives served as control. Two different carrier materials, maltodextrin and potato starch, were used to obtain different formulations.

In a first approach, maltodextrin was tested with the following protectants: 10 % sucrose, 20 % sucrose, 10 % skimmed milk (SM), 10 % sucrose plus 10 % SM and 20 % sucrose plus 10 % SM. Subsequently, the best protectants obtained were tested using potato starch instead. Additionally, the protectants 5 % and 10 % maltodextrin and 10 % glucose syrup were included.

CPA-8 survival after formulation

Three replicate samples (0.5 g) of each dried product were rehydrated with 5 mL of distilled water, shaken vigorously for 1 min and then allowed to rehydrate for other 9 min in static. Ten-fold dilutions of each suspension were made and plated on NYDA to determine the CFU g^{-1}.

The relative cell viability was calculated for each preparation by the difference between the cell concentration after drying and the initial cell concentration. The rate of surviving cells was calculated as follows:

$$\text{Relative cell viability} = \text{Log}_{10} (N_f / N_i)$$

Where N_i represents the CFU in the suspension of CPA-8 before being formulated: population of the suspension (CFU mL^{-1}) x amount of solution (mL), and N_f is the CFU of CPA-8 obtained after drying: population of the powder (CFU g^{-1}) x amount of powder (g).

Moisture content, a_w, and shelf-life of CPA-8 formulations

To calculate the moisture content of the CPA-8 dried products, duplicate samples of 0.5 g each were placed in aluminium-weighing boats and dried in a convection oven at 100 °C for 24 h. The dry matter was calculated based on the weight loss after drying and expressed as relative humidity percentage (% RH). The water availability (a_w) of each formulation was checked with an Aqualab (Decagon Devices Inc, Pullman, WA, USA) a_w-meter to an accuracy of ± 0.003. Finally, CPA-8 formulations were stored in 250 mL plastic flasks (leak-resistant wide mouth translucent HDPE bottle and cap, Fisherbrand, Fisher Scientific S.L, Madrid, Spain) at 4 and 22 °C and the shelf-life was determined monthly for 15 months. Three replicate samples (0.5 g) of each dried formulation and temperature were sampled, rehydrated in 5 mL of distilled water and plated on NYDA (CFU g^{-1}).

Antagonistic activity of CPA-8 formulations against *M. laxa* and *M. fructicola* in stone fruit

Antagonistic activity of CPA-8 formulations to control brown rot caused by *M. laxa* and *M. fructicola* was evaluated in a wide range of stone fruit. Treatments prepared from no stored formulations and from those kept for 10 months at either, 4 or 22 °C were tested in 'Ruby Rich' peaches, 'Noracila' nectarines, 'UFO-4' flat peaches and 'Early Bigi' cherries. 'Flopria' apricots and 'Saphire' plums were used to test formulations stored for 6 months at both temperatures (4 and 22 °C). Efficacy was compared to 72 h-old fresh cells and water as the control treatment (CK).

Fruit with no visible injuries and similar in size and maturity was selected, wounded in the equator with a sterile nail (3 mm wide and 3 mm deep) and then inoculated with 15 µL of a pathogen conidial suspension adjusted at 10^3 conidia mL^{-1}. Conidia of each pathogen were transferred to 5 mL of sterile distilled water amended with Tween-80 (one drop per litre). After air-drying, 15 µL of each CPA-8 formulation suspended in distilled water (10^7 CFU mL^{-1}) were applied. Five fruits constituted a single replicate and each

treatment was replicated four times. With cherry fruit, there were three replicates with 10 fruits each. The percentage of fruit decayed (disease incidence) and the mean lesion diameter (cm) of brown rot (disease severity) were determined after 5 or 7 days of storage at 20 ºC and 85 % RH when *M. fructicola* or *M. laxa* were used, respectively. In the case of cherry fruit, only the disease incidence was evaluated after 7 days of storage.

Statistical analysis

Data from CPA-8 grown in different culture conditions and the shelf-life assays of CPA- 8 formulated products were log-transformed (log_{10} CFU mL^{-1} and log_{10} CFU g^{-1}, respectively) and plotted in figures where the error was represented by the mean standard deviation (±SD) of three replications of each sampling data. CFU mL^{-1} of CPA-8 heat resistance tests were also log-transformed. After formulation trials, the relative cell viability of CPA-8 was estimated on the basis of CFU counted before (fresh cells) and after being dried (formulated cells). Finally, for the efficacy evaluation of the CPA-8 formulated products, brown rot incidence and brown rot severity were analysed. The formulation and efficacy trials were evaluated using analysis of variance (ANOVA) with the JMP®8 statistical software (SAS Institute, Cary, NC, USA). In case of no homogeneity of variances, the Wilcoxon test was applied. Statistical significance was judged at the level $P<0.05$. When the analysis was statistically significant, the Tukey's HSD test was used for separation of means.

RESULTS

CPA-8 culture media optimisation

Growth curves for CPA-8 in low cost media with three different nitrogen sources are shown in Figure 1. After 6 h, the medium with protein PROSTAR 510A at 20 g L^{-1} provided higher CPA-8 growth (6.3 log CFU mL^{-1}) compared to protein PROSTAR 510A at 10 g L^{-1} and the modified DSF medium (5.7 and 5.6 log CFU mL^{-1}, respectively), indicating a faster exponential growth phase. A decrease was observed after 22-30 h (time in which maximum CPA-8 growth was observed) when protein PROSTAR 510A was used at low concentration and it was even more pronounced in the modified DSF medium curve. However, this effect was not detected when protein PROSTAR 510A was used at 20 g L^{-1}. Although all media provided good CPA-8 growth after 72 h of culture, achieving stable CPA-8 concentrations around 10^9 CFU mL^{-1} (8.9-9.0 log

CFU mL^{-1}), the medium including protein PROSTAR 510A at 20 g L^{-1} was selected for CPA-8 production in later trials.

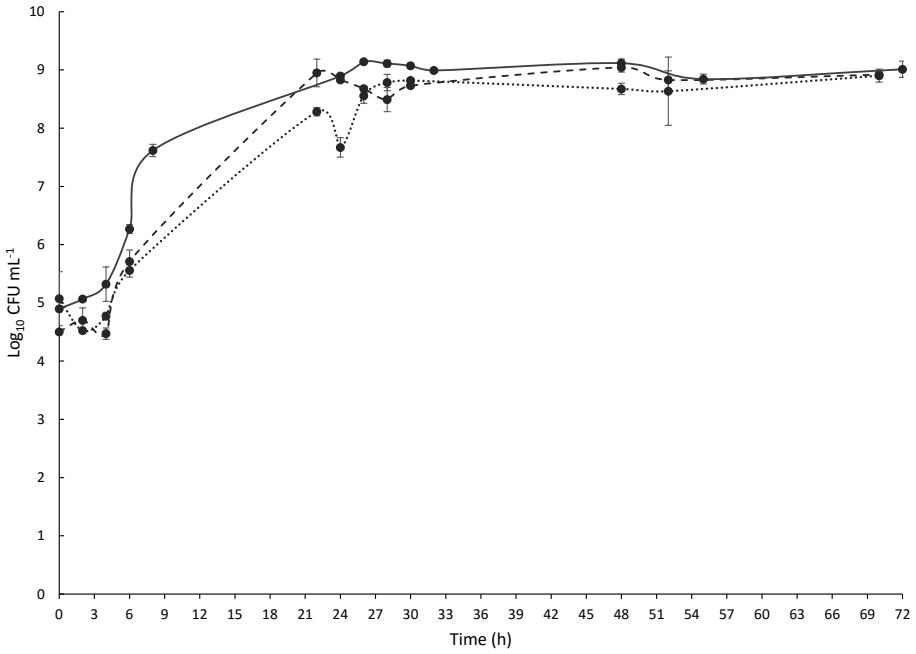

Figure 1. CPA-8 growth curves in low cost media using different nitrogen sources: Defatted soy flour (DSF) extract (·····●·····), protein PROSTAR 510A at 10 g L^{-1} (- - ● - -) and protein PROSTAR 510A at 20 g L^{-1} (—●—). Values are the averages of three determinations and bars indicate the standard deviation.

Heat resistance of CPA-8 by endospore production

The heat resistance of CPA-8 cells grown in the medium including protein PROSTAR 510A at 20 g L^{-1} and incubated at 80 ºC for 12 min is shown in Figure 2. Although a significant interaction was observed between the two repetitions of the experiment, a clear tendency in CPA-8 endospore production was detected. While the number of viable cells (vegetative cells and endospores) remained practically unchanged after 24, 48, and 72 h of culture (3.9-6.2 $\cdot 10^9$ CFU mL^{-1} and 1.9-3.6 $\cdot 10^9$ CFU mL^{-1} in the first and second repetition, respectively), the number of viable cells previously exposed to heat at 80 ºC was higher as the age of the culture increased. Therefore, CPA-8 endospores after 72 h of culture (4.3 $\cdot 10^8$ and 4.0 $\cdot 10^7$ CFU mL^{-1} in the first and second repetition, respectively) where higher than after 48 (5.2 $\cdot 10^7$ and 7.9 $\cdot 10^6$ CFU mL^{-1}) and 24 h (2.7 $\cdot 10^5$ and 1.1 $\cdot 10^6$ CFU mL^{-1}). In the first repetition, the differences between samples

exposed or not to heat incubation ranged from 4.3 to 2.0 and 1.0 log units reduction, whereas in the second repetition populations decreased 3.3, 2.7 and 1.8 log units compared to the initial concentration after being cultured 24, 48, and 72 h, respectively.

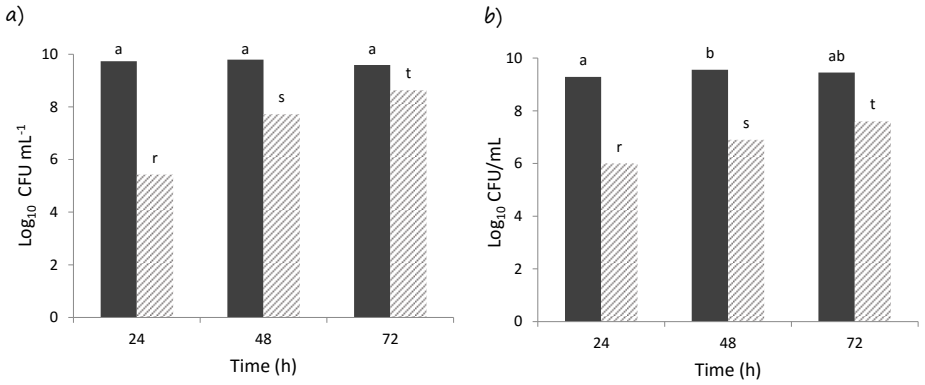

Figure 2. Endospore production of CPA-8 from 24-, 48- and 72-h-old cultures grown in low cost medium using protein PROSTAR 510A at 20 g L^{-1} as nitrogen source. The figure shows CPA-8 cells (■) and CPA- 8 endospores after incubation at 80 ºC for 12 min (▨). (a) First repetition of the experiment and (b) second repetition of the experiment. Within the same figure, different letters in the same column pattern indicate significant differences (P<0.05) according to Tukey's HSD test.

Fluid- bed spray-drying CPA-8 formulations

Maltodextrin as carrier material

To obtain good formulated products, the protective ability of sucrose and SM was evaluated and compared against cells formulated without protectants (control) (Fig. 3a). After drying, good survival of CPA-8 was generally observed, obtaining reductions in cell viability lower than 0.4 log unit. Compared to the control, the best formulations obtained included 20 % sucrose plus 10 % SM and 10 % sucrose plus 10 % SM as protectants. Moreover, the formulation with 20 % sucrose plus 10 % SM allowed to obtain products with the highest final concentration ($3.4 \cdot 10^9$ CFU g^{-1}) and large quantity of powder recovered from the dryer (341.1 g) (Table 1).

Potato starch as carrier material

A second formulation approach was conducted using 300 g of potato starch as carrier material instead of maltodextrin. In this case, non-amended CPA-8 cells (control)

and cells mixed with 20% sucrose plus 10 % SM and with 10 % sucrose plus 10 % SM were formulated. Moreover, two more formulations were obtained using 5 % maltodextrin and 10 % glucose syrup as protectants. When the concentration of maltodextrin was increased at 10%, the poor solubility triggered clogging the spray nozzle and it was not found appropriated for CPA-8 formulation (data not shown). Regarding the relative cell viability ratio, all protectants provided good CPA-8 survival after drying except 5 % maltodextrin, which could not improve the ratio obtained in the control (Fig. 3b). Once more, the yield of the process was considerably improved when the combination 20 % sucrose plus 10 % SM was used as protectant. In this case, 257.7 g of product with $8.6 \cdot 10^9$ CFU g^{-1} were obtained (Table 2).

Consequently, CPA-8 formulated products with 20 % sucrose plus 10 % SM using either, maltodextrin or potato starch as carrier material, were analysed for residual moisture content and a_w and then selected for subsequent assays of shelf-life and biocontrol efficacy.

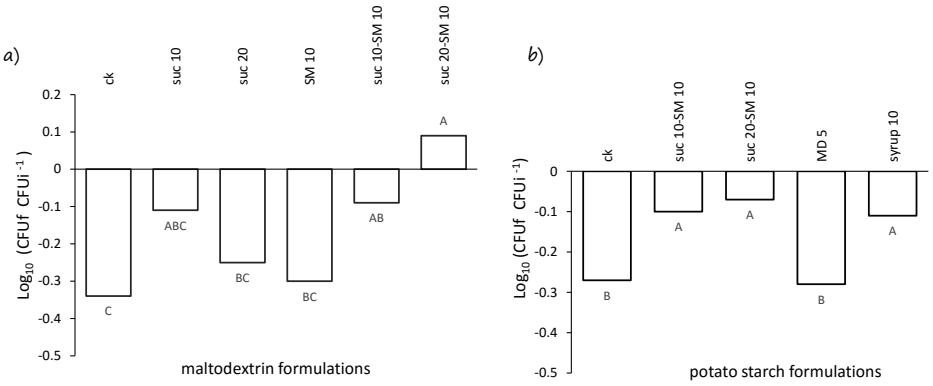

Figure 3. Survival of CPA-8 cells after fluid-bed spray-drying with different protective substances: non-amended cells (CK, control), 10 % sucrose (suc 10), 20 % sucrose (suc 20), 10 % skimmed millk (SM 10), 10 % sucrose plus 10 % skimmed milk (suc 10-SM 10), 20 % sucrose plus 10 % skimmed milk (suc 20-SM 10), 5 % maltodextrin (MD 5), and 10 % glucose syrup (syrup 10). (a) CPA-8 formulations by using maltodextrin as carrier material and (b) CPA-8 formulations by using potato starch as carrier material. For each figure, different letters indicate significant differences (P<0.05) according to Tukey's HSD test.

Table 1. Powder recovery (g of dried product) and final concentration (CFU g^{-1} and total CFU) of the resulting formulations of CPA-8 after fluid-bed spray-drying using maltodextrin as carrier material.

Protectant	Powder recovered (g)	Concentration (CFU g^{-1})	CFU
Ck, control without protectants	314.1	$1.4 \cdot 10^9$	$4.3 \cdot 10^{11}$
10 % sucrose	315.0	$2.3 \cdot 10^9$	$7.3 \cdot 10^{11}$
20 % sucrose	331.0	$1.6 \cdot 10^9$	$5.3 \cdot 10^{11}$
10 % skimmed milk (SM)	314.0	$1.0 \cdot 10^9$	$4.8 \cdot 10^{11}$
10 % sucrose – 10 % SM	330.2	$2.3 \cdot 10^9$	$7.6 \cdot 10^{11}$
20 % sucrose – 10 % SM	341.1	$3.4 \cdot 10^9$	$1.2 \cdot 10^{12}$

Table 2. Powder recovery (g of dried product) and final concentration (CFU g^{-1} and total CFU) of the resulting formulations of CPA-8 after fluid-bed spray-drying using potato starch as carrier material.

Protectant	Powder recovered (g)	Concentration (CFU g^{-1})	CFU
Ck, control without protectants	205.3	$6.8 \cdot 10^9$	$1.4 \cdot 10^{12}$
10 % sucrose – 10 % skimmed milk (SM)	236.3	$8.7 \cdot 10^9$	$2.0 \cdot 10^{12}$
20 % sucrose – 10 % SM	257.7	$8.6 \cdot 10^9$	$2.2 \cdot 10^{12}$
5 % maltodextrin	227.1	$3.3 \cdot 10^9$	$7.4 \cdot 10^{11}$
10 % glucose syrup	235.8	$3.1 \cdot 10^9$	$7.3 \cdot 10^{11}$

Residual moisture content, a_w, and shelf-life evaluation

The residual moisture content and the a_w values for 20 % sucrose plus 10 % SM CPA- 8-based products formulated with either, maltodextrin or potato starch, were in the range between 7.0-9.8 % and 0.33- 0.36, respectively (Table 3). The viability of CPA-8 in each formulation at different shelf-life periods and storage temperatures was also studied (Fig 4). In general, the viabilities were unchanged during 15 months of storage regardless of the temperature; after this period, cell concentrations between $7.8 \cdot 10^9$ and $1.2 \cdot 10^{10}$ CFU g^{-1} were maintained.

Table 3. Average residual moisture contents (% RH) and water availability (water activity, a_w), of the most suitable CPA-8 formulations after fluid-bed spray-drying.

Carrier	Protectant	% RH	a_w
Maltodextrin	20 % sucrose – 10 % skimmed milk (SM)	7.0	0.33
Potato starch	20 % sucrose – 10 % SM	9.8	0.36

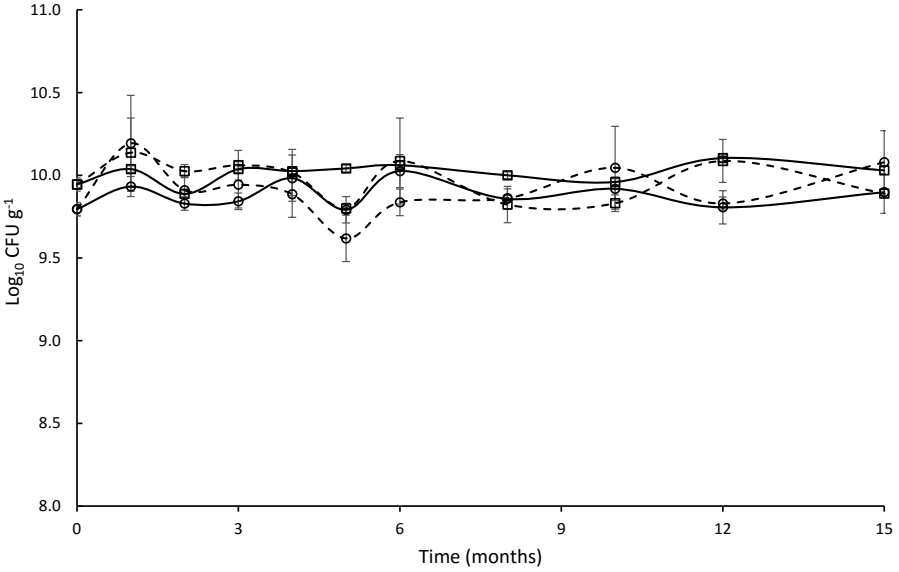

Figure 4. Shelf life of the most suitable CPA-8 products after long-term storage at different temperatures: 20 % sucrose plus 10 % skimmed milk CPA-8 formulations with maltodextrin as carrier material stored at 4 (—o—) and 22 ºC (-o-) and 20 % sucrose plus 10 % skimmed milk CPA-8 formulations with potato starch as carrier material stored at 4 (—□—) and 22 ºC (--□--). Values are the averages of three determinations and bars indicate the standard deviations.

Biocontrol efficacy assay of CPA-8 formulations

Fluid-bed spray-dried CPA-8 products were effective in controlling brown rot caused by *M. laxa* and *M. fructicola* in artificially inoculated peaches, nectarines, flat peaches, and cherries regardless of the age of the formulation and temperature of storage (Fig. 5). The formulations were as effective as fresh cells, exhibiting disease incidence from 35 to 0 % and from 50 to 0 % for *M. laxa* and *M. fructicola*, respectively, compared to values generally higher than 80 % in the untreated controls (Fig. 5a-d). In flat peaches inoculated with *M. laxa*, the disease incidence in the control was low (53.3 %); however, it was noticeably different compared to the treatments (0-10 %) (Fig 5c). Regarding the disease severity for both pathogens, *M. laxa* and *M. fructicola*, the decayed fruit in the untreated control showed mean lesion diameters from 4.4 to 2.1 cm, which were much bigger than those obtained in fruit treated with CPA-8 formulations (1.5-0 cm) (Fig. 5a-c). When compared to the control, all the formulations applied revealed disease percentage reductions ranging from 44.4 to 100 % in disease

incidence and from 46.2 to 100 % in disease severity. In general, higher percentages of decayed fruit and bigger lesion diameters were observed in fruit inoculated with *M. fructicola* than with *M. laxa*. The efficacy of CPA-8 formulations stored for 6 months against *M. laxa* and *M. fructicola* was also tested on artificially inoculated apricots and plums. The percentage of disease reduction compared to the control ranged from 45.0 to 95.0 % and from 45.5 to 100 % regarding disease incidence and severity, respectively, except in apricots treated with CPA-8 formulated with maltodextrin (in which no more than 25 % reduction of disease incidence could be observed). These data indicate that drying and storage conditions (at low or ambient temperature) did not have any negative effect on the biocontrol efficacy of the CPA-8-based products.

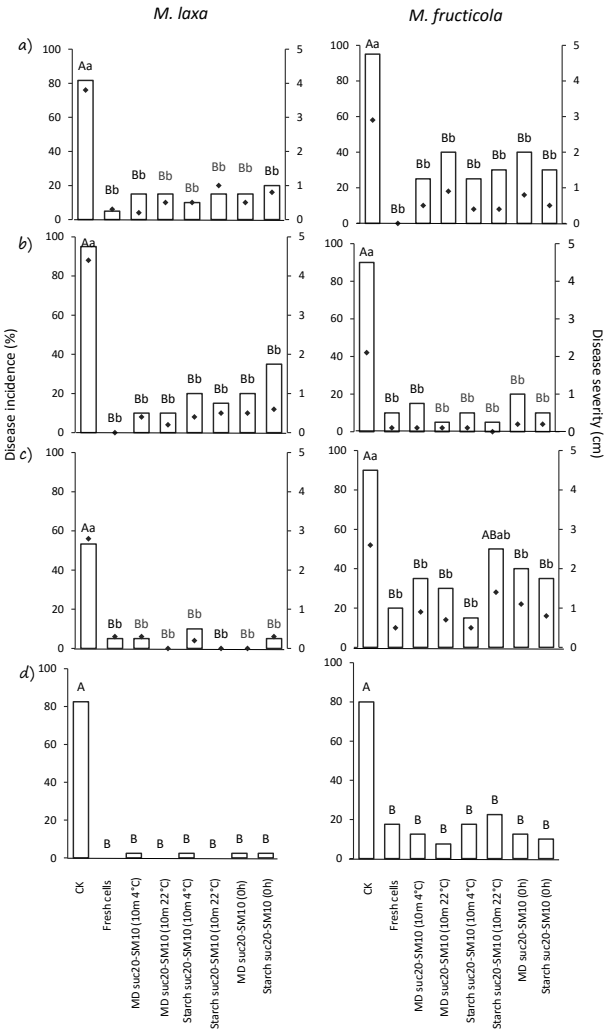

Figure 5. Antagonistic activity of CPA-8 formulations and fresh cells against artificial infection with *M. laxa* and *M. fructicola* on peaches (a), nectarines (b), flat peaches (c) and cherries (d). The percentage of fruit decayed (disease incidence) and the mean lesion diameter (cm) of brown rot (disease severity) were determined after 5 or 7 days of storage at 20 ºC and 85 % RH when *M. fructicola* or *M. laxa* were used, respectively. In the case of cherry fruit, only the disease incidence was evaluated after 7 days of storage. Within the same figure, different letters indicate significant differences (P<0.05) according to Tukey's HSD test. Uppercases and bars refer to disease incidence (%) and lowercases and diamonds refer to disease severity (cm). The treatments tested were: CK (control, without CPA-8); 72 h-old CPA-8 fresh cells; CPA-8 fluid-bed spray-dried products stored for 10 months (10 m) at 4 and 22 ºC and non-stored CPA-8 fluid-bed spray dried products (0 h). The most suitable CPA-8 formulations were used: CPA-8 cells mixed with 20 % sucrose plus 10 % skimmed milk, using maltodextrin (MD suc20-SM10) or potato starch (Starch suc20-SM10) as carrier materials.

DISCUSSION

In this work, two optimised CPA-8 products with long-term shelf-life and effective against brown rot caused by *M. laxa* and *M. fructicola* on a wide range of stone fruit have been developed. These biocontrol products are particularly suitable for further applications in commercial trials under field conditions.

Economical production of microorganisms in a culture medium requires intensive investigation to determine the optimum conditions that ensures a large, stable, and effective microbial population prior to the formulation process (Hynes & Boyetchko, 2006). The work conducted by Yánez-Mendizábal *et al.* (2012c) showed that the use of commercial products and by-products such as DSF in combination with molasses and mineral trace supplements provided high CPA-8 growth while maintaining its biocontrol efficacy. However, the use of crude soy proved to be a source of contamination due to its high microbial load. Furthermore, its low solubility during the medium preparation was a major drawback in the production and scaling–up process. To solve these hurdles, three different nitrogen sources were tested. After 22-30 h of fermentation, a slight decrease in CPA-8 growth was observed when the BCA was grown in media using the boiled extract from DSF and protein PROSTAR 510A at 10 g L^{-1}. This effect could be due to the endospores production, which occurs in high cell density populations with stressful conditions (during the stationary growth phase when nutrients are exhausted). This stress probably mean the moment in which CPA-8 derives its metabolic energy to produce endospores rather than keep growing (Posada-Uribe *et al.*, 2015). Nevertheless, the results revealed better CPA-8 growth curves when the isolated soy protein PROSTAR 510A was used at 20 g L^{-1}. Although maximum CPA–8 growth was obtained when the concentration of protein PROSTAR 510A was high (20 g L^{-1}), the low cost of this substrate allows its use without reducing its suitability for a commercial production. Moreover, the medium preparation is not time consuming as there is no need in boiling previously the flour.

Similarly to the work conducted by Yánez-Mendizábal *et al.* (2012a), high CPA-8 endospore production ($4.3 \cdot 10^8$ CFU mL^{-1}) was obtained with the new optimised medium based on protein PROSTAR 510A. After heat incubation, CPA-8 cells (and consequently, CPA-8 endospores) significantly increased from 24 to 72 h, suggesting

that the ability of CPA-8 to survive the drying process greatly depends on the age of the culture. These results indicate that, similar to many other *Bacillus* species, CPA-8 produced endospores as a mean of surviving temperatures up to 80 ºC (Baril *et al.*, 2012; Nguyen Thi Minh *et al.*, 2011). This ability is important for the formulation of the BCAs, especially in drying systems that use high temperatures.

In previous works, CPA-8 has been successfully formulated by using both, liquid and dried forms (Gotor-Vila et al., 2017b; Yánez-Mendizábal *et al.* 2012b). Among the drying technologies used, fluid-bed spray-drying resulted the most suitable one (Gotor-Vila et al., 2017b), obtaining larger quantities of the product with lower operating costs and shorter process times. Once the best drying process was defined for CPA-8 formulation, different processing parameters to obtain high performance under commercial conditions need to be optimised. In the current work, we focussed our attention on the effect of protectants and carrier materials.

Many authors have reported the protective efficiency of certain substances in response to osmotic and ionic stress that desiccation causes on cells (Abadias *et al.*, 2001; Costa *et al.*, 2000). After fluid-bed spray-drying, the combined use of 20 % sucrose plus 10 % SM resulted in the best CPA-8 formulations when either, maltodextrin or potato starch, was used as carrier. The damage caused by the drying process may be reduced by incubating bacterial cells with some carbohydrates as their presence during desiccation resulted in higher survival rates than those of non-treated cells (Strasser *et al.*, 2009). Several physical principles such as the ability of sucrose to stabilise cell membranes have been underlined as a mechanism of cells stabilisation in the dry state (Fu & Chen, 2011; Morgan *et al.*, 2006). The use of SM at concentrations of 1-10 % has often been used for cell's viability preservation during drying, but even more frequently in mixtures with other protectants (Navarta *et al.*, 2011; Santivarangkna *et al.*, 2007). Moreover, the protective effect of SM during the cell rehydration process has been considered very amenable (Abadias *et al.*, 2001; Costa *et al.*, 2000). The appropriate water binding capacities of the non-reducing disaccharide sucrose also enabled residual moisture and a_w contents that are particularly favourable for survival and storability of CPA-8. Moisture content in the dry product plays a key role in the maintenance of cell viability, suggesting that high levels tend to decrease the cell survival during the storage period (Fu & Chen, 2011). In this work, products with moisture contents lower than 10 % and a_w values between 0.33-

0.36 enabled good final concentrations ($7.8 \cdot 10^9$-$1.2 \cdot 10^{10}$ CFU g^{-1}), extending the viability of CPA-8 for up to 15 months at both temperatures of storage studied, 4 and 22 ^oC.

In the fluidised-bed process, liquid mixtures of cells and protectants are deposited on a solid substance and subsequently dried resulting in homogeneous powders with uniform thickness that exerts specific functions desired in the final products (Srivastava & Mishra, 2010). The spherical carrier material is loaded into the chamber of the dryer and then fluidised by the air flow applied. In this work, two different carriers were tested in order to obtain formulated products with different flowability and dispersability. The polysaccharides maltodextrin and potato starch were chosen for enhancing powder recovery with no agglomerates and also for being low-cost commercial products. These two materials differ in the Dextrose Equivalent (DE) measure, which indicates the degree of polymerisation from starch to sugars. Maltodextrin is typically composed of an amount of reducing sugars between 3-20 % (whereas starch is close to zero) resulting in products with higher solubility (Shamekh *et al.*, 2002). Moreover, maltodextrin also shows larger size of particles that avoid losses during drying and thus, major quantities of dried product were recovered improving the yield of the process.

Although these two formulations provide good quality requirements, it is noteworthy that biocontrol efficacy needs to be retained as one of the most important requisites for commercial purposes. Based on this, our results confirmed the biocontrol activity of CPA-8 formulations to reduce brown rot caused by *M. laxa* and *M. fructicola* in different stone fruit such as peaches, nectarines, flat peaches, cherries, apricots, and plums. These results provided a broad host range effect, suggesting an attractive opportunity for marketing biopesticides as products with an added value (Glare *et al.*, 2012; Usall *et al.*, 2016b). The biocontrol ability of this bacterium was previously reported by Casals *et al.* (2012) and by Yánez-Mendizábal *et al.* (2011) not only against *Monilinia* spp. in stone fruit ('Baby Gold 9' peaches, 'Andros' peaches and 'Big Top' nectarines) but also against *Botrytis cinerea* in 'Golden Delicious' apples, demonstrating the capability of this BCA to control different postharvest pathogens. Additionally, this study demonstrated the long-term storability (even at room temperature) of the two CPA-8-based products developed, which did not show any negative effect in the biocontrol efficacy of CPA-8, providing suitable product delivery.

In this study, the complete development of two effective biocontrol products has been optimised. An easily handling and low cost growth medium for CPA-8, which ensures an efficient endospore production and therefore heat tolerance during the bacteria drying process, has been described. This work also proved that the use of maltodextrin or potato starch as carriers combined with 20 % sucrose plus 10 % SM as protectants is a good tool for reaching an adequate stability and efficacy of CPA-8 formulated products. These results supported the suitability of the fluid-bed spray-drying technology as a promising way to preserve the BCA CPA-8 and consequently, other microorganism-based products. Further commercial trials with these two effective products are now the next research step. In conclusion, this work demonstrates that products based on biological formulations of *B. amyloliquefaciens* CPA-8 could be a suitable approach to the management of postharvest brown rot control.

Acknowledgments

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Chapter VI

Environmental stress responses of the *Bacillus amyloliquefaciens* CPA-8-formulated products on nectarines and peaches

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ABSTRACT

The efficacy of the biocontrol agent *Bacillus amyloliquefaciens* CPA-8 against brown rot caused by *Monilinia* spp. has been described and suggested as an effective alternative to chemical applications. This study aimed to describe the population dynamics of CPA-8 on the surface of nectarines and peaches after being exposed to unfavourable environmental conditions. Two CPA-8-formulated products were obtained by fluid-bed spray-drying and then applied on fruit. Although both products included 20 % sucrose plus 10 % skimmed milk as protecting agents, they differ in the carrier material used during the formulation process: maltodextrin (CPA-8-formulated product called BA3) or potato starch (CPA-8-formulated product called BA4). CPA-8 has demonstrated wide tolerance to different factors such as temperature, relative humidity and simulated rainfall. The minimal antagonist population obtained after exposure was generally higher than 10^4 CFU cm^{-2} of fruit surface, which ensures high treatment efficacy. The results also indicated that peaches were, in general, more suitable for the CPA-8 survival than nectarines. Moreover, the properties of the two CPA-8-formulated products influenced the population dynamics of the bacterium, suggesting that the BA4 CPA-8-formulated product provided higher degree of ecological fitness of CPA-8 over the fruit than the BA3 CPA-8-formulated product. The data obtained in this work led us to conclude that the integration of these CPA- 8-formulated products into cropping systems is a promising strategy to achieve higher levels of brown rot control and hence contribute to a successful handling of postharvest diseases in stone fruit.

Keywords: *Bacillus*; biocontrol; population dynamics; temperature; relative humidity; rainfall.

INTRODUCTION

Controlling fruit decays with ecologically friendly techniques is becoming popular. Using microbial antagonists has been proposed in the last decades an effective alternative to reduce or replace the chemicals applied for control of pre- and postharvest diseases (Droby *et al.*, 2016). The biological control is safe for the appearance of fungicide-resistant population of pathogens and also for the possibility of involve toxicological risks for consumers' health. The efficacy of the biocontrol agent (BCA) *Bacillus amyloliquefaciens* CPA-8, formerly known as *Bacillus subtilis* (Gotor-Vila *et al.*, 2016), has been previously described against brown rot caused by *Monilinia* spp. (Casals *et al.*, 2012; Yánez-Mendizábal *et al.*, 2011), the wound-invading fungus that causes economically important loses (reaching even as high as 80 %) of stone fruit worldwide (Mari *et al.*, 2016; Usall *et al.*, 2015). The key mode of action of CPA-8 is based on fengycin-like lipopeptides production (Yánez-Mendizábal et al., 2012) and the emission of effective volatile organic compounds (Gotor-Vila et al. 2017a). Moreover, data regarding registration purposes, such as molecular marker design (Gotor-Vila et al, 2016) and safety tests (Gotor-Vila et al., unpublished results), have been recorded. However, while an abundance of effective beneficial microorganisms has been widely studied to control postharvest diseases, few microorganism-based products are already available in the market (Glare *et al.*, 2012).

The main goal for developing a commercial microorganism-formulated product is to obtain large quantities of the microorganism that ensures a reasonable shelf-life (preferentially stored at room temperatures for at least twelve months) and maintains efficacy compared to fresh cells on a wide range of hosts (Droby *et al.*, 2016; Teixidó *et al.*, 2011). Recently, two efficacious CPA-8-formulated products have been developed in a powder state by fluid-bed spray-drying (Gotor-Vila et al., 2017c). While both products contained 20 % sucrose plus 10 % skimmed milk as protecting agents, they mainly differ in the carrier material (maltodextrin or potato starch) used during the formulation process. However, to guaranty the biocontrol efficacy of such BCA-based products under field conditions, the technical application thresholds have to be determined.

An antagonist applied in the field presents a number of difficulties because BCAs would have to withstand exposure to variable and frequently hostile environmental conditions for long periods of time (Cañamás *et al*., 2008; Köhl & Fokkema, 1998; Lahlali *et al*., 2008).

Additives are often incorporated during mass production, formulation, and storage or added later to spray tank mixes in order to enhance the activity of the microorganism at the target side (Andrews, 1992; Burges, 1998; Sui *et al*., 2015). Moreover, another way of improving survival rates for microorganisms is by understanding microbial stress response mechanisms and using this knowledge to improve resistance to unfavourable environmental conditions (Cañamás *et al*., 2008). Teixidó *et al*. (2006) demonstrated that modifying water potential in the culture medium can result in cells with improved tolerance to desiccation.

Few studies have evaluated the effect of abiotic factors interfering with the survival of BCAs, such as temperature, relative humidity (RH) or UV radiation (Calvo-Garrido *et al*., 2014a; Cañamás *et al*., 2008; Lahlali *et al*., 2011). In the orchard, microbial populations are subjected to daily fluctuations of the mentioned factors which could be controlled during postharvest storage (Calvo-Garrido *et al*., 2014a; Magan, 2001; Teixidó *et al*., 2010). However, the effect of other weather phenomena such as rainfall events on BCAs has been barely studied. Calvo-Garrido *et al*. (2014b) specifically evaluated the population dynamics of the yeast *Candida sake* exposed to simulated rainfall with different rain intensities, rain volumes, and time length between rain events. These factors have been described for influencing wash off of agrochemicals on different types of crop plants (Fife & Nokes, 2002; Hunsche *et al*., 2007).

Effective colonisation, high population and viability of BCAs on fruit surfaces have been considered important aspects in the successful control of postharvest diseases. If appropriate environmental conditions are not consistently available, BCA populations may fail to reduce disease incidence and severity, and may not recover as rapidly as pathogen populations when conducive conditions occur (Garrett *et al*., 2006). The environmental conditions mentioned directly influence the capability for growth and establishment of BCAs on the fruit surface. Thus, it is important to identify the environmental niche in which an individual BCA can actively grow as

this enables abiotic threshold criteria and hence design application programs to achieve high treatment efficacy (Teixidó *et al.*, 1998).

The aim of this work was to assess the persistence of the BCA *B. amyloliquefaciens* CPA-8 on the surface of nectarines and peaches after being treated with two CPA-8-formulated products and exposed to different environmental conditions. In order to do this, we studied the main factors which could affect the CPA-8 survival under field conditions: (i) temperature, (ii) relative humidity, and (iii) wash-off caused by simulated rainfall.

MATERIALS AND METHODS

Microorganism and culture conditions

B. amyloliquefaciens CPA-8 was isolated from a nectarine surface and belongs to the Postharvest Pathology Group Collection of IRTA (Lleida, Catalonia, Spain). Bacteria were subcultured on nutrient yeast dextrose agar (NYDA: 8 g L^{-1} nutrient broth, 5 g L^{-1} yeast extract, 10 g L^{-1} dextrose and 20 g L^{-1} agar) at 30 °C for 24 h when required.

Fresh bacteria cultured overnight at 30 ^{o}C in NYDA plates and suspended in potassium phosphate buffer (PB, 70 mL KH_2PO_4 0.2 M; 30 mL K_2HPO_4 0.2 mol L^{-1} and 300 mL deionized water v/v/v pH 6.5) were used to prepare an appropriate volume of inoculum to inoculate a 2 L (BioFlo/CelliGen 115, Eppendorf, New Brunswick, Canada) and 5 L (BIOSTAT-A modular fermenters, Braun Biotech International, Melsungen, Germany) laboratory scale bioreactors containing growth medium previously described by Yánez-Mendizábal *et al.* (2012a) and optimised by Gotor-Vila, *et al*. (2017c). The initial concentration was adjusted at 10^6 CFU mL^{-1}. CPA-8 cells were grown for 68-72 h at 30 °C to obtain high endospore concentration (Gotor-Vila *et al.* 2017c). Agitation was set to 300 rev min^{-1}, the air feeding rate was 0.33 vvm and antifoam (1 mL per litre) was added if needed (30 % Simethicone emulsion USP, Dow Corning®, USA).

CPA-8-formulated products

CPA-8 cells were harvested by centrifugation at 9820 *g* for 12 min at 10 ^{o}C in an Avanti J-20 XP centrifuge (Beckman Coulter, CA, USA) and resuspended approximately at 10^{10} CFU mL^{-1} in the same CPA-8 supernatant medium to include the antifungal

lipopeptides synthesised by the bacterium during the production process (Yánez-Mendizábal *et al*., 2012b). Two CPA-8 formulated products were obtained by using a fluid-bed spray-dryer (HüttlinGmbH, Bosch Packaging Technology Company, Schopfheim, Germany) according to the protocol developed by Gotor-Vila, *et al*. (2017b) and by Gotor-Vila, *et al*. (2017c). Briefly, CPA-8 cells were mixed with the protective substances 20 % sucrose plus 10 % skimmed milk and then fluid-bed spray-dried with 300 g of powdered carrier material previously loaded into the drying camera. Two different carriers were used: maltodextrin (CPA-8-formulated product called 'BA3') and potato starch (CPA-8-formulated product called 'BA4').

Persistence of CPA-8-formulated products on nectarines and peaches under different environmental conditions

Treatment of fruit with the CPA-8-formulated products

For each treatment, condition and sampling time assessed, 20 fruits were randomly selected without visible injuries and rots and as much as homogeneous in maturity and size. Each setup consisted of four replicates with five fruits each. CPA-8 suspensions in water were prepared from the CPA-8-formulated products BA3 or BA4 and adjusted at 10^7 CFU mL^{-1}. The suspensions were sprayed on nectarines and peaches until run off by using a manual backsprayer (ARPI 18 L, CA Bovi, Lleida, Catalonia, Spain) and then air-dried for 2 h at room temperature. The spray system was kept close to the fruit to reduce bacterial movement by aerosol from the application side.

Effect of temperature

The effect of two temperatures of storage (0 and 20 ºC) on CPA-8 populations was evaluated on 'Rome Star' peaches and 'Big Top' nectarines previously treated with the BA3 and BA4 CPA-8-formulated products as described above. 20 fruits (peaches or nectarines) per treatment, condition, and sampling time were placed on packing trays and stored in two climatic chambers programmed at the temperatures mentioned. The population dynamics of CPA-8 was assessed after 0, 1, 2, 5 and 7 days or after 0, 2, 7, 15, 30 and 60 days in fruit stored at 20 and 0 ºC, respectively.

Effect of RH

The effect of three different values of RH (40, 60 and 85 %) on CPA-8 populations was evaluated on 'Gladys' peaches and 'Fantasia' nectarines previously treated with the BA3 and BA4 CPA-8-formulated products as described above. 20 fruits (peaches or nectarines) per treatment, condition, and sampling time were placed on packing trays, covered with plastic chambers, sealed, and stored in climatic chambers programed at 20 ^{o}C. The distinct RH values inside the plastic chambers were achieved by placing a dehumidifier (FDC32S, FRAL, Carmignano di Br., PD, Italy), and monitoring RH during storage with an external data logger (Testo 175H1, Testo Inc., Sparta Township, NJ, USA). The population dynamics of CPA-8 cells under the mentioned storage conditions was assessed after 0, 1, 2, 3, 6 and 9 days on peaches and after 0, 1, 2, 3, 8 and 10 days on nectarines.

Effect of simulated rainfall

Two different trials were conducted in order to evaluate the population dynamics of CPA-8 on 'Summer lady' or 'Sweet Dream' peaches and 'Diamond Ray' or 'Alba Red' nectarines exposed to simulated rainfall. 20 fruits (peaches or nectarines) were used per treatment, condition, and sampling time. (i) In a first approach, the effect of three rain intensities (60, 100 and 150 mm h^{-1}) and four rain volumes (0 'non-exposed', 20, 60 and 120 mm) were analysed on fruit previously treated with the BA3 and BA4 CPA-8-formulated products as described above. (ii) In a second approach, the effect of an establishment time of the CPA-8 cells on the surface of the fruit (0, 1, 3 and 7 days of incubation at 20 ^{o}C and 85 % RH) prior exposition to the most stressful rainfall conditions (determined in the first experiment) was evaluated.

Rainfall simulation was performed based on the protocol reported by Calvo-Garrido, *et al.* (2014b) using a rainfall simulator consisting of a metallic box 100x50x20 cm with a drop generation system at the bottom (2.5 mm diameter drops and 50 mm separation among each drop). Water fell freely 1.5 m above the metal grids in which the fruit was located. To avoid the continuous impact of rain drops on the same fruit part, a moving fan was placed in front of the rain curtain. Rain intensity was regulated by maintaining a constant water layer above the droppers and its uniformity was measured regularly before and after each rain event.

CPA-8 population dynamics

Five fruits were sampled per replicate and four replications were analysed from each treatment per condition tested. 25 peel disks were randomly removed with a cork borer (16 mm in diameter) from the surface of each fruit. Then, the 125 peel disks were placed together into sterile plastic filter bags (BagPage 400 mL, Interscience BagSystem, St Nom la Brètech, France) and mixed with 100 mL of PB. Each bag was homogenised in a stomacher blender (Masticator Basic 400 mL, IUL SA, Torrent de l'Estadella, Barcelona, Catalonia, Spain) set at 12 strokes sec^{-1} for 90 s. Serial ten-fold dilutions of the washings were made and plated on NYDA medium. Colonies were counted after incubation for 24 h at 30 ºC. Population dynamics of CPA-8 were collected as CFU mL^{-1} and finally expressed as CFU cm^{-2} of fruit surface.

Statistical analysis

Data from CPA-8 populations under each evaluated condition was log-transformed and expressed as Log_{10} (CFU cm^{-2}) to achieve a normal distribution. In case of the establishment of the CPA-8 cells before wash-off caused by simulated rainfall, CPA-8 population reduction was finally expressed as Log_{10} (N/N_0), where N = populations in the sample exposed to rainfall (CFU cm^{-2}) and N_0 = mean value of the population in the four replicates non exposed to rainfall (CFU cm^{-2}). For every trial, different conditions were evaluated using analysis of variance (ANOVA) with the JMP®8 statistical software (SAS Institute, Cary, NC, USA). In case of no homogeneity of variances, the Wilcoxon test was applied. Statistical significance was judged at the level $P<0.05$. When the analysis was statistically significant, the Tukey's HSD test was used for separation of means.

RESULTS

Effect of temperature on the persistence of CPA-8-based products on nectarines and peaches

The population dynamics of CPA-8 on nectarines and peaches after storage at two different temperatures are shown in Fig. 1. The viabilities were practically unchanged regardless of the temperature. After 7 days of storage, fruit kept at 20 ºC did not show significant differences between neither the fruit nor the treatments (Fig. 1a). Along this time, the maximum reduction in CPA-8 viability (0.21 log units) was observed in

peaches treated with the BA3 CPA-8-formulated product (from $1.16 \cdot 10^5$ to $7.15 \cdot 10^4$ CFU cm^{-2}) (Fig. 1a). After 60 days of storage at 0 ºC, no differences were observed in peaches. However, CAP-8 populations on nectarines moved from $9.4 \cdot 10^4$ to $6.22 \cdot 10^4$ (0.18 log units) and from $1.01 \cdot 10^5$ to $6.81 \cdot 10^4$ CFU cm^{-2} (0.17 log units) in the case of BA3 and BA4 products, respectively (Fig. 1b). CPA-8 populations obtained after 60 days of storage at 0 ºC were lower in nectarines than in peaches, decreasing even more in the case of nectarines treated with the BA3 CPA-8-formulated product ($6.22 \cdot 10^4$ CFU cm^{-2}) (Fig. 1b).

Effect of RH on the persistence of CPA-8-formulated products on nectarines and peaches

CPA-8 populations were generally maintained after storage at three different RH values: 85, 60 and 40 % (Fig. 2). When the nectarines were treated with the BA3 CPA-8-formulated product, a reduction from $2.85 \cdot 10^4$ to $1.59 \cdot 10^4$ CFU cm^{-2} (0.25 log units) and from $2.85 \cdot 10^4$ to $1.36 \cdot 10^4$ CFU cm^{-2} (0.32 log units) was observed in fruit stored for 10 days at 85 and 60 % of RH, respectively (Fig. 2a). Similarly, when the nectarines were treated with the BA4 CPA-8-formulated product and stored for 10 days, the CPA-8 populations were reduced from $3.69 \cdot 10^4$ to $1.66 \cdot 10^4$ CFU cm^{-2} (0.35 log units) in case of fruit exposed to 85 % of RH and from $3.69 \cdot 10^4$ to $1.69 \cdot 10^4$ CFU cm^{-2} (0.34 log units) when the RH was set at 60 % (Fig. 2b). Conversely, no differences were observed on peaches at the mentioned relative RH values (Fig. 2c-d). Moreover, when either nectarine or peaches were subjected to 40 % of RH, the CPA-8 populations remained unchanged after 10 or 9 days of storage, respectively (Fig 2). On the whole, peaches provided better CPA-8 maintenance than nectarines. It is noteworthy the growth profile of CPA-8 on peaches, in which CPA-8 seems to need a preadaptation step of 3 days prior stabilisation. Moreover, peaches treated with the BA3 CPA-8-formulated product and kept for 9 days at 40 % of RH ($6.31 \cdot 10^4$ CFU mL^{-1}) showed lower CPA-8 populations than peaches exposed to either, 85 or 60 % of RH ($1.06\text{-}1.19 \cdot 10^5$ CFU mL^{-1}) (Fig. 2c).

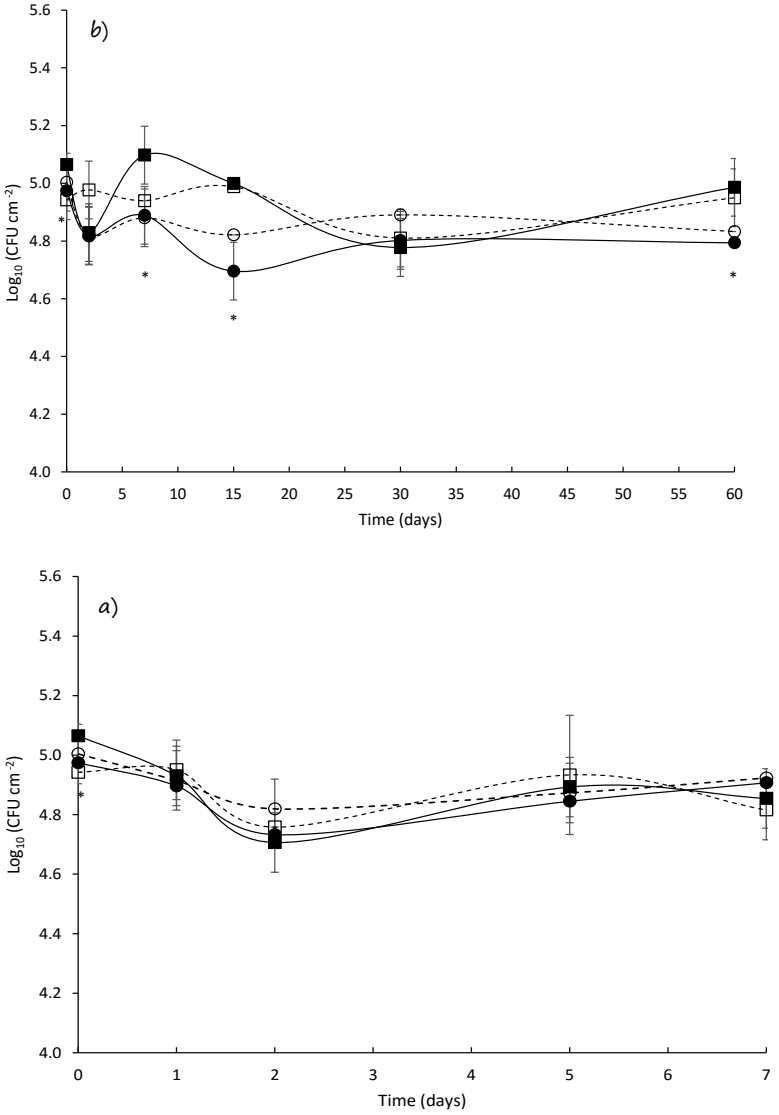

Figure 1. Effect of temperature on the population dynamics of CPA-8 at different sampling times on nectarines and peaches after storage at 20 (a) and 0 ºC (b). Nectarines treated with the BA3 CPA-8-formulated product (—●—) and with the BA4 CPA-8-formulated product (- - ⊖ - -) and peaches treated with the BA3 CPA-8-formulated product (—■—) and with the BA4 CPA-8-formulated product (- - ⊟ - -). Values are the averages of four determinations and bars indicate the standard deviation.
(*) means that these values are statistically significant according to Tukey's HSD test.

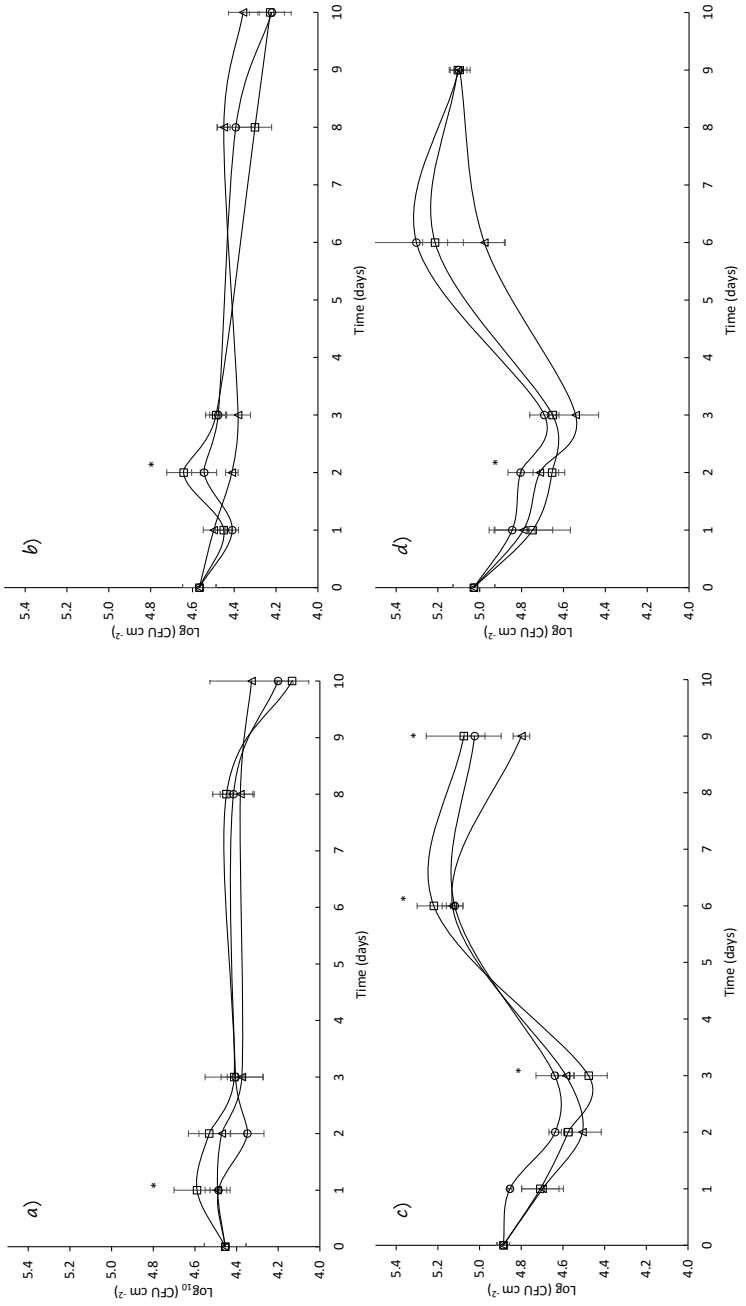

Figure 2. Effect of relative humidity (RH) on the population dynamics of CPA-8 at different sampling times on nectarines and peaches after storage at 20 ºC and 85 (—O—), 60 (—□—) and 40 % (—Δ—) RH. Nectarines treated with the BA3 CPA-8-formulated product (a) and with the BA4 CPA-8-formulated product (b) and peaches treated with the BA3 CPA-8-formulated product (c) and with the BA4 CPA-8-formulated product (d). Values are the averages of four determinations and bars indicate the standard deviation. (*) means that these values are statistically significant according to Tukey's HSD test.

Chapter VI

Effect of simulated rainfall on the persistence of CPA-8-formulated products on nectarines and peaches

Effect of intensity and rain volume

CPA-8 populations on peaches and nectarines were evaluated after exposing the treated fruit to different quantities of simulated rain (rain volume) and rain intensities. In general, good CPA-8 populations were obtained after wash-off caused by simulated rainfall ($6.01 \cdot 10^3$-$2.87 \cdot 10^4$ and $1.53 \cdot 10^4$-$3.71 \cdot 10^4$ CFU cm^{-2} on nectarines and peaches, respectively). The effect of the rain volume was not different depending on each intensity. The most substantial losses were always detected after 20 mm of rain exposure whereas higher volumes caused little additional CPA-8 removal. Moreover, it is worth mentioning the dynamic populations of CPA-8 on peaches, which reached levels much higher than those obtained on nectarines.

In detail, we could observe that for nectarines, the effect of 60 mm of rain volume was also significant when an intensity of 60 or 150 mm h^{-1} was applied to fruit treated with the BA3 or BA4 CPA- 8-formulated products, respectively (Fig 3a). When the two products were compared at each rain volume, the results proved that higher CPA-8 populations were obtained in nectarines treated with the BA4 CPA-8-formulated product regardless of the rain volume applied (20, 60 or 120 mm). The results showed losses in CPA-8 populations between 0.83-1.01 and 0.32-0.53 log units for nectarines treated with the BA3 and BA4 CPA-8-formulated products, respectively, maintaining approximately 0.5 log units difference among products (Fig. 3a). Similar results were obtained for peaches, indicating that fruit treated with the BA4 CPA-8-formulated product achieved higher CPA-8 populations (losses of 0.21-0.45 log units) than that treated with the BA3 CPA-8-formulated product (losses of 0.38-.046 log units) (Fig 3b). Moreover, the effect of 60 mm of rain volume resulted again significant when either CPA-8 formulated products, BA3 or BA4, were applied on peaches and subjected to 60 or 150 mm h^{-1} (Fig. 3b). When the rain intensity was set at 100 mm h^{-1}, higher rain volumes (120 mm) also produced a significant effect (Fig. 3b).

Consequently, restrictive conditions of simulated rainfall (intensity of 150 mm h^{-1} and rain volume of 60 mm) were chosen for subsequent assays.

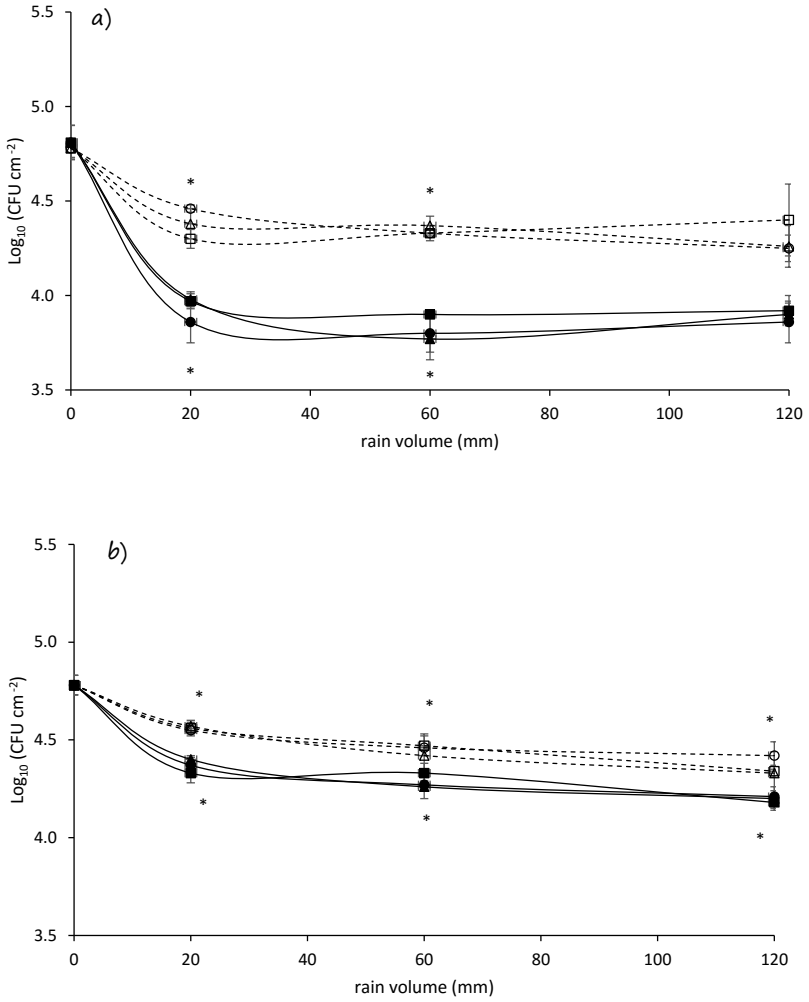

Figure 3. Population dynamics of CPA-8 on nectarines and peaches after exposure to simulated rainfall at different rain volumes (mm) and intensities (mm h $^{-1}$). (a) Nectarines treated with the BA3 CPA-8-formulated product and exposed to 150 (—●—), 100 (—■—) or 60 mm h $^{-1}$ (—▲—) and nectarines treated with the BA4 CPA-8-formulated product and exposed to 150 (- -●- -), 100 (- -□- -) or 60 mm h $^{-1}$ (- -▲- -). (b) Peaches treated with the BA3 CPA-8-formulated product and exposed to 150 (—●—), 100 (—■—) or 60 mm h $^{-1}$ (—▲—) and peaches treated with the BA4 CPA-8-formulated product and exposed to 150 (- -●- -), 100 (- -□- -) or 60 mm h $^{-1}$ (- -▲- -). Values are the averages of four determinations and bars indicate the standard deviation. (*) means that these values are statistically significant according to Tukey's HSD test.

Effect of the establishment of CPA-8 cells prior exposure to rainfall

The occurrence of the establishment period of CPA-8 on nectarines and peaches prior exposure to simulated rainfall (intensity of 150 mm h^{-1} and rain volume of 60 mm) significantly affected the BCA survival (Fig. 4). In order to minimise the losses of CPA- 8 on treated fruit, an establishment time of 3 days was needed when the BA3 CPA-8-formulated-product was applied on nectarines, reaching a population reduction of -0.26 log units (Fig. 4a). However, in case of nectarines treated with the BA4 CPA-8-formulated product, the establishment time had to be extended up to 7 days to achieve a population reduction of -0.22 log units (Fig. 4b). When the BA3 CPA-8-formulated-product was applied on peaches, an establishment time of 7 days was also needed to minimise the CPA-8 population reduction until -0.33 log units (Fig. 4c). However, no effect was observed on peaches treated with the BA4 CPA-8-formulated-product, obtaining population reductions between -0.26 and -0.30 log units after 0, 1, 3 and 7 days of establishment (Fig. 4d).

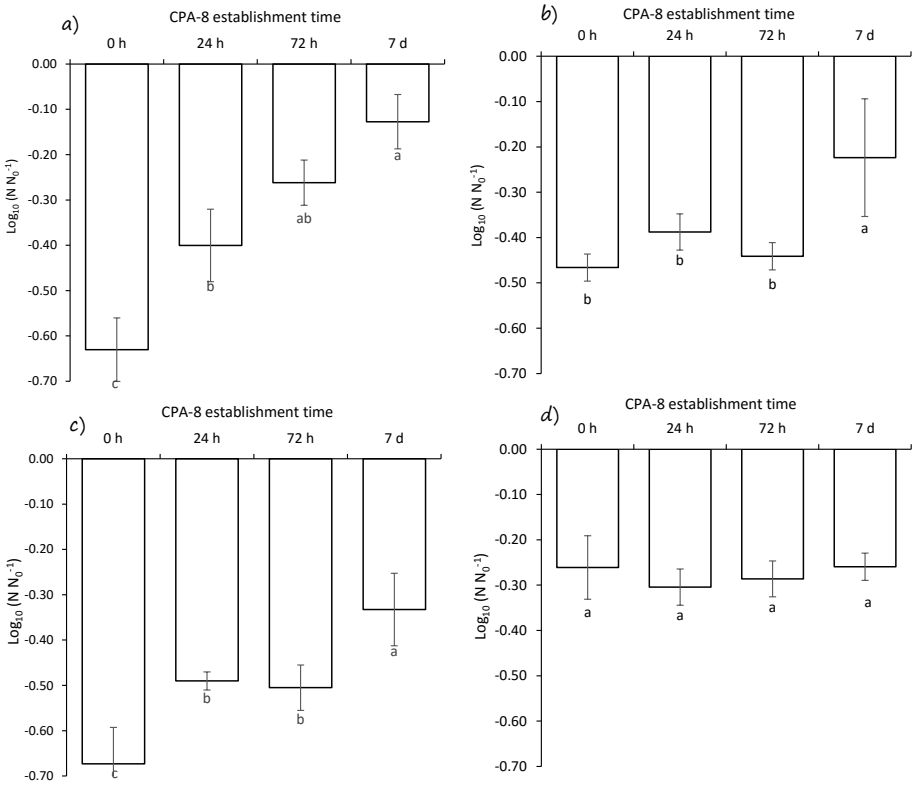

Figure 4. Population dynamics of CPA-8 on nectarines and peaches incubated for 0, 1, 3 and 7 d at 20 ºC and 85 % relative humidity (RH), prior to exposure to simulated rainfall at 150 mm h^{-1} and 60 mm of rain volume. (a) Nectarines treated with the BA3 CPA-8-formulated product; (b) nectarines treated with the BA4 CPA-8-formulated product; (c) peaches treated with the BA3 CPA-8-formulated product and (d) peaches treated with the BA4 CPA-8-formulated product. Values are the averages of four determinations and bars indicate the standard deviation. Within the same figure, different letters indicate significant differences (P<0.05) according to Tukey's HSD test.

DISCUSSION

The present work provides decisive information regarding the influence of the main abiotic factors on the persistence of CPA-8-formulated products in stone fruit, which may directly affect the efficacy of the treatments. The implementation of the data obtained into the management of field strategies may provide higher levels of disease control and would then contribute to a successful handling of postharvest diseases.

The BCA CPA-8 has demonstrated a wide tolerance to different environmental conditions. Temperature is one of the major environmental stresses experienced by microorganisms. Once the antagonist has been applied on a fruit surface, exposure to temperature stress can significantly reduce the survival of the BCAs, especially during preharvest applications under field conditions or even during postharvest storage, distribution, and marketing period (Sui *et al.*, 2015). While the optimum temperature of CPA-8 ranges from 37 to 30 ºC (Gotor-Vila et al., unpublished results), this study demonstrated that CPA-8 also tolerate exposure to 20 ºC and to 0 ºC (which simulates the temperature of the chambers conditioned for fruit storage), thus exhibiting a high degree of thermotolerance on nectarines and peaches. In general, the viabilities remained practically unchanged regardless of the temperature and treatment, reaching CPA-8 populations between $6.22 \cdot 10^4$ and $9.68 \cdot 10^4$ CFU cm^{-2} after 7 days or 60 d of storage, when fruit was exposed to room or cold temperatures, respectively. These results proved that the temperatures tested (0 and 20 ºC) do not restrict the storage conditions for fruit treated with the two CPA-8-formulated products assessed. These data agree with the previous work reported by Yánez-Mendizábal *et al.* (2012a) in which non-formulated CPA-8 cells also had good survival and efficacy on fruit after storage at 20 and 0 ºC.

The sensitivity of the CPA-8-formulated products to dry conditions on treated nectarines and peaches was evaluated at three different RH values: 85 (suitable RH for the development of the microorganism), 60 (intermediate) and 40 % (average RH in the field in the summer time). Although bacteria are generally very sensitive to the absence of free water in the phyllosphere (Köhl & Fokkema, 1998), CPA-8 populations were largely maintained, reaching values over $1.36 \cdot 10^4$ CFU cm^{-2} in all the conditions tested. Conversely, *Pantoea agglomerans* populations rapidly declined in chambers with low RH contents (Cañamás *et al.*, 2008). Moreover, the work conducted by Calvo-Garrido *et al.* (2014a) also revealed that the population of *C. sake* on treated grape berries drastically decreased under suboptimal regimes of temperature and RH, suggesting that temperature is one of the major environmental stresses experienced by yeasts.

Regarding the mentioned factors of temperature and RH, we could observe that in general, peaches were more suitable for the CPA-8 development than nectarines. Comparing the results obtained, a slight decrease of CPA-8 populations could be observed in nectarines after 60 days of storage at 0 ºC and in nectarines exposed to the

highest RH values (85 and 60 %). These results could be due to the dissimilar morphology of the peel of the fruit, which exhibits different sensibility to stress. Moreover, it was noticeable that the two CPA-8-formulated products used in this study had a considerable influence on the population dynamics of this bacterium, probably due to the composition of each carrier material used during formulation. These results indicated that the BA3 CPA-8-formulated product was more restrictive for the CPA-8 maintenance over the fruit than the BA4 CPA-8-formulated product.

The differences above observed were even more accentuated when the effect of simulated rainfall on CPA-8 populations was evaluated. The occurrence of rain had the greatest effect upon the activity of the BCAs applied (Behle *et al.*, 1997; Norris *et al.*, 2002; Pietrarelli *et al.*, 2006). Factors such as the dilution, redistribution, physical removal, and extraction from the fruit tissue should be specially contemplated (Hunsche *et al.*, 2007). In this work, wash-off of CPA-8-formulated products from the surface of nectarines and peaches was evaluated after 0 (non-exposed), 20, 60, and 120 mm of heavy rain. Results showed that CPA-8 was washed-off easily from the surface of the fruit due to the impact of the first 20 mm of rain, while higher volume of rain caused little additional removal. As it was expected, rain washed easier on the surface of nectarines than on peaches and the CPA-8 cells from the BA4 CPA-8-formulated product remained better on fruit. However, the data obtained revealed loses in CPA-8 as much as 1 log unit, reaching viabilities between $6.01 \cdot 10^3$ and $1.53 \cdot 10^4$ after rain exposure. The establishment time of CPA-8 cells on fruit between treatment and rainfall has significantly affected the wash-off of the BCA. Depending on the properties of each product applied and the type of fruit assessed, different establishment times were needed to achieve a reduction in CPA-8 cells between -0.22 and -0.33 log units, which was the minimum loss observed in all the experiments conducted. The positive effect of an establishment time prior exposure to rainfall was also observed in *C. sake* (Calvo-Garrido *et al.*, 2014b), which significantly reduced population loss and removed the effect of rain intensity. These practical observations underline an advantage of BCAs compared to chemical pesticides as treatment persistence may improve due to the colonisation ability of the microorganisms (Calvo-Garrido *et al.*, 2014b).

The two CPA-8-formulated products used in this work included the same proportion of protectants (sucrose and skimmed milk) but differed in the carrier materials

employed in the formulation process. Maltodextrin and potato starch were chosen for enhancing powder recovery with no agglomerates and also for being low-cost commercial products. The composition of these two polysaccharides was different regarding the content of reducing sugars, which provided maltodextrin (Dextrose Equivalent (DE) between 3-20 % whereas starch is close to zero) with higher degree of solubility in water (Shamekh *et al.*, 2002). That property could probably determine the differences observed in the population dynamics of CPA-8.

In order to enhance biological control of postharvest diseases, the antagonist needs to possess effective mechanisms to cope with the abiotic stresses to which the microorganisms are commonly subjected (Sui *et al.*, 2015). This study provides for the first time exhaustive information to describe the persistence of two CPA-8-formulated products on nectarines and peaches directly exposed to environmental factors which most affect the survival of BCAs under field conditions: temperature, RH and rainfall. The results obtained led us to conclude that the minimal antagonist population level on fruit surface to subsequently obtain efficient control (10^4 CFU cm^{-2}) has been achieved. However, it is important to note that this study was a small-scale experiment and that run off dynamics may change on a field scale under a natural environment. Moreover, more studies should be considered to establish the impact of other stresses caused by environmental factors such as solar radiation (UV light), wind or oxidative stress probably associated with controlled-atmosphere storage.

While potato starch appears to be an essential component in the formulation of CPA- 8, further studies are now the next research step in order to better clarify this issue. These data demonstrate that the two CPA- 8-formulated products evaluated suppose a promising way to devise possible strategies to maximise the efficacy of brown rot control in stone fruit under field applications and thus widespreading the commercialisation of BCAs.

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Chapter VII

Biological control of brown rot in stone fruit using *Bacillus amyloliquefaciens* CPA-8 under field conditions

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ABSTRACT

Different treatments based on the biocontrol agent (BCA) *Bacillus amyloliquefaciens* CPA-8 to control brown rot under field conditions were evaluated as alternative to chemical applications. As part of a well-designed disease program that enables the integration of BCAs into cropping systems, testing of the sensitivity of *Monilinia laxa* and *Monilinia fructicola* at different doses of CPA-8 were conducted in stone fruit. CPA-8 dose of 10^7 CFU mL^{-1} reduced more than 60.0 and 75.5 % of brown rot incidence and severity, respectively. Once in the orchard, different degree of biocontrol activity was obtained depending on the inoculum pressure, which was mainly associated with meteorological conditions. Under drastic disease pressure, neither CPA-8 treatment nor the chemicals controlled the disease at harvest and only the chemical treatment reduced postharvest brown rot incidence. However, when *Monilinia* spp. incidence was close to the standard levels recorded in the area, treatments based on CPA-8 formulations proved to be efficacious. At harvest, BA3, BA4 treatments (CPA-8 optimised products) and PF+BA3 treatment (CPA-8 combined with *Penicillium frequentans*) reduced *Monilinia* spp. incidence compared to the control (54.7-64.1 %) although less than the chemicals (90.6 %). At postharvest, almost all CPA-8-based treatments (except PF+BA3) controlled the pathogen with BA4 treatment being as much effective as the chemicals (50.3 % of disease reduction). Finally, the population dynamics of CPA-8 on treated fruit surface remained after treatment application, at harvest and at postharvest shelf-life (>10^4 CFU cm^{-2}). This study highlights the potential of *B. amyloliquefaciens* CPA-8 as alternative or complementary strategies to control *Monilinia* spp.

Keywords: Postharvest, biocontrol, formulation, dose of application, timing, population dynamics.

INTRODUCTION

Brown rot caused by *Monilinia*. spp. is the most significant postharvest disease of stone fruit causing losses as high as 80 % of the production in years when the climate conditions are favourable to disease development (Usall *et al.*, 2015). The risk of infection increases considerably during harvest, transport, and storage processes and synthetic fungicides such as cyprodinil, fenhexamid, fludioxinil, boscalid, and triazole-like compounds have been traditionally used during bloom or preharvest to prevent postharvest decays. However, the proliferation of fungicide-resistant population of pathogens and public concerns about health risk and environmental contamination have promoted the search for alternative methods to chemical applications (Droby *et al.*, 2016; Usall *et al.*, 2016a). Among the control strategies, the use of antagonistic microorganisms has been the focus of considerable research over the last decades (Droby *et al.*, 2016; Usall *et al.*, 2016b) although its use is not already routinely applied in fruit industry.

Among the biological control agents (BCAs), *Bacillus amyloliquefaciens* CPA-8, has been reported as an effective antagonist against postharvest brown rot caused by *Monilinia* spp. (Casals *et al.*, 2012; Yánez-Mendizábal *et al.*, 2011) based on its capability of production of powerful antifungal metabolites such us fengycin-like lipopeptides (Yánez-Mendizábal *et al.*, 2012b). Moreover, the volatile organic compounds (VOCs) emitted by this strain have been recently described to play an important role in controlling postharvest fruit pathogens such as *Monilinia* spp. and *Botrytis cinerea* (Gotor-Vila *et al.*, 2017a). As part of the genus *Bacillus*, its spore forming ability also provides high resistance to extreme environmental conditions, making CPA-8 a good candidate for developing stable and efficient BCA products (Gotor-Vila *et al.*, 2017c ; Yánez-Mendizábal *et al.*, 2012a).

Microbial antagonists are applied either, before or after harvest. However, the control of postharvest diseases should be focused on the orchard as the application of BCAs after harvest may be too late to effectively compete with the decay pathogens already established in fruit (Moretto *et al.*, 2014). Moreover, the efficacy of the microbial antagonist(s) can be enhanced if they are used with alternative strategies such as low risk chemical compounds (i.e. inorganic salts, plants extracts or volatile and essential oils) or physical treatments like hot water dipping, radio frequency, microwave

energy or irradiation with far UV-light, at postharvest (Karabulut *et al.*, 2010; Mari *et al.*, 2016; Palou *et al.*, 2016; Sisquella *et al.*, 2014). Otherwise, mixed cultures of microbial antagonists could provide better control of postharvest diseases over individual cultures or strains (Sharma *et al.*, 2009). Other microorganisms have been reported as effective antagonists against brown rot disease. Guijarro *et al.*, (2007) demonstrated the effective control of *Penicillum frequentans* strain 909 against *Monilinia* spp. mainly based on competition for space and nutrients (Guijarro *et al.*, 2017). Thus, the efficiency of one BCA would be enhanced with the addition of other BCAs to finally obtain a combined action which could be additive or synergistic.

Economical production of large quantities of microorganism and formulation strategies that ensure reasonable shelf-life and maintain efficacy during long-term storage are fundamental factors in the process of developing and commercialisation of BCAs (Droby *et al.*, 2016; Teixidó *et al.*, 2011). Recently, CPA-8 has been successfully formulated by fluid-bed spray-drying (Gotor-Vila *et al.*, 2017b; Gotor-Vila *et al.*, 2017c) an innovative technology (commonly used in the pharmaceutical industry) which operates with a large air volume and low temperatures. Besides, two optimised formulations proved to be efficacious against *Monilinia* spp. in a wide range of stone fruit (i.e. peach, nectarine, apricot, plum, flat peach, cherry), which enables to broaden the spectrum of action of CPA-8 making the process of BCA's commercialisation more successful (Gotor-Vila *et al.*, 2017c).

Nevertheless, an antagonist applied in the field is frequently subjected to severe environmental conditions that may drastically limit BCAs establishment on a host target site (Cañamás *et al.*, 2008). Few studies have evaluated the effect of abiotic factors interfering with the survival of CPA-8 such as temperature, relative humidity (RH) and wash-off caused by simulated rainfall (Gotor-Vila et al., unpublished results). However, small-scale experiments may change under a natural environment. The step wise screening of microorganisms for commercial use in biological control needs full field testing (including disease control in crops with complete common plant protection schedules) and a well-designed disease program that enables the integration of BCAs into cropping systems (Köhl *et al.*, 2011). In order to do this, this work aimed to assess the potential of different formulations of *B. amyloliquefaciens* CPA-8 for brown rot control under commercial peach production. Specifically, we studied (i) the suitable CPA-8 dose of treatment, (ii) the CPA-8 population dynamics on fruit once applied in the

orchard, (iii) the CPA-8-based treatments efficacy in controlling *Monilinia* spp. incidence at harvest and postharvest time and (iv) the possibility to combine CPA-8 with *P. frequentans* 909.

MATERIALS AND METHODS

BCA isolation, production and formulation

B. amyloliquefaciens CPA-8, formerly *Bacillus subtilis* (Gotor-Vila *et al*., 2016), was originally isolated from a nectarine surface and belongs to the Postharvest Pathology Group Collection of IRTA (Lleida, Catalonia, Spain). Bacteria were produced and formulated by fluid-bed spray-drying according to the works conducted by Gotor-Vila *et al.* (2017b) and Gotor-Vila *et al.* (2017c). CPA-8 was mass produced in 2 L (BioFlo/CelliGen 115, Eppendorf, New Brunswick, Canada) laboratory scale bioreactors containing optimised growth medium based on extracted defatted soy flour, DSF (100 g L^{-1}) or protein PROSTAR 510A (20 g L^{-1}). Therefore, CPA-8 cells were harvested by centrifugation at 9820 *g* for 12 min at 10 ºC in an Avanti J-20 XP centrifuge (Beckman Coulter, CA, USA) and resuspended approximately at 10^{10} CFU mL^{-1} in the same CPA-8 supernatant medium to include the antifungal lipopeptides synthesised by the bacterium during the production process. Then, the protecting agents were added to the cell solution, mixed with 3.5 g of pregelatinised potato starch as binder, and fluidised with 300 g of carrier material. The dried product was obtained by using a fluid-bed spray-dryer (HüttlinGmbH, Bosch Packaging Technology Company, Schopfheim, Germany) with a 0.8 mm nozzle in bottom-spray position and applying a spraying air pressure of 80 kPa. Inlet air temperature was set to 65 ºC which resulted in a maximal product temperature of 42 ºC depending on the spraying rate, which ranged between 4 and 4.5 g min^{-1}.

CPA-8 fresh cells (treatment called BA) and formulated CPA-8 cells (non-amended or amended with $MgSO_4 \cdot 7H_2O$ as protectant) were dried by using potato starch as carrier material and applied in the field trials conducted in 2014 (treatments BA1 and BA2, respectively). For the growing season of 2015, optimised formulations were developed based on the protective combination of 20 % sucrose 10 % skimmed milk and fluidised with either, maltodextrin (treatment BA3) or potato starch (treatment BA4), as carrier material.

CPA-8 dose of treatment optimisation

A laboratory assay to determinate the most suitable dose applicable for CPA-8 treatments was conducted on 'Big Top' nectarines (*Prunus persica* var. nectarine (Ait.) Maxim.) and 'Corona' peaches (*P. persica* (L.) Batch) against *Monilinia laxa* or *Monilinia fructicola*, respectively. The treatments were based on the optimised CPA-8 formulations (BA3 and BA4) adjusted at 10^6, $5 \cdot 10^6$ and 10^7 CFU mL^{-1}. Their efficacy was compared to untreated fruit (water as control, CK). Fruit with no visible injuries and similar in size and maturity was selected, wounded in the equator with a sterile nail (3 mm wide and 3 mm deep) and then inoculated with 15 µL of a pathogen conidial suspension adjusted at 10^3 conidia mL^{-1}. After air-drying, a 15 µL suspension of each treatment was applied. Five fruits constituted a single replicate and each treatment was replicated four times. The percentage of fruit infected (disease incidence) and the mean lesion diameter (cm) of brown rot (disease severity) were determined after 5 days of storage at 20 ^{o}C and 85 % RH.

Field trials and experimental design

Four field trials were carried out in four peach commercial orchards located in Lleida area (Catalonia, Spain): Alguaire, Alfarràs, Sudanell and Albesa over two growing seasons, 2014 and 2015 (Table 1).

Table 1. Characteristics of the orchards used in 2014 and 2015 seasons for CPA-8 field experiments.

Orchard	Location	Coordinates	Variety	Growing season
Orchard01	Alguaire	41.755150N-0.591865E	'Jesca' Peaches	2014
Orchard02	Alfarràs	41.833624N-0.531087E	'Xuclà' Peaches	
Orchard03	Sudanell	41.551752N-0.574049E	'Red Jim' Nectarines	2015
Orchard04	Albesa	41.779151N-0.629172E	'Roig d'Albesa' Peaches	

Different cultivars of peach and nectarine were used (Table 1). Plots were distributed in a completely randomised block design with four replicates per treatment. Each replicate consisted in 3-4 trees (depending on the number of fruits per tree). Barrier trees (non-treated trees) were used to separate treatments and replicates. Treatments consisted of CPA-8 formulations, alone or combined with *P. frequentans* (PF) formulations, and two control treatments: one based on chemical applications (Q) and another one based on non-

treated trees (CK). The CPA-8 concentration was adjusted according to CPA-8 dose optimisation (section above). During 2014 season, a treatment based on CPA-8 fresh cells was also applied. *P. frequentans 909*, originally obtained from peach twigs in an experimental orchard in Madrid (Spain) was identified and provided by the Department of Plant Protection of INIA (Madrid, Spain). Oil diluted PF formulations used in this study were provided by Bayer CropScience Biologics GmbH, (Malchow, Germany). The PF dose of application in the orchard was adjusted at 10^6 conidia mL^{-1}. The treatments and timing of applications are summarised in Table 2.

All treatments were applied four times following the recommended schedule for controlling brown rot in the area: 30, 14, 7 and 3 days approximately before harvest. The date of the applications was arranged according to the weather events. All treatments were applied in the morning with a backpack sprayer (operating pressure of 15 bar and hollow cone nozzle of 1.8 mm). Each tree was sprayed for 16 s (approximately 3 L). Orchards received the standard cultural and crop protection practices until 45 days before harvest. A weather station (Decagon Services Inc., Pullman, WA, USA) was placed into the field trials to hourly record weather observations of temperature (T), relative humidity (RH) and rainfall (mm).

Table 2. Treatments applied in CPA-8 field experiments.

Orchard	Treatment	Treatment description	Date of application
Orchard01	CK	No treatment applied	-
	Q	Cyproconazole	04.09.14
		Iprodione	09.09.14
		Tebuconazole	25.09.14
	BA	CPA-8 fresh cells	04.09.14/09.09.14/25.09.14
	BA1	Non-amended CPA-8 formulated cells	04.09.14/09.09.14/25.09.14
	BA2	CPA-8 formulated cells with 10 % $MgSO_4 \cdot 7H_2O$ as protectant and potato starch as carrier	04.09.14/09.09.14/25.09.14
Orchard02	CK	No treatment applied	-
	Q	Cyproconazole	05.09.14
		Iprodione	19.09.14
		Tebuconazole	26.09.14
	BA	CPA-8 fresh cells	05.09.14/19.09.14/26.09.14
	BA1	Non-amended CPA-8 formulated cells	05.09.14/19.09.14/26.09.14
	BA2	CPA-8 formulated cells with 10 % $MgSO_4 \cdot 7H_2O$ as protectant and potato starch as carrier	05.09.14/19.09.14/26.09.14

Table 2. (continued)

Orchard	Treatment	Treatment description	Date of application
Orchard03	CK	No treatment applied	-
	Q	Cyproconazole	15.07.15
		Tebuconazole	29.07.15
		Tebuconazole	05.08.15
		Fembuconazole	12.08.15
	BA3	CPA-8 formulated cells with 20 % sucrose plus 10 % skimmed milk as protectants and maltodextrin as carrier	15.07.15/29.07.15/05.08.15/ 12.08.15
	BA4	CPA-8 formulated cells with 20 % sucrose plus 10 % skimmed milk as protectants and potato starch as carrier	15.07.15/29.07.15/05.08.15/ 12.08.15
	PF	Oil diluted formulation of *P. frequentans*	15.07.15/29.07.15/05.08.15/ 12.08.15
	BA3+PF	BA3 was applied in the 1st and 2nd applications; PF was applied in the 3rd and 4th applications	15.07.15/29.07.15/05.08.15/ 12.08.15
	PF+BA3	PF was applied in the 1st and 2nd applications; BA3 was applied in the 3rd and 4th applications	15.07.15/29.07.15/05.08.15/ 12.08.15
Orchard04	CK	No treatment applied	-
	Q	Cyproconazole	19.08.15
		Tebuconazole	02.09.15
		Tebuconazole	07.09.15
		Fembuconazole	10.09.15
	BA3	CPA-8 formulated cells with 20 % sucrose plus 10 % skimmed milk as protectants and maltodextrin as carrier	19.08.15/02.09.15/07.09.15/ 10.09.15
	BA4	CPA-8 formulated cells with 20 % sucrose plus 10 % skimmed milk as protectants and potato starch as carrier	19.08.15/02.09.15/07.09.15/ 10.09.15
	PF	Oil diluted formulation of *P. frequentans*	19.08.15/02.09.15/07.09.15/ 10.09.15
	BA3+PF	BA3 was applied in the 1st and 2nd applications; PF was applied in the 3rd and 4th applications	19.08.15/02.09.15/07.09.15/ 10.09.15
	PF+BA3	PF was applied in the 1st and 2nd applications; BA3 was applied in the 3rd and 4th applications	19.08.15/02.09.15/07.09.15/ 10.09.15

CPA-8 population dynamics

To compare CPA-8 populations on fruit among the different treatments applied in the field during 2015 season, 20 fruits (five fruits from each replicate) were sampled according to Table 3. Samples were taken periodically in the orchard, at harvest, and at postharvest (harvested fruit stored for 4-6 days of shelf-life at 20 ºC and 85 % RH). 25 peel disks were randomly removed with a cork borer (16 mm in diameter) from the surface of every fruit. Then, the 125 peel disks of each replicate were placed together into sterile plastic filter bags (BagPage 400 mL, Interscience BagSystem, St Nom la Brètech, France) and mixed with 100 mL of phosphate buffer (PB, 70 mL KH_2PO_4 0.2 mol L^{-1}; 30 mL K_2HPO_4 0.2 mol L^{-1} and 300 mL deionised water v/v/v pH 6.5). Each bag was homogenised in a stomacher blender (Masticator Basic 400 mL, IUL SA, Torrent de l'Estadella, Barcelona, Catalonia, Spain) set at 12 strokes sec^{-1} for 90 s. Serial ten-fold dilutions of the washings were made and plated on nutrient yeast dextrose agar medium (NYDA: 8 g L^{-1} nutrient broth, 5 g L^{-1} yeast extract, 10 g L^{-1} dextrose and 20 g L^{-1} agar). Colonies were counted after incubation for 24 h at 30 ºC. Population dynamics of CPA-8 were collected as CFU mL^{-1} and finally expressed as CFU cm^{-2} of fruit surface.

Table 3. CPA-8 Population dynamics schedule.

Orchard	Date of sampling	Harvest	Shelf-life
Orchard03	15.7.15/17.07.15/22.07.15/28.07.15/29.07.15/31.07.15/ 04.08.15/05.08.15/07.08.15/12.08.15/12.08.15/14.08.15	14.08.15	20.08.15
Orchard04	19.08.15/21.08.15/26.08.15/01.09.15/03.09.15/04.09.15/ 07.09.15/07.09.15/09.09.15	14.09.15	18.09.15

Efficacy trials

Disease incidence in the field

At the commercial harvest time, all fruits from one full tree (250 fruits on average) for each treatment and replicate were evaluated taken into account the total number of fruits (healthy and affected by *Monilinia* spp.) in the tree and also in the ground. The evaluation was carried out in the tree in the middle in case of three trees per replicate or when there were four trees per replicate, one tree of the middle ones was selected.

Disease incidence after harvest

At harvest time, 100 healthy fruits per replicate were randomly collected from each treatment and placed in packing trays (20 fruits each) to avoid contact between them and consequent contaminations. The number of fruits affected by *Monilinia* spp. were then recorded after 5-7 days of shelf-life storage at 20 ^{o}C and 85 %, conditions that favour rot development.

Statistical analysis

Data from *Monilinia* spp. incidence (and *Monilinia* spp. severity in the case of CPA- 8 dose trials) were submitted to an analysis of variance (ANOVA) with the JMP®8 statistical software (SAS Institute, Cary, NC, USA). In case of no homogeneity of variances, the Wilcoxon test was applied. Statistical significance was judged at the level $P<0.05$. When the analysis was statistically significant, the t-student LSD test was used for separation of means (though the LSD test control the comparison–wise type I error rate rather than experiment-wise type I error rate). To achieve a normal distribution, the arcsine square root transformation of the data from efficacy trials was performed, if needed, prior to analysis. Non-transformed means are presented. Finally, data from CPA- 8 populations was log-transformed and expressed as Log_{10} (CFU cm^{-2}) and plotted in figures where the error was represented by the mean standard deviation (±SD) of four replications of each sampling date.

RESULTS

Optimisation of the CPA-8 dose of treatment

CPA-8 dose affected brown rot caused by *M. laxa* and *M. fructicola* on artificially infected nectarine and peach fruit, respectively. Results showed that in general, higher CPA-8 doses ($5 \cdot 10^6$ and 10^7 CFU mL^{-1}) better controlled the pathogen (Fig.1). When CPA-8 was applied at 10^7 CFU mL^{-1}, the percentage of disease reduction compared to the control ranged, in peaches and nectarines, from 60.0 to 77.8 % and from 75.5 to 80.0 % according to the two disease parameters studied, disease incidence and disease severity, respectively. Subsequently, CPA-8 treatments were prepared at 10^7 CFU mL^{-1} for further applications under field conditions.

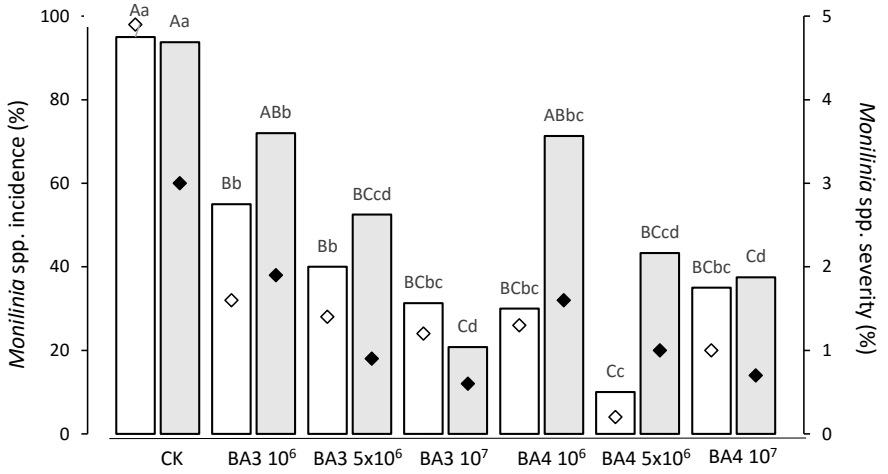


Figure 1. Antagonistic activity of CPA-8 treatments at different doses (CFU mL^{-1}) on nectarines and peaches artificially infected with *Monilinia* spp. and stored at 20 ºC and 85 % RH for 5 days. *M. laxa* in nectarines is represented by (□) and (◇) and *M. fructicola* in peaches by (■) and (◆). Uppercases and bars refer to disease incidence (%) and lowercases and diamonds refer to disease severity (cm). Within the same pattern, different letters indicate significant differences (P<0.05) according to the LSD test. The treatments were: CK (control, without CPA-8); BA3 (fluid-bed spray-dried CPA-8 cells formulated with sucrose and skimmed milk as protectants and maltodextrin as carrier); BA4 (fluid-bed spray-dried CPA-8 cells formulated with sucrose and skimmed milk as protectants and potato starch as carrier).

Meteorological data

Meteorological conditions occurred during the field experiments were collected and showed in Fig 2. The average temperature in the Orchard01 and Orchard02 were 20.2 and 19.9 ºC, respectively (2014 season late ripening varieties), lower than those obtained in the Orchard03 and Orchard04 (25.9 and 21.1 ºC) in 2015 (middle and late ripening varieties, respectively). The maximum temperature was recorded in the Orchard04, reaching 40 ºC. Regarding the RH recorded, both seasons showed similar values in average (68.8-83.0 %), although higher percentages were registered in 2014. Among the four orchards studied, the Orchard03 recorded the lowest % RH on average (68.8 %). Moreover, the 2014 season, was consistently wetter and colder than 2015 season, with more than ten times rain volume registered (96 and 64 mm of rain in the Orchard01 and Orchard02 opposed to less than 9 mm in the Orchard03 and Orchard04). It is worth mentioning the hail and heavy localised rain happened in the Orchard01 (05.09.2014) and Orchard03 (31.07.2015), damaging considerably the fruit.

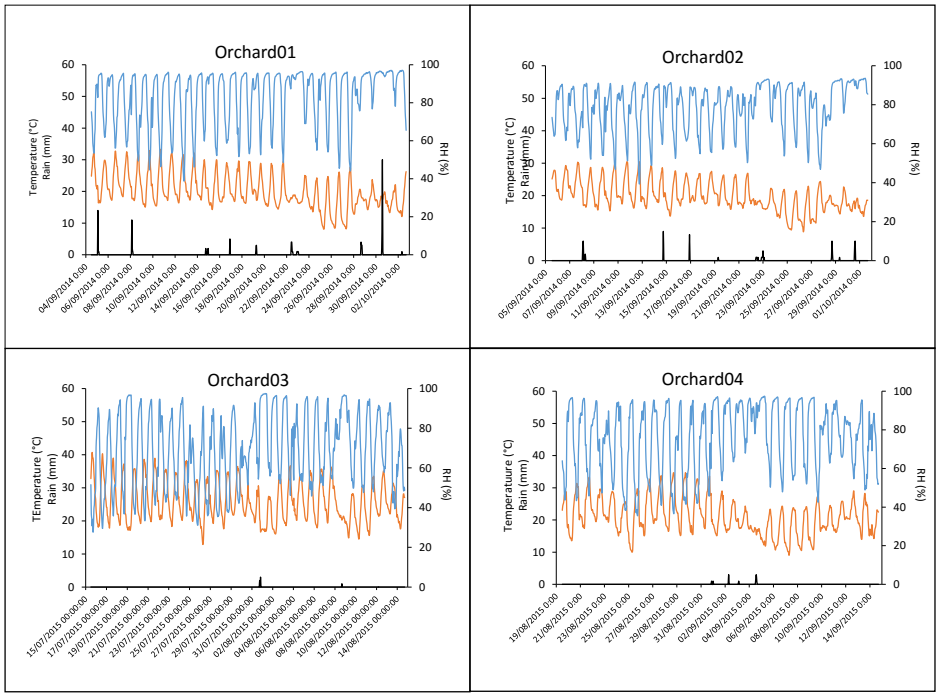


Figure 2. Meteorological data in the orchard during the treatments' application. The figure represents the temperature (ºC) in red, the RH (%) in blue and the rain volume (mm) in black.

CPA-8 population dynamics

The population dynamics of CPA-8 on the fruit surface was estimated periodically after each treatment application in the orchard, at harvest, and after postharvest shelf-life (2015 season) (Fig. 3). In this season, CPA-8 treatments were applied on middle-season nectarines (Orchard03) and late-season peaches (Orchard04). In general, CPA-8 was largely maintained on fruit. CPA-8 CFU cm^{-2} were high immediately after application and then slightly declined over time until next application.

Populations of CPA-8 in the Orchard03 (Fig. 3a) were similar when comparing the different CPA-8 treatments and ranged from 2.77-3.43 $\cdot 10^4$ CFU cm^{-2} (after the first application) to 4.10-4.95 $\cdot 10^4$ CFU cm^{-2} (at harvest), except in the combined treatment BA3+PF, in which CPA-8 population decreased 1.18 log units during that time (probably due to both, physical removal caused by the heavy rain recorded in July

2015, and the fact that non-additional CPA-8 treatment was applied after the second application). In the Orchard04 (Fig. 3b), fruit was not exposed to heavy rain so CPA- 8 cells better remained on the surface of treated fruit after the second application. Once the treatments were applied, CPA-8 populations between 5.46 and 6.62 $\cdot 10^4$ CFU cm^{-2} were obtained and maintained until harvest without exceptions (8.26-9.51 $\cdot 10^4$ CFU cm^{-2}). After harvest, (shelf-life of 4-6 days at 20 ºC and 85 % RH) the CPA-8 cells remained in all treatments (Orchard03 and Orchard04), reaching values generally higher than 4 $\cdot 10^4$ CFU cm^{-2} (except treatment BA3+PF in the Orchard03).

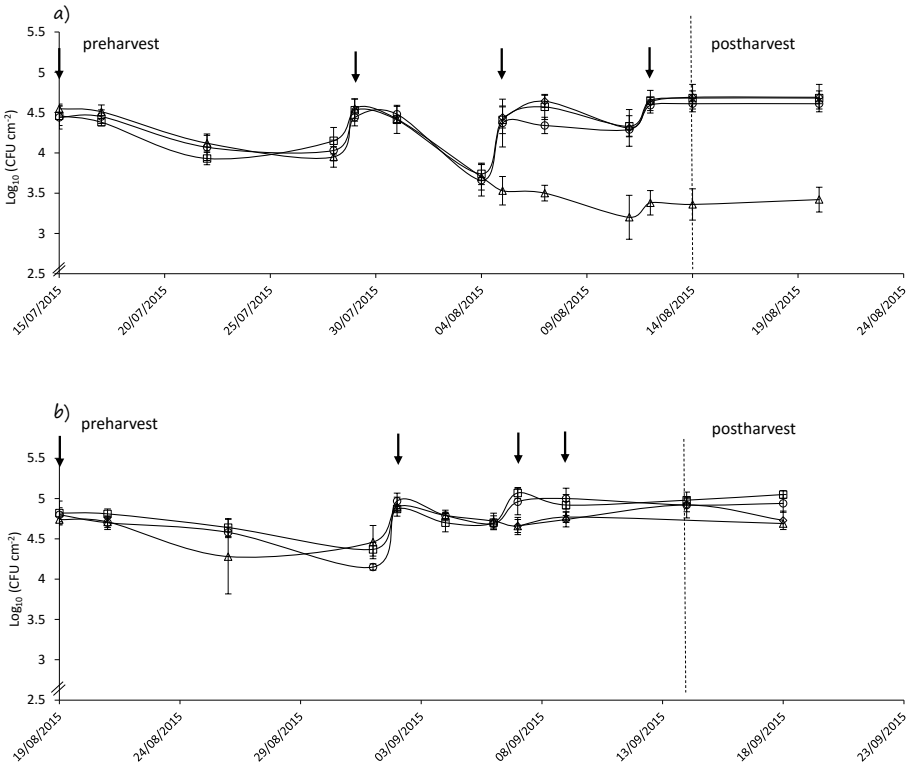
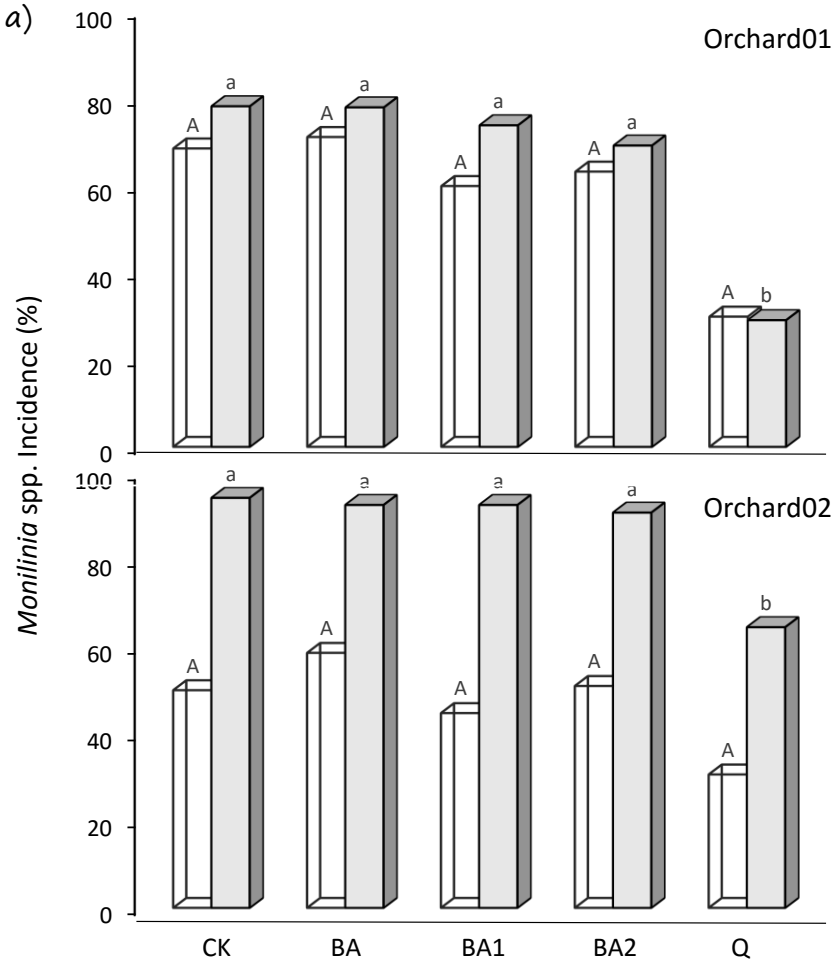


Figure 3. Population dynamics of CPA-8 cells (Log_{10} CFU cm^{-2}) on fruit surface after treatment application in the orchard, at harvest, and after shelf-life (2015 season). a) Orchard03 and b) Orchard04. Arrows indicate the moment of each treatment's application and treatments were BA3 "fluid-bed spray-dried CPA- 8 cells formulated with sucrose and skimmed milk as protectants and maltodextrin as carrier" (-o-), BA4 "fluid-bed spray-dried CPA-8 cells formulated with sucrose and skimmed milk as protectants and potato starch as carrier (-□-), BA3+PF "the first two applications with BA3 and the last two with the BCA *P. frequentans*" (-△-) and PF+BA3 "the first two applications with the BCA *P. frequentans* and the last two with BA3 (-◇-). Values are the averages of four determinations and bars indicate the standard deviation.

Efficacy trials

The efficacy of CPA-8 preharvest treatments applied in four orchards in Lleida area during 2014 and 2015 seasons is described in Fig. 4. Different degree of biocontrol activity was obtained mainly due to the inoculum pressure, which is greatly depending on the meteorological conditions. When the presence of *Monilinia* spp. in the field was higher than 50 % (2014 season and the late ripening variety of 2015 season), brown rot disease could not be controlled at harvest time, not even when fruit was treated with chemical applications. Although 56.3 % disease reduction could be observed in the chemical treatment in the Orchard01 compared to the control (untreated trees), it was not statistically significant (P=0.09). It was not the case detected at postharvest (5-7 days of shelf-life storage at 20 ºC and 85 % RH), in which despite the high presence of inoculum, the chemicals applied successfully decreased brown rot decay, exhibiting 31.5-62.7 % of disease reduction compared to the untreated control.

Otherwise, when disease pressure was in the range of the standard levels recorded in the area (< 10 % and < 30 % at harvest and postharvest, respectively), treatments based on CPA-8 proved to be efficacious (Orchard03, middle season variety in 2015). At harvest (5.3 % disease pressure), BA3 and BA4 treatments and the PF+BA3 combined treatment significantly reduced disease incidence compared to the untreated control (54.7-64.1 % disease reduction). However, their efficacy was inferior compared to the one obtained by the synthetic fungicides applied (90.6 % disease reduction). Otherwise, at postharvest (disease pressure of 17.3 %) while all treatments controlled the pathogen (except the PF+BA3 combination), the BA4 treatment significantly controlled *Monilinia* spp. even statistically similar to chemical applications. In this case, 50.3 % of disease reduction was obtained, compared to the untreated control. It is also worth mentioning that the efficacy obtained when combining both BCAs (CPA-8 and *P. frequentants)* did not improve the efficacy of the bacterium when used alone.


a)
Orchard01
Orchard02
Monilinia spp. Incidence (%)
100
80
60
40
20
0
A
a
b
CK
BA
BA1
BA2
Q

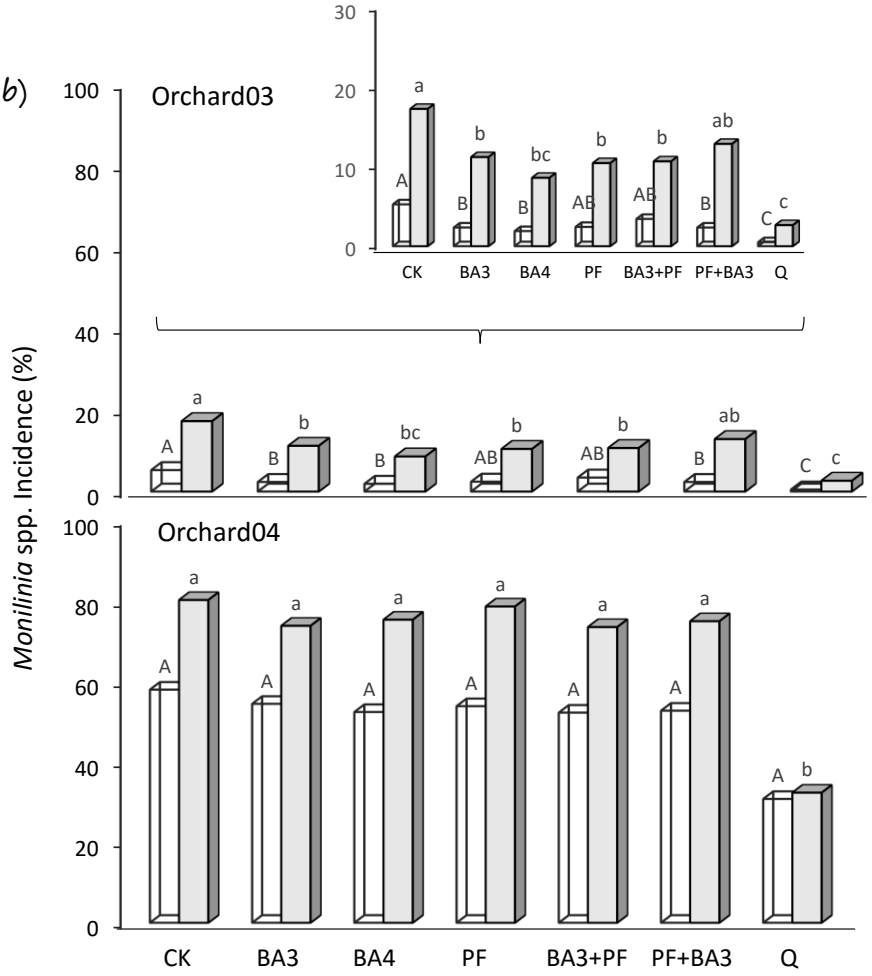


Figure 4. Efficacy trials of CPA-8 treatments during 2014 (a) and 2015 (b) season. (□) represents *Monilinia* spp. incidence (%) at harvest time and (□) represents *Monilinia* spp. incidence (%) at postharvest (after 5- 7 days of storage at 20 ºC and 85 % HR). The treatments tested were BA 'CPA-8 fresh cells', BA1 'non-optimised fluid-bed spray-dried CPA-8 cells formulated without protectants', BA2 'non-optimised fluid-bed spray-dried CPA-8 cells formulated with $MgSO_4$ as protectant and potato starch as carrier', BA3 'fluid-bed spray-dried CPA-8 cells formulated with sucrose and skimmed milk as protectants and maltodextrin as carrier', BA4 'fluid-bed spray-dried CPA-8 cells formulated with sucrose and skimmed milk as protectants and potato starch as carrier', PF '*P. frequentans* formulation', BA3+PF 'the first two applications with BA3 and the last two with the BCA *P. frequentans*', PF+BA3 'the first two applications with the BCA *P. frequentans* and the last two with BA3', Q 'chemical control' and CK 'no treatment applied'. Within the same pattern, different letters indicate significant differences ($P<0.05$) according to the LSD test. If needed, the arcsine square root transformation of the data was performed. Non-transformed means are presented.

Chapter VII

DISCUSSION

The present study represents the first full field testing on BCA *B. amyloliquefaciens* CPA-8 applications. Key factors for brown rot control have been identified, contributing to the development of disease management strategies in the Mediterranean area. Moreover, this work also illustrated aspects on *P. frequentans* 909 efficacy against *Monilinia* spp. complementing few studies already available (Guijarro *et al*., 2007; Guijarro *et al*., 2008).

As part of a well-designed disease program that enables the integration of BCAs into cropping systems, testing of the efficacy of CPA-8 against *M. laxa* and *M. fructicola* at different doses were conducted under laboratory conditions. The results showed that the highest BCA dose tested (10^7 CFU mL^{-1}) was the most effective against brown rot development, showing more than 60.0 and 77.8 % suppression of disease incidence and disease severity, respectively. Although doses of $5 \cdot 10^6$ CFU mL^{-1} also worked well, it was preferably to apply the highest one to thus ensuring biocontrol efficacy. The established CPA-8 dose of treatment (10^7 CFU mL^{-1}) was easily achievable (around ten times lower than those applied for the commercialised *Bacillus subtilis* Serenade Max, Bayer CropScience, Germany), thus faciliting the biomass production and formulation procedures.

BCA timing experiments conducted in four fields and over two seasons gave consistent information about the relevance of *Monilinia* spp. incidence. As it was observed, different degree of biocontrol activity was obtained depending on the inoculum pressure. Brown rot incidence was lower in the Orchard03 than in the Orchard04 and in both orchards studied in 2014 season, as it was evidenced by minor disease incidence in the untreated control. This difference may be associated with meteorological conditions. Since outbreaks of brown rot are dependent on prevailing environmental conditions, Gell *et al.* (2008) demonstrated that temperature and wetness durations were the two most important weather factors that contribute to the incidence of latent infections caused by *M. laxa* and *Monilinia fructigena* in Spanish peach orchards and that could account for more than 90 % of brown rot. They also suggested that RH is even more influential than temperature. Thus, longer fruit wetness duration most likely accounted for the significantly higher incidence of *Monilinia* spp. on fruit in 2014

season and in the Orchard04 in 2015. Under such drastic circumstances (> 50 % *Monilinia* spp. incidence), not even four chemical applications were sufficient for an effective control of brown rot at harvest time. Otherwise, when *Monilinia* spp. incidence was in the range of the standard levels recorded in the area (Orchard03), treatments based on CPA-8 formulations proved to be efficacious. Such standard levels have been considered over four growing seasons (2013-2016) including 26 field trials comprising early and late varieties. These data revealed that 90 % of the cases showed disease incidence at harvest lower than 10 %. Regarding postharvest studies (shelf-life at 20 ºC and 85 % RH), the percentage of trials which presented < 30 % of *Monilinia* spp. incidence raised the 77 % (Casals, personal communication). Therefore, results obtained from the Orchard03 could be included into these standard ranges of disease. At harvest (5.3 % *Monilinia* spp. incidence), BA3 and BA4 treatments and the PF+BA3 mixed culture significantly reduced *Monilinia* spp. brown rot compared to the untreated control (54.7-64.1 % disease reduction). In addition, almost all treatments controlled the pathogen at postharvest (except PF+BA3). At this moment, the BA4 treatment effectively reduced brown rot disease (50.3 % compared to the untreated control), even statistically similar to the chemical applications.

Moreover, the interaction between CPA-8 and PF did not improve the BCA effectiveness in controlling brown rot disease. Therefore, despite recommending a combination of strategies with multiple modes of action to ensure more consistent disease control and overcome fluctuations in external factors, a synergistic or additive effect was unlikely to occur. Combinations of BCAs would not have been necessary in this case.

The main differences observed in the efficacy tests among CPA-8 treatments are probably due to the formulation process. BA3 and BA4 treatments differ in the composition of the two polysaccharides used in fluidification: maltodextrin, with higher degree of solubility in water (Shamekh *et al.*, 2002), and potato starch. It seems that potato starch is an essential component in the formulation of CPA-8 since the BA4 treatment demonstrated higher efficacy than BA3 treatment at postharvest time. However, further studies should be conducted in order to better clarify this issue.

All CPA-8 formulations used in this work (except BA1 used in 2014 season) have been previously designed using diverse protective substances ($MgSO_4$, sucrose or

skimmed milk) and carrier materials (maltodextrin and potato starch) with the aim to improve biocontrol ability (Gotor-Vila *et al*., 2017b; Gotor-Vila *et al*., 2017c). These compounds were added to extend the shelf-life of the product over a period of up to 15 months and to increase the CPA-8 adherence over the fruits. The antagonists need to possess effective mechanisms to daily cope with the abiotic stresses to which they are commonly subjected in the orchard (Sui *et al*., 2015). Gotor-Vila *et al*., (unpublished results) described the persistence of BA3 and BA4 formulations directly exposed to abiotic factors that most affect the survival of BCAs under field conditions: temperature, RH and rainfall. Although satisfactory results were then reported, it is important to note that run off dynamics may change in the field under a natural environment. In this work, colonisation of treated fruit by CPA-8 appears to follow a general pattern in which high CPA-8 populations were obtained just after treatment application (>10^4 CFU cm^{-2}) and largely maintained until harvest and shelf-life evaluation at postharvest. These results led us to conclude that enough antagonist population level on fruit surface to subsequently obtain efficient control was achieved. Similar results were generally obtained in 2014 season, in which CPA-8 population dynamics (BA, BA1 and BA2 treatments) did not suffer losses between first application and harvest (data not shown). It is worth mentioning the ability of CPA-8 to largely survive on the fruit surface after preharvest application. It suggests that with less number of applications, the efficacy obtained for CPA-8 treatments would be the same. In contrast, many other BCAs such as the yeast *Candida sake* CPA-1, drastically declines over time once applied in the orchard (Calvo-Garrido *et al*., 2013).

The approach to biocontrol research has evolved toward being more ecologically holistic and more oriented toward both production strategies and industry's concerns. Biocontrol products will be the alternative not only to demonstrate disease control but also to be an environment-friendly strategy that do not imply residues on fruit. However, more research is needed in integrating BCAs into cropping systems such as in rotating biocontrol with chemical pesticides and in considering these into forecast models to choose whether to apply a chemical pesticide or biocontrol (Fravel, 2005).

Application of the CPA-8-based products resulted in high efficacy when the pressure of the pathogen ranged between the standard values recorded in the area, indicating that this BCA is a good candidate for future larger-scale field applications

in different regions producers of stone fruit. Thus, even if the contribution of treatments to brown rot reduction at harvest is dependent on meteorological conditions, this study highlights the potential of *B. amyloliquefaciens* CPA-8 as alternative or complementary strategies to control *Monilinia* spp. Disease control could be achieved by combining BCAs with non-chemical control or prevention methods already existing or under development (Köhl *et al.*, 2011). Therefore, continued research in biocontrol is needed to contribute to the movement toward sustainable agriculture and to ensure available alternatives.

Acknowledgments

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General Discussion

General Discussion

After considering the results detailed in Chapters I-VII, subject under study of the present Ph.D. thesis, many criteria regarding the successful development of BCA- based products have been achieved.

Two optimised CPA-8- formulated products with long-term shelf-life have been developed, which demonstrated high efficacy against brown rot caused by *Monilinia* spp. on a wide range of stone fruit under laboratory conditions. Moreover, such formulations have been applied at large scale, involving full field testing trials during two different growing seasons. The biocontrol potential of CPA-8 has been highlighted, thus suggesting the introduction of this strain into well-designed disease programs that integrate the use of biopesticides into diverse pest management strategies.

The main reason for conducting postharvest biocontrol research is to reduce or replace the use of synthetic chemicals because of the concerns regarding their impact on the human health and the environment (Droby *et al.*, 2009). However, prospects of developing successful biocontrol products were greater than advances already consolidated. In most cases, product development was successfully conducted but their full commercial potential was not achieved (Droby *et al.*, 2016; Usall *et al.*, 2016). Compared to the traditional chemicals, BCAs have the ability to better regulate the physical environment and to act directly on the target location. Nevertheless, despite the advantages noted, the performance of successful biocontrol products is still subjected to significant variability, thus limiting their acceptance among disease control strategies (Droby *et al.*, 2009).

The complete development of microorganism-based products is a long and costly process. Therefore, the most important points regarding the obtaining of CPA- 8-based products are largely discussed below:

1. CHARACTERISATION OF CPA-8

Among the diverse reasons which could explain the lack of success of biological control, one is related to the legal status of BCAs, which must be registered according to guidelines originally developed for chemical pesticides (Alabouvette & Cordier,

2011). In Europe, the insertion of plant protection products in the market is regulated by EC regulation 1107/2009, which entails the procedures for chemicals, semiochemicals, plant extracts, and microorganisms approval. Data requirements in the EU are dictated by Commission regulations Nº 283/2013 and 284/2013, which provide a list of data required for active substances and products, including microorganisms (e.g. bacteria, fungi, protozoa), and viruses.

1.1. Molecular marker design

According to the EU requirements, the regulation clearly states that the identification together with the characterisation of the microorganisms provides crucial information for decision making regarding risk assessment (Alabouvette & Cordier, 2011). Scientists agree that identification must be at the strain level. However, such identification frequently involves many difficulties. In this thesis, two molecular markers have been developed to accurately identify the BCA CPA-8 among other strains belonging to related species or even the same (**Chapter I**).

The first approach was to design a SCAR marker, a sequence-characterised region derived from a previous RAPD analysis. Random markers (RAPDs) have been widely used because of the simplicity of the method and its ability to discriminate closely related microorganisms without prior knowledge of the target sequence (Nunes *et al.*, 2008). However, they need to be converted into more specific SCAR markers, in order to improve the reproducibility and applicability of the method. The developed SCAR-4 F/R amplified a 665 bp fragment which was partially specific for CPA-8 since twelve related isolates among the whole collection (including 77 strains) were also detected. Nevertheless, all of them were phenotypically different from strain CPA-8 and could be totally distinguished by plating methods prior DNA amplification.

CPA-8 was firstly identified by 16S rDNA partial analysis by the Netherland Culture Collection of Bacteria as a member of the *B. subtilis* species complex. However, after considering partial sequence alignments among related strains, it was renamed as *Bacillus amyloliquefaciens* instead of *Bacillus subtilis*, which was its first name in the literature.

General Discussion

For many years, most of the available evidence concerning genotypic variation among different *B. subtilis* group isolates came from the assessment of phenotypic variation. Moreover, it was not until the 1990s that loci other than 16S rDNA genes were examined among multiple strains (Earl *et al.*, 2008). 16S rDNA-based taxonomy seemed like a clear way forward, except in the case of closely related species groups such as *Bacillus*, where insufficient divergence in 16S rDNA prevented the resolution of strain and species relationship (Borriss *et al.*, 2011; Maughan & Van der Auwera, 2011). Therefore, it is highly informative to determine which genes exhibit limited or not variability. The subsequent use of housekeeping genes that are essential and not lost from genomes, but evolve more quickly than 16S rDNA, has proven to be useful for taxonomic classification (Maughan & Van der Auwera, 2011; Palys *et al.*, 1997). Hossain *et al.* (2015) compared the genome of different *Bacillus* related strains and provided insights into genomic features involved in control activity. Chen *et al.* (2009) and Yssel *et al.* (2011) also shown the efficacy of genes involved in chemotaxis regulation in resolving closely related taxa of the *B. subtilis* group. Such interpretations likely reveal the ecological diversity within the *Bacillus* species, thus providing a true picture of the genus ecology and evolution (Maughan & Van der Auwera, 2011). Sequencing of the *Bacillus* genomes allowed us to choose gene sequences unique for a particular strain and to design specific PCR reactions.

In this work, the genes *RBAM 007760* and *trpE (G)* were chosen due to its significance adaptive traits such as surface adhesion and biofilm formation or to its participation in metabolic pathways, respectively (Chen *et al.*, 2007; Johansson *et al.*, 2014). These interesting sequences were blasted against different related *B. amyloliquefaciens* strains available in the NCBI GenBank and the most diverse regions were selected. Oligonucleotide primers where then designed based on the sequence of the type strain *B. amyloliquefaciens* subsp. *plantarum* FZB42. Tests of gene product formation were conducted on the whole bacteria collection. Finally, a band of 265 bp was specifically amplified for CPA-8 by using the primer pair *RBAM 007760* F2/R2 and any similar pattern was obtained within all the heterologous DNA samples.

The availability of the genome of few *B. amyloliquefaciens* species harbours an array of gene clusters involved in the synthesis of lipopeptides and polyketides with antimicrobial activity, mainly responsible for the biological control and highly

conserved within related species and strains (Blom *et al.*, 2012; Chen *et al.*, 2008; Chen *et al.*, 2007; Niazi *et al.*, 2014a; Niazi *et al.*, 2014b). Members of *B. amyloliquefaciens*, including CPA-8, are industrially important and have been described as efficacious BCAs of fruit pathogens (Calvo *et al.*, 2017; Wei *et al.*, 2015; Zhang *et al.*, 2015). Therefore, information regarding comparative analysis of sequenced genomes is crucial to identify which genes and mechanisms are involved in CPA-8's ecology and biology. The figure 1 illustrates de relative abundance (%) of the different subsystem categories in different genomes of the *Bacillus* genus, *B. subtilis* group, *B. amyloliquefaciens* species and *B. amyloliquefaciens* subsp. *plantarum* (Hossain *et al.*, 2015).

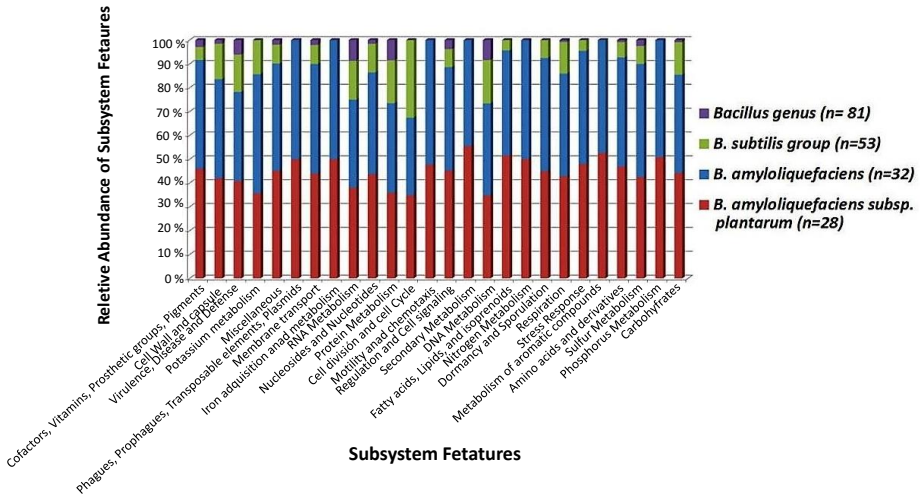

Figure 1. Distribution of different subsystem categories of four different core genomes specific to genus *Bacillus*, *B. subtilis* group, *B. amyloliquefaciens* species and *B. amyloliquefaciens* subsp. *plantarum* (Hossain *et al.*, 2015).

Having identified the BCA CPA-8 at the strain level, it allows to track accurately the strain after being released in the environment. According to EC regulations, the distribution and fate of the BCAs in the field needs to be addressed as indispensable requisite for registration purposes (Soto-Muñoz *et al.*, 2015). Therefore, trials on environmental monitoring of CPA-8 once the strain is applied in stone fruit at preharvest time have been considered.

The molecular markers developed serve now as the basis for developing a qPCR method that not only identify but also quantify CPA-8. This methodology would be a potential tool to quickly and specifically monitor CPA-8 populations in large-scale experiments immediately after its application. Thus, the use of more efficient DNA-based methods greatly facilitates the investigation of BCAs' fate and activity (Soto-Muñoz *et al.*, 2014).

1.2. Genetic stability

As part of the quality protocol system, the genotypic stability of CPA-8 was evaluated over time to thus determining its integrity during long-term storage in CRIOBILLES AEB 400100 (AES Laboratory, Combourg, France). The genetic integrity during the storage conditions (from 2005 to 2012) was evaluated through the detection of DNA amplification methods (DNA fingerprint patterns) that can highlight specific variations. Moreover, this methodology was also applied to confirm that the drying methods used in CPA-8 formulation do not alter the genetic integrity of the bacterium (unpublished results).

Interspersed repetitive sequences in prokaryotic genomes can be used as oligonucleotide primer binding sites for PCR reactions. Rep-PCR methods are based on sequences lying between these elements. Multiple amplicons of different size can be fractionated by electrophoresis and enable the establishment of DNA fingerprint patterns specific for individual bacterial strains (Versalovic *et al.*, 1994).

Examples of evolutionary conserved repetitive DNA sequences are BOX, ERIC, REP, and $(GTG)_5$. BOX elements consist of differentially conserved subsequences, originally defined as different subunits (boxA, 57 bp; boxB, 43 bp and boxC, 50 bp). REP sequences are 38 bp palindromic units which contain a 5 bp variable loop, and ERIC sequences are 126 bp elements which possess central, conserved palindromic structures (Versalovic *et al.*, 1994). $(GTG)_5$ PCR fingerprinting consists of five repetitions of the trinucleotide, providing the broadest band pattern (Freitas *et al.*, 2008).

PCR tests for BOX A1R and $(GTG)_5$ were applied, as it was described by Valerio *et al.* (2012), to evaluate the genetic stability of CPA-8 after long-term storage or

drying conditions. Differences ranging from a single fragment position to multiple fragment changes were compared between CPA-8 isolates. As figure 2 demonstrates, the pattern of CPA-8 is unchanged. Therefore, the possibility that storage or formulation conditions would derive in genotypically different CPA-8 subpopulations is considered remote.

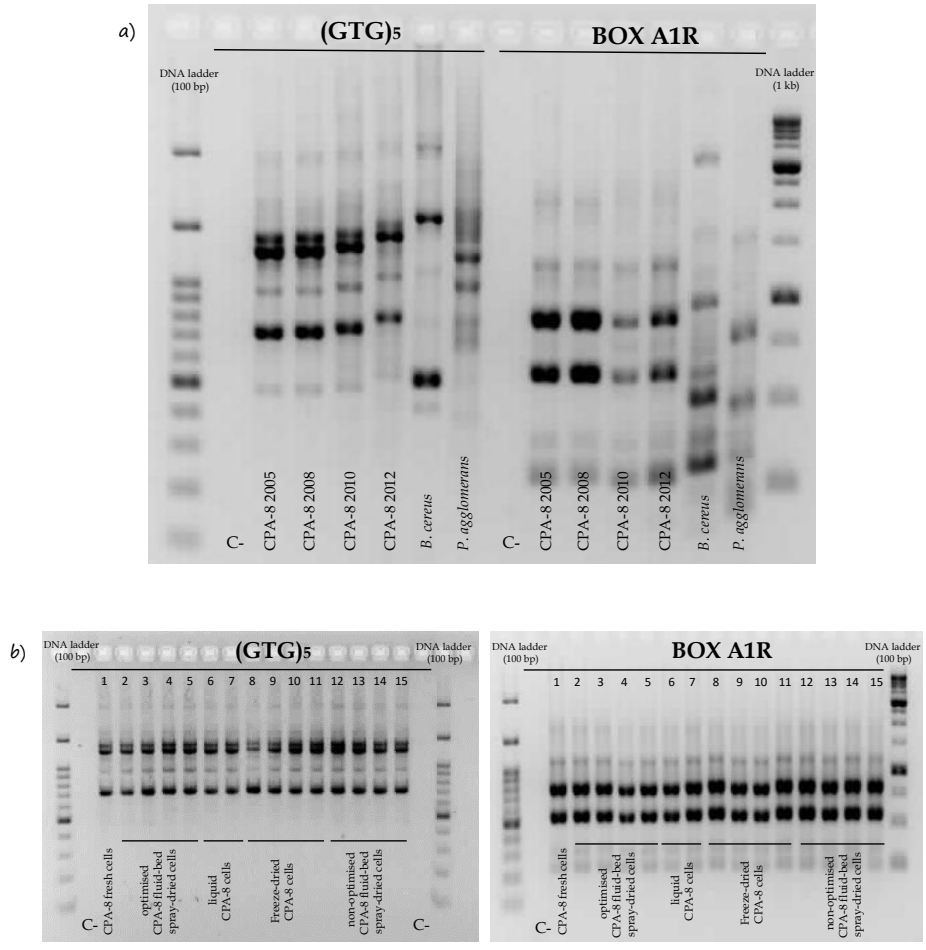

Figure 2. Genetic stability of CPA-8 after long-term storage at -80 ºC (a) and after different formulation approaches (b). CPA-8 formulations (two replicates each) consist of optimised CPA-8 fluid-bed spray-dried cells amended with sucrose and skimmed milk as protectants and fluidised with either, maltodextrin or potato starch as carriers; liquid CPA-8 formulated cells without protectants; freeze-dried CPA-8 cells formulated with $MgSO_4$ and $MgSO_4$ plus skimmed milk as protectants; and non-optimised CPA-8 fluid-bed spray-dried cells non amended or amended with $MgSO_4$ as protectant and fluidised with potato starch as carrier.

1.3. Metabolites production: Enterotoxins

This point, which is obviously very important in regard to risk assessment, needs to be assessed strain by strain, as microorganisms behave very different in the environment. It concerns sensitisation, acute toxicity, pathogenicity, and infectiveness and genotoxicity (Alabouvette & Cordier, 2011).

Regarding the toxicity trials, the EC regulation clearly states that relevant metabolites must be purified and identified. But, which of them should be considered hazardous in relation to toxicity studies? Those compounds responsible of the mode of action of the BCA must be studied per se. However, most of the microorganism are able to produce many secondary metabolites at different times of their life cycle and depending on the target organism (Alabouvette & Cordier, 2011). In the University of Technology of Graz, Austria (unpublished results), the presence of the metabolites fengycin, iturine, surfactin, and subtilisin, was analysed in CPA-8 formulated samples (out of this thesis but part of the BIOCOMES European Project). These products were chosen for its key role in the biocontrol efficacy of CPA-8 (Yánez-Mendizábal *et al.*, 2012d). Exact masses and fragment masses of the selected substances as well as the corresponding spectra were compared to the data available in the literature (Tang *et al.*, 2010; Wang *et al.*, 2004) and in the ChemSpider library (http://www. chemspider.com/Chemical-Structure.1906.html).

Moreover, consumer concerns regarding toxins entering the food chain has promoted closer scrutiny of the BCA's ability to produce toxic metabolites, since biocontrol products are based on living microorganisms capable to remain in or on plant products.

Bacillus cereus is a very common food borne pathogen responsible for two types of food poisonings. Diarrheal type is associated with production of diarrheal enterotoxins such as hemolysin BL, nonhemolityc enterotoxin NHE, cytotoxin K, enterotoxin BceT and other toxic proteins such as phospholipase C and lecithinase (Hansen & Hendriksen, 2001; López & Alippi, 2010; Moravek *et al.*, 2004; Ngamwongsatit *et al.*, 2008; Oltuszak-Walczak & Walczak, 1997). The emetic type of food poisoning is associated with the ability of some *B. cereus* to produce a cyclic depsipeptide called cereulide. The dominating type of disease caused by *B. cereus*

differs from country to country. In Japan, the emetic type is about ten times more frequently than the diarrheal type, while in Europe and North America the diarrheal type is the most regularly reported (Granum & Lund, 1997).

However, despite the high degree of phylogenetic relatedness among members of the genus *Bacillus*, few researches have reported the presence of foodborne illnesses associated with *Bacillus* spp. other than *B. cereus* (From *et al.*, 2005; Phelps & McKillip, 2002).

In this thesis, the presence of components from both, HBL and NHE *B. cereus* protein complexes, has been analysed in CPA-8 (**Chapter II**). These proteins are highly present within *B. cereus* strains, being considered the most important factors of virulence in this specie (López & Alippi, 2010; Phelps & McKillip, 2002). Among the different primer pairs used, the results revealed that only the subunit NheA of the three component NHE operon amplified for CPA-8, when the primers described by Hansen & Hendriksen, (2001) were used. This suggested that CPA-8 is not hazardous for human health since all the components are necessary for cytotoxic activity (Lindbäck *et al.*, 2004; López & Alippi, 2010). However, further assays should be needed to totally ensure the safe use of CPA-8 (e.g. different genes, western-blot). Ideally, all BCAs should be sequenced and analysed before considered for application, given the low cost of sequencing and the amount of microbial genetic information currently available in the databases.

Again, the Directive requires that those consequences regarding the non-target microorganisms already exposed to the BCA, must be also described. Possible non-target effects need to be identified and reported as part of the risk assessment protocols. This will be a complicate task because the composition and dynamics of a microbial community depend on multiple factors (ecological niche of the microorganism, past and current environmental conditions, plants species and physiological state, etc.) that would influence on the survival and development of a foreign BCA within this community (Massart *et al.*, 2015).

Out of this thesis but part of the BIOCOMES European Project, the INIA group (National Institute of Agrifood Research, in Madrid, Spain) evaluated the possible changes in population dynamics within the epiphytic community of stone fruit after

application of CPA-8 in the field (Guijarro *et al*. unpublished results). The effect on the number, activity, biomass and microbial community structure was measured by using two methods already available for extraction, identification, and detection of host natural microbiota diversity: Denaturing Gradient Gel Electrophoresis (DGGE) and Fluorescein DiAcetate (FDA) hydrolysis. Preliminary results demonstrated that microbial diversity (fungi and bacteria) on fruit surface was slightly modified by CPA-8 treatments, especially the fungal populations. However, these effects need to be validated in further assays during the next growing season.

1.4. Antibiotic sensibility pattern

The CPA-8 sensibility (or resistance) to an extended spectrum of antibiotics was evaluated by using a relatively simple, reliable, and rapid test (**Chapter II**). The sensibility (or resistance) is based on the size of a zone of inhibition that surrounds the bacterium incubated with a specific concentration of an antibiotic. CPA-8 growth was evaluated against chloramphenicol, ampicillin, gentamicin, nalidixic acid, streptomycin, and hygromycin.

It was found that CPA-8 was susceptible to most of the antibiotics investigated. CPA-8 could not grow in the presence of chloranphenicol and streptomycin when they were used at concentrations higher than 80 and 200 ppm, respectively. Moreover, the sensibility to gentamicin, ampicillin, and nalidixic was manifested at all concentrations used, detecting larger halo diameters as the antibiotic concentrations increased. However, this study showed a clear resistance to hygromicyn. Such information could be further used to avoid bacterial contamination during different stages of the CPA-8-based products development, such as, formulation process or shelf-life storage.

1.5. Ecophysiological responses

Biocontrol activity of BCAs is heavily influenced by abiotic factors. In this thesis, two approaches have been performed regarding CPA-8. The first of them was based on *in vitro* testing (**Chapter II**); the other one (**Chapter VI**) will be discussed latterly, in the section referred to the definition of the control strategy. Effects of pH, water

activity (a_w), and temperature in culture media were assessed on CPA-8 growth. These three factors interact and are commonly considered of key importance to identify the environmental niche in which a BCA can actively grow and successfully act as antagonist (Costa *et al.*, 2002; Teixidó *et al.*, 1998).

Temperature is one of the major environmental stresses experienced by microorganisms. The minimum temperature at which a BCA is able to grow is an intrinsic property that allows the organism to grow when growth conditions other than temperature are non-limiting (Ratkowsky *et al.*, 1982). The adaptation of the BCAs to high temperatures is essential, as it provides an advantage when they have to be applied at field conditions and need to be formulated by drying technologies (Sui *et al.*, 2015).

The ability of adaptation to different ambient pH is also crucial for a BCA to be successful in agricultural systems, for example where the addition of N- or P-fertilisers are likely to affect BCAs due to the associated change in soil or plant pH (Daryaei *et al.*, 2016). Moreover, the pH on or in the fruit is also a changing factor inherent to the fruit development. Besides that, the availability of water (a_w) greatly influences microbial growth and negatively affects BCAs development when it is suboptimal.

On the whole, pH, a_w, and temperature requirements for microbial metabolism and growth meaningfully differ between and within species (Daryaei *et al.*, 2016) and should therefore be estimated experimentally. In this work, two parameters were considered for CPA-8: the maximum growth rate (μ_{max}) and lag phase duration (λ), both obtained by fitting the data to the primary model of Baranyi & Roberts (1994). Estimation of bacterial growth parameters was performed by the ln-transformation of absorbance measurements, which have the advantage of being rapid, non-destructive, inexpensive and relatively easy to automate when compared to classical viable count methods (Dalgaard & Koutsoumanis, 2001).

Data on pH-temperature analysis revealed that the optimum growth for CPA-8 was observed at 37 ^{o}C and pH between 7 and 5. On the contrary, the slowest growth was recorded at 20 ^{o}C and pH 4.5. Moreover, the type of solute used to modify a_w greatly influenced the minimum a_w at which the CPA-8 was able to grow. It was 0.950

and 0.960 when the solutes used to modify the media were either, glycerol or glucose, respectively. In general, the lowest a_w for CPA-8 growth increased when the temperature decreased to 20 ºC, at which CPA-8 was not able to grow at less than 0.990 a_w, regardless of the type of solute. Based on this, CPA-8 grew better under warmer temperatures and pH neutral conditions, which is consistent with its description as mesophilic and neutrophilic bacterium. Although CPA-8 could not grew at temperatures lower than 10 ºC and pH lower than 4.5, its maintenance was confirmed. These data agree with the previous work reported by Yánez-Mendizábal *et al.* (2012c), in which CPA-8 survived on wounded nectarines, with pH lower than 4.5, and stored at 0 ºC.

The relationship between microbial growth rate and environmental conditions involves different response mechanisms, which may trigger a series of energy resource pathways in the cell as it attempts to maintain cytoplasmic homeostasis (Magan, 2001). For instance, the accumulation or synthesis of intracellular compatible solutes (amino acids, sugars or ions) could improve the tolerance to the dehydration process, which is a key factor in cell viability during the formulation of microorganisms (Cañamás *et al.*, 2007; Csonka & Epstein, 1996; Mossel *et al.*, 1995; Pascual *et al.*, 2003). Regarding a_w media modification, Teixidó *et al.* (2005) demonstrated that *Pantoea agglomerans* accumulated substantial amounts of the compatible solutes glycine–betaine and ectoine when the ionic solute NaCl was introduced. These compounds played a critical role in the environmental stress tolerance of *P. agglomerans* cells. Subjection to a mild stress can make cells resistant to a subsequent lethal challenge of the same stress and can also render cells resistant to other stress conditions (cross protection) (Teixidó *et al.*, 2005). This approach could provide a method for improving the physiological quality of inocula and could have implications in the formulation and shelf-life of CPA-8.

In summary, this study has provided ecological data on the growth profiles of CPA-8 in relation to pH-temperature and a_w-temperature. From an ecological point of view, CPA-8 presents high tolerance against a wide range of pH, a_w, and temperature conditions.

Moreover, sigmoidal models other than the one traditionally used may have better potential to describe CPA-8 growth process (logistic model, Gompertz or

Richard's model). Under many circumstances, primary modelling yields reliable information on the value of μ_{max} and λ. However, secondary modelling would be of practical importance to predict how this bacterium behaves under diverse hypothetic environments.

1.6. Mode of action

Antagonists, as living organisms, can display a wide range of modes of action, which could be different depending on the stage of their activity, the host, and the pathogen. Sometimes, different modes act simultaneously and it is therefore difficult to establish if their effect is due to either synergic, additive or a single action (Di Francesco *et al.*, 2016). Regarding the main biocontrol mechanism of CPA-8, Yánez-Mendizábal *et al.,* (2012d) provided experimental evidence about the strong antifungal effect of this bacterium against *Monilinia* spp., based on the production of fengycin-like lipopeptides. However, the synthesis of these compounds could not be considered as the only mechanism of action.

In this thesis, the CPA-8 volatile organic compounds (VOCs) emission was analysed (**Chapter III**) with a double petri dish assay against mycelial and colonial growth of three postharvest fruit pathogens: *Monilinia laxa*, *Monilinia fructicola* and *Botrytis cinerea*. The results obtained clearly demonstrated that the VOCs generated by the antagonist significantly inhibited the mycelium development of the target pathogens. Those compounds emitted by CPA-8 were then identified by head-space (HS)-SPME coupled with (GC-MS) as 1,3 pentadiene, acetoin (3-hydroxy-2-butanone), thiophene, and ethylacetate; being the pure compound thiophene the most effective one, showing even 82 % suppression of mycelial growth with EC_{50} values lower than 6.67 $\mu L\ mL^{-1}$ headspace. However, it did not manifest any effect under *in vivo* conditions.

Despite the great variability obtained when the VOCs emitted by CPA-8 were applied on artificially infected cherry fruit, *M. fructicola* was significantly controlled. Moreover, it was observed that for all pathogens, the presence of spores on the fruit surface was significantly reduced. Data from *in vitro* and *in vivo* trials meaningfully differed probably because VOCs production is highly influenced by different parameters such as microorganism growth stage, culture mode, temperature air exchange or media

composition (Chaves-Lopez *et al.*, 2015; Di Francesco *et al.*, 2015; Mercier & Jimenez, 2004). Moreover, the antifungal action of the VOCs produced by CPA-8 largely depended on the nature of the pathogenic fungi against which the VOCs were tested. In this work, VOCs effect could be enhanced by the addition of different nutrients to the culture medium.

VOCs produced by CPA-8 may play an essential role in the biocontrol efficacy of this BCA. The antimicrobial mechanism of the VOCs is another important consideration to effectively design a biofumigation program. Volatiles act by changing protein expression and the activity of specific enzymes (Wheatley, 2002) or by increasing the cellular membrane permeability, thus reducing the activity of membrane-associated enzymes and impairing nutrient uptake (Chaves-Lopez *et al.*, 2015; Fialho *et al.*, 2011). This is of practical importance since the procedures needed for registration purposes of non-living biocontrol products would be considerably shortened.

Many *Bacillus* strains recognised as effective BCAs not only prevent plant diseases by producing antifungal substances (known as antibiotics) or VOCs, but also improve plant growth by fixing or solubilising efficiently the nutrients, producing plant growth hormones and siderophores, being involved in biofilm formation systems or inducing systemic resistance in plants (Raza *et al.*, 2016; Spadaro & Droby, 2016).

Competition is the consequence of the requirement for the same macro and micronutrients (like sugars, vitamins and minerals) or space by two or more microorganisms (Di Francesco *et al.*, 2016). As the main postharvest fungal pathogens are wound parasites, the ability of the antagonist to rapidly colonise the niches and increase their populations at the expense of the pathogen is strategic for the success of the disease control (Di Francesco *et al.*, 2016). Among nutrients, iron is one of the most critic microelements for fungal development. Many *Bacillus* spp. and *Pseudomonas* spp. are able to produce specific compounds called siderophores which have the ability to compete for the iron available in the environment and interfere with the pathogen germination, growth, and virulence. In iron starvation conditions, fungi have lower catalase activity and lower reactive oxygen species (ROS) protection (Di Francesco *et al.*, 2016; Spadaro & Droby, 2016).

Siderophores, which form tight and stable complexes with ferric ion, can be divided into three main classes depending on the chemical nature of the oxygen donator for Fe^{3+} coordination: carboxylate, hydroxamate, and catecholate; the latter two being produced by bacteria (Beneduzi *et al.*, 2012; Miethke & Marahiel, 2007; Spadaro & Droby, 2016). To quickly and efficiently screen if CPA-8 is a siderophore-producing bacterium, the Chrome Azuoral Sulphonate (CAS) agar plate assay firstly described by Schwyn & Neilands (1987) was used with modifications (Milagres *et al.*, 1999). In the CAS agar plate assay, microorganisms able to produce siderophores form colonies with an orange or violet halo, depending on the type of siderophore. This occurs because when the rate of iron supply limits the microbial growth, it promotes the siderophore secretion and iron is chelated from the original blue CAS-Fe (III) complex (Yu *et al.*, 2011). The figure 3 illustrates the siderophore production of CPA-8 on CAS agar dual plates (unpublished results).

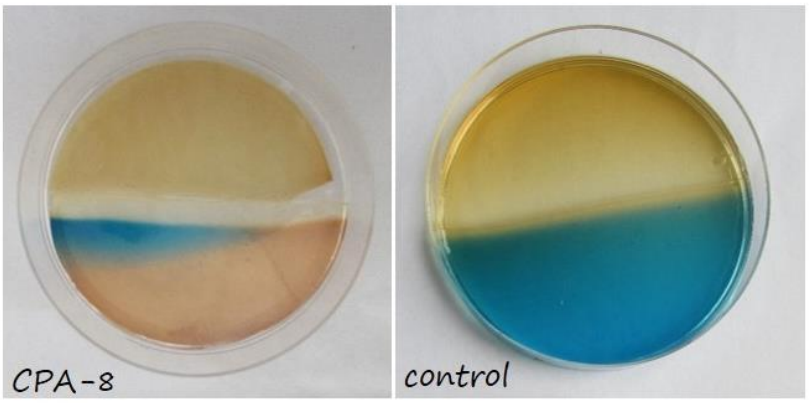

Figure 3. Siderophore production of CPA-8 on CAS agar dual plates. CPA-8 was inoculated on the border of the NYDA (Nutrient Yeast Dextrose Agar) medium and incubated for 21 days at 30 ºC.

The amount and type of the siderophores produced by an organism depends on the availability of organic and inorganic nutrients. Therefore, bacteria are likely to respond different when colonising each target site (Alexander & Zuberer, 1991). Nevertheless, optimised protocols to specifically analyse siderophore production, based on spectrophotometry measurements and curves with standard solutions, are available in the literature (Calvente *et al.*, 2001; Wang *et al.*, 2009a; Wang *et al.*, 2009b).

Apart from the above mentioned mechanisms, among the other strategies involved in biocontrol efficacy, the biofilm formation and the induction of resistance (ISR, induced systemic resistance) will be hereafter highlighted. To successfully colonise intact and injured fruit surfaces, the antagonist should have the ability to use specific features facilitating its adherence, colonisation and multiplication (Spadaro & Droby, 2016). In most cases, these features are associated with the formation of a biofilm, made by multicellular communities in which cells are held together by an extracellular matrix that is composed mainly by exopolysaccharides, proteins, and nucleic acids (Chen *et al.*, 2013; Spadaro & Droby, 2016; Zeriouh *et al.*, 2014). Recently, it was found that the colonising behaviour and biofilm formation of *Bacillus* strains belonging to the *B. subtilis sensu lato* group, depend on the production of surfactines, a family of lipopeptides characteristic of these bacteria that has the ability to reduce the surface tension and is involved in the swarm motility (Mousivand *et al.*, 2012; Navarta *et al.*, 2011; Ongena & Jacques, 2008; Zeriouh *et al.*, 2014). In the future, it would be interesting to characterise how CPA-8 senses and responds to the signals of stimulating biofilm formation. Regarding ISR, it is the state of an enhanced defensive ability developed by plants when they are appropriately stimulated (Beneduzi *et al.*, 2012). Bacteria of diverse genera have been identified as Plant Growth Promoting Rizhobacteria (PGPR), of which *Bacillus* strains, *including B. amyloliquefaciens*, are predominant (Beneduzi *et al.*, 2012). The direct promotion of plant growth by PGPR entails either providing the plant with essential compounds or facilitating the uptake of certain nutrients from the environment (Beneduzi *et al.*, 2012). According to the wide range of antagonistic functions exerted by CPA-8, it would be of practical interest to further determine whether this bacterium belongs to PGPR to thus leading to a more efficient use of biocontrol strategies.

Understanding, as much as possible, the mode of action of an antagonist greatly facilitates the registration requirements for commercial use. The availability of more efficient DNA-based and proteomic technologies, along with bioinformatics, has provided new opportunities and tools to gain deeper and more accurate insights about the interactions already indicated (Massart *et al.*, 2015; Spadaro & Droby, 2016). Moreover, knowledge of the modes of action involved in biocontrol efficacy is of crucial importance to further develop appropriated protocols regarding the production and formulation processes and delivery systems of the BCAs (Di Francesco *et al.*, 2016; Spadaro & Droby, 2016).

2. PRODUCTION AND FORMULATION OF CPA-8

2.1. Mass production

Production of BCAs is a fundamental step along the development of successful biocontrol products for commercial use. Mass production has the objective of obtaining the greatest quantity of efficacious cells in the shortest period of time (Nunes, 2012). However, one factor limiting commercial interest in BCAs is the high cost involved in the process. Therefore, considerable research has focused on the use of alternative nitrogen or carbon sources based on agricultural wastes and by-products from the food industry (Nunes, 2012).

Yánez-Mendizábal *et al.* (2012c) reported an exhaustive investigation regarding growth media optimisation for CPA-8. This work focused on five nitrogen sources (yeast extract, peptone, whole soy flour, defatted soy flour, and ground soy seed) and two carbon sources (molasses and sucrose), which were prepared at different concentrations. After analysing CPA-8 growth data with the Baranyi equation (Baranyi & Roberts, 1994), the use of defatted soy flour (DSF) (44 %) with the addition of molasses, resulted an excellent substrate with high cell density. DSF was obtained from the food industry. Molasses, which are by-products widely used in the production of microorganisms, provided high sugar content (approx. 50 % w/w sucrose, glucose, and fructose), colloidal suspension, heavy metals, vitamins and nitrogen compounds (Costa *et al.*, 2001; Yánez-Mendizábal *et al.*, 2012c).

Although by-products have a low price, they present some hurdles that must be considered. By-products are not as standardised as purified products and may contain impurities that need to be removed during media preparation (Stambury *et al.*, 1995; Teixidó *et al.*, 2011). Furthermore, their composition may vary according to the season and origin (Costa *et al.*, 2001). The non-homogeneity of this kind of products can limit their usefulness in an industrial scale-up process (Zhang & Greasham, 1999). In this thesis (**Chapter V**), such inconveniences had to be faced when CPA-8 was mass produced prior to designing suitable formulation strategies.

First of all, the microbial load inherent to the soy flour proved to be a source of contamination. Furthermore, its low solubility during the medium preparation was also a negative factor. Therefore, it needed to be filtered before autoclaving to discard the insoluble fraction. To solve these hurdles, alternative nitrogen sources were evaluated. In a first approach, the DSF medium was slightly modified using the same soy flour extracted by boiling for 10 minutes (100 g L^{-1} extracted DFS). As the media preparation was highly time-consuming, the isolated soy flour protein PROSTAR 510A (90 % protein content) was also evaluated at two different concentrations (10 and 20 g L^{-1}). The results revealed that while all media provided good CPA-8 growth after 72 h of culture, achieving stable CPA-8 concentrations around 10^9 CFU mL^{-1} (8.9-9.0 log CFU mL^{-1}), the best CPA-8 growth curves were obtained when protein PROSTAR 510A was used at 20 g L^{-1} (**Chapter V**). Apart from indicating a faster exponential growth phase, the decrease on CPA-8 growth after 22-30 h of growing (probably caused by the nutrients limitation) was hardly detected in the medium based on protein PROSTAR 510A at 20 g L^{-1}, compared to the other two media. Moreover, the use of this nitrogen source did not suppose any appreciable increment in the cost of the substrate for CPA-8 production, thus maintaining its suitability for commercial purposes.

The effect of mineral supplements as trace elements has been referred as an important factor in the production of metabolites of many *Bacillus* species (Cooper *et al.*, 1981). It has been observed a stimulatory effect of some minerals, specially Mn^{2+}, Fe^{2+}, and Mg^{2+} ions, which provide a significant improvement in cell growth (Abdel-Mawgoud *et al.*, 2008). Consequently, some of them were included in the CPA-8 growth media optimisation (Yánez-Mendizábal *et al.*, 2012c).

The results here obtained, supported again the possibility to produce commercial amounts of CPA-8 at low cost. Moreover, main aspects other than substrate composition on CPA-8 growth conditions (aeration, stirring speed, and antifoam addition) have been described. Temperature and pH have significant effects on CPA-8. Jacques *et al.* (1999) showed that for *B. subtilis* strain S499, maximum cell growth, endospore and lipopeptides production by liquid fermentation occurred between 25 and 37 ^{o}C and optimum pH of 7, which concords with the CPA-8 ecophysiological responses discussed above.

2.2. Endospore production

CPA-8 survival after drying is directly related with its ability to produce endospores (Yánez-Mendizábal *et al.*, 2012a). This is so because the spores are exceptionally resistant and stable, which means that they can remain viable during a long period of time under extreme conditions (Nagorska *et al.*, 2007). This additional property makes the genus *Bacillus* high profitable from a technological point of view. Thereby, the endospore production, which is a crucial factor in the production process of BCAs, requires to be done in a relatively easy and inexpensive way. The figure 4 shows a microphotography in which CPA-8 vegetative cells and endospores could be distinguished by staining procedures.

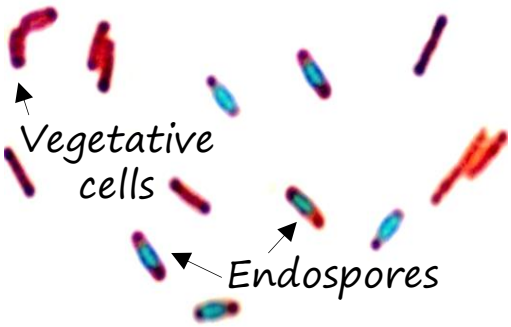

Figure 4. Microphotograph of vegetative cells and endospores of CPA-8 from rehydrated spray-dried formulates (Yánez-Méndizábal, 2012).

Once the medium for CPA-8 growth was optimised, the dependence between the age of the culture and the final concentration of endospores must be estimated, since Yánez-Mendizábal *et al.* (2012a) pointed out to be of practical importance. In this regard, CPA-8 cells after heat incubation (CPA-8 endospores) significantly increased from 24 to 72 h of culture, suggesting that the ability of CPA-8 to survive up to 80 ºC is highly related with the age of the culture. While the number of viable cells (vegetative cells and endospores) remained practically unchanged after 24, 48, and 72 h of culture (3.9-6.2 $\cdot 10^9$ CFU mL^{-1} and 1.9-3.6 $\cdot 10^9$ CFU mL^{-1} in the first and second repetition of the experiment conducted in **Chapter V**, respectively), the number of viable cells previously exposed to heat at 80 ºC was higher as the age of the culture increased.

Therefore, CPA-8 endospores after 72 h of culture ($4.3 \cdot 10^8$ and $4.0 \cdot 10^7$ CFU mL^{-1} in the first and second repetition, respectively) where higher than after 48 ($5.2 \cdot 10^7$ and $7.9 \cdot 10^6$ CFU mL^{-1}) and 24 h ($2.7 \cdot 10^5$ and $1.1 \cdot 10^6$ CFU mL^{-1}). It is fair to say that this correlation was also demonstrated after 96 and 120 h (Yánez-Mendizábal *et al.*, 2011). However, having obtained enough CPA-8 endospores to successfully survive the formulation process or extreme field conditions, cultures ages longer than three days were not considered convenient.

As described Errington (2003), *Bacillus* sporulation is very much dependent upon the decline of nutrients during the stationary phase. It has also been reported that the addition of salts to the growth media improves sporulation. Elements such as Ca, Mn, Mg, Fe, and Zn, included in CPA-8 growth medium (in appropriate concentrations), are essential to the spore formation, since they are present in the spore layers (Posada-Uribe *et al.*, 2015).

2.3. Formulation

After determining which operating conditions, on economic and technological aspects, allow the large-scale production of CPA-8, a meticulous plan regarding the formulation of BCAs by multidisciplinary approaches is required to optimise the yield, efficacy, and storage of the product developed (Hynes & Boyetchko, 2006). Formulation involves knowledge of microbial-plant ecology and pathology to clarify the interactions among BCA, pest, and environment. Moreover, microbial physiology needs to be considered in order to optimise the variables of the process. Last but not least is chemistry, which is also important to select those formulation components that sustain and promote an optimum activity of the microorganism (Hynes & Boyetchko, 2006).

Formulations are mainly composed by (i) an active ingredient, cells or spores; (ii) carriers, often formed by inert materials that support and deliver the active ingredient in the target site; and (iii) protectants or adjuvants, which sustain the function of the active ingredient by protecting it from high temperatures, desiccation, and ultraviolet radiation and promote the spread and dispersal of the product in the intended environment (Hynes & Boyetchko, 2006).

As it has been discussed above, the fengycin-like lipopeptides synthesised by CPA-8 play a key role in the antagonistic effect of this strain. This is the reason why CPA-8 is dissolved on its own culture medium after harvesting the cells from the bioreactor. Otherwise, the liquid could be filtrated with the final purpose to eliminate the cells from the supernatant. This might work because the cell free supernatant was effective enough against *Monilinia* spp. on peaches (Yánez-Mendizábal *et al.*, 2012d). However, it has been demonstrated that CPA-8 also involves different mechanisms of control that could lead different reactions in its antagonistic activity. Therefore, CPA-8 cells must be included in the biocontrol product. In this thesis, apart from the already described spray-drying technique (Yánez-Mendizábal *et al.*, 2012b), three different approaches were firstly carried out to formulate CPA-8: liquid, freeze-drying and fluid-bed spray-drying (**Chapter IV**).

CPA-8-based products effective against *Monilinia* spp. were obtained with satisfactory final concentrations (ranging from $1.93 \cdot 10^9$ to $2.98 \cdot 10^9$ CFU mL^{-1} and from $4.76 \cdot 10^9$ to $1.03 \cdot 10^{10}$ CFU g^{-1}, in case of liquid and dry formulations, respectively) and stable shelf-life.

Although no protectants were necessary to successfully formulate CPA-8 in a liquid form, this procedure requires the manipulation of high volumes with no protection from contamination. Moreover, the obtained liquid products needed to be conserved under refrigeration conditions, which significantly increased the costs of storage. For this reason, this system was not finally chosen to formulate CPA-8.

Regarding drying formulations, the table 1 below reports both, the fixed and manufacturing costs inherent to different formulation technologies, all referred to freeze-drying, which is by far, the most expensive one.

Table 1. Costs of drying processes referenced to freeze-drying (Santivarangkna *et al.*, 2007).

Drying process	Fixed costs (%)	Manufacturing costs (%)
Freeze-drying	100.0	100.0
Vacuum-drying	52.2	51.6
Spray-drying	12.0	20.0
Drum-drying	9.3	24.1
Fluidised-bed drying	8.8	17.9
Air-drying	5.3	17.9

After freeze-drying CPA-8, it was observed that those undesirable effects caused by the drying process could be partially alleviated by the addition of protectants. In this work, sucrose, $MgSO_4$, and skimmed milk (SM) were tested. Although the addition of SM (10 %) did not improve the cell viability ratio (based on the difference between the CFU after and before drying) obtained for the control (non-amended CPA-8 cells), the use of 10 % $MgSO_4$ plus 10 % SM showed the best results in CPA-8 viability after freeze-drying process (0.17-log cell viability reduction) and no significant differences were observed when it was compared to products formulated with either 10 % sucrose or 10 % $MgSO_4$. Moreover, the stability of those products conserved at 4 and 22 ^{o}C was demonstrated. Although a minor reduction in cell viability could be observed (0.24-0.34 log units) after four months of storage, it was not considered decisive. Besides the high cost that freeze drying involves, this process was considered very time-consuming. Therefore, it was also discarded to formulate CPA-8.

Apart from the above mentioned processes, the fluid-bed spray-drying method for the granulation of particles is well-known in the pharmaceutical and other industries as a one-step, enclosed operation (Hemati *et al.*, 2003; Planinsek *et al.*, 2000; Srivastava & Mishra, 2010). Because several ingredients can be mixed in the same vessel, this approach reduces material handling and shortens the process duration (Srivastava & Mishra, 2010). Besides that, the operating costs would be reduced up to five times, compared to freeze drying. Taking into account the considerations already mentioned and those discussed in **Chapter IV**, fluid-bed spray-drying appeared to be the most suitable technology for CPA- 8 formulation. Briefly, this approach offers high level of microbial survival due to less damage of the membrane and good control over residual moisture content, water activity, powder flowability and particle size.

In this work, different carriers and binder materials were evaluated, in a preliminary way, to enhance CPA-8 powder recovery with no undesirable agglomerates that triggered clogging the spray nozzle. The combination of potato starch as carrier and pregelatinised potato starch as binder was finally chosen. Moreover, the CPA-8 viability was experimented with two cell suspensions: one without additives "BA1" (control) and other one amended with 10 % $MgSO_4$ as protectant "BA2". Both formulations provided high bacterial viability after drying with cell viability ratios of 0.06-0.14-log units. Moreover, they were found shelf-stable in all the storage temperatures tested (22, 4 and -20 ^{o}C), extending the viability of CPA-8 for up to twelve months. The results obtained are

important for subsequent CPA-8 distribution and application, considering that commercial products must have a long storage life and also should not require special cold conditions.

Among the advantages of this technology, this equipment offers three different patterns of fluidification, mainly based on the spray nozzle position/location: top spray, bottom spray, and tangential spray. These different applications allow to obtain dried products with particular characteristics. On the whole, the fluid-bed top-spray method produces highly dispersible granules with a specific porous structure that enhances wettability. The tangential mode, also known as rotary mode, has been used for granulating and pelleseting with subsequent coating. Finally, the bottom position, which is the one used in CPA-8 formulation, is commonly applied for active layering and granulation as it produces a superior film compared with other agglomerating techniques (Srivastava & Mishra, 2010). The advantage of granulation is the resulting open, coarse, porous, and homogenous product, which is almost dust-free, free-flowing and easily solubilised (Strasser, 2008).

To obtain CPA-8 fluid-bed spray-dried products, each bacterial suspension, amended or not with protectants, was sprayed by a 0.8 mm nozzle using a peristaltic pump and applying a spraying air pressure of 80 kPa. For an application that requires a high degree of reproducibility and high granulation efficiency, the bottom spray method is advantageous. The figure 5 illustrates the process principle of spray agglomeration, which is the basis of the fluid-bed spray-drying technology.

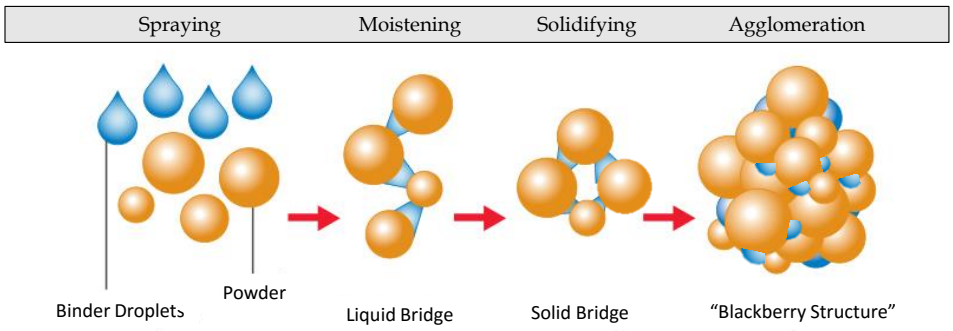

Figure 5. Process principle of spray agglomeration (Srivastava & Mishra, 2010).

In the case of particle growth by agglomeration, the wetted particles are held together by liquid binding bridges which are converted into solid binding bridges when the solvent is evaporated (Hemati *et al.*, 2003; Link & Schlunder, 1997). The selection of a suitable fluid-bed process for a particular application is based on several criteria: the capacity requested, the physical properties of the raw materials, the requirements of the finished product, the productivity (yield) of the process, and the coating or loading level achieved (Srivastava & Mishra, 2010). The factors that should be considered in the process selection are compared in the table 2 below.

Table 2. Comparison of the three fluid-bed spray-drying methods (where 1 = least desirable and 3 = most desirable) (Srivastava & Mishra, 2010).

Parameters	Fluid-bed spray-drying method		
	Top-spray	Bottom-spray	Tangential-spray
Process considerations			
Simplicity	3	2	1
Nozzle access	3	1	2
Scale-up issues	3	2	1
Mechanical stress	3	2	1
Product considerations			
Surface morphology	1	3	3
Coating uniformity	2	3	3
Layering efficiency	1	3	3
Product coating capacity	2	3	3
Economic considerations			
Space requirement	2	1	3
Equipment capacity	3	2	1
Equipment cost	3	2	1

Moreover, the figure 6 shows a diagrammatic representation of the three modes of the fluid-bed spray-drying process: top, bottom and tangential position.

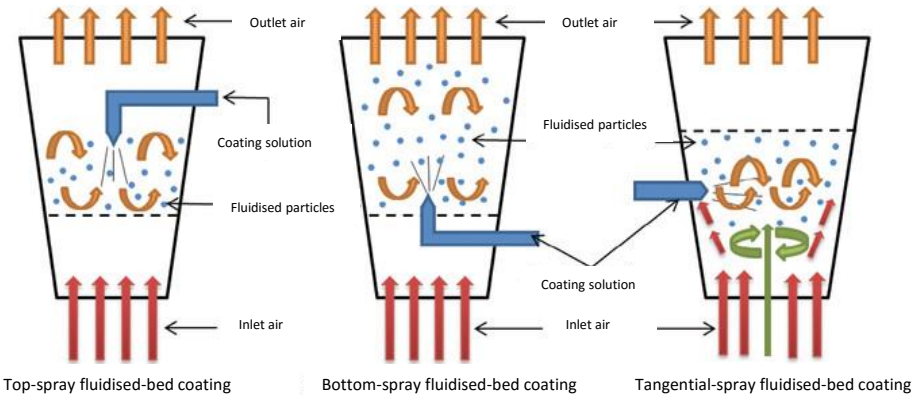

Figure 6. Schematic diagram to show top, bottom, and tangential-spray fluidised-bed spray-drying (Bakry *et al*., 2016).

Although the preliminary results described in **Chapter IV** are promising (CPA-8 fluid-bed spray-drying ensured the efficacy, stability and low-cost easily application of the BCA), this approach needed to be further studied, thus improving the development of a commercially available microorganism-based product. Many factors may affect the outcome of a wet granulation process. They can be divided into process (or intrinsic) variables (inlet air temperature and humidity, outlet air temperature and humidity, size of the expansion chamber, spraying and airflow rates, etc.); and variables of the entering materials (extrinsic factors) such as, carrier material, particle size distribution, physicochemical properties, surface free energy, binder solution concentration, etc. (Hemati *et al*., 2003; Planinsek *et al*., 2000). In the figure 7, the major processing steps during the BCA's formulation have been highlighted.

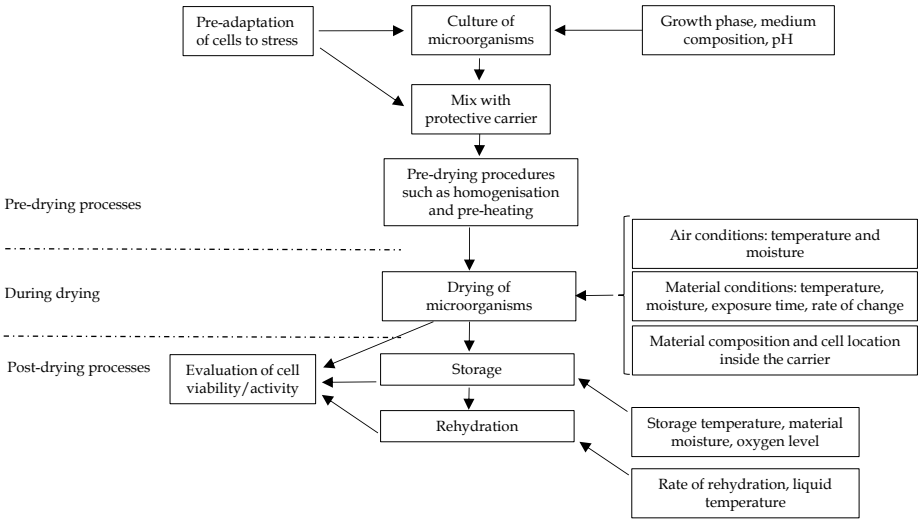

Figure 7. Major processing steps and the important extrinsic factors during the drying of microorganisms. Adapted from Fu & Chen (2011).

Unfortunately, not all the microorganisms are able to survive the drying conditions imposed. In order to maintain cell viability, the fundamental requirement is to keep essential cellular structures intact after drying and fully functional after rehydration (Fu & Chen, 2011). To avoid undesirable effects on CPA-8, a series of protectants added to the formulation medium was assessed. They mainly have two protective mechanisms on cells: to provide a dry residue and thus acting as a receptor in the rehydration process and also to act as a physical shield to alleviate the heat and osmotic stress caused during the drying and dehydration processes (Abadias *et al.*, 2001a; Abadias *et al.*, 2001b; Melin *et al.*, 2011; Sabuquillo *et al.*, 2010).

In this thesis, two optimised CPA-8-based products have been developed (**Chapter V**). Among all the protective substances tested, the combined use of 20 % sucrose plus 10 % SM resulted in the best CPA-8 formulations when either, maltodextrin "BA3" or potato starch "BA4" was used as carrier material. Compared to those non-treated CPA-8 cells (without protecting agents), the addition of these components to the formulation media resulted in higher CPA-8 survival rates after drying. Moreover, they also allowed to obtain large amounts of dry product. Santivarangkna *et al.* (2007) reported that synergetic effects may be acquired from the combination of different protectants.

Non-reducing sugars are effective protectants against dehydration damage. Compounds such as sucrose depress the melting temperature (T_m) and stabilise the cell membrane in the liquid crystalline state during drying, thus limiting the potential leakage of the membranes during such phase transition (Fu & Chen, 2011; Leslie *et al.*, 1995; Melin *et al.*, 2011; Strasser, 2008). They also offer thermotolerance against protein denaturation (Morgan *et al.*, 2006; Stephan *et al.*, 2016). Moreover, the protective effect of the SM in cell reconstitution is fairly reputed (Abadias *et al.*, 2001b; Costa *et al.*, 2000; Navarta *et al.*, 2011). It was suggested that its effective protection may be related to the lactose, which interacts with the cell membrane and helps to maintain the membrane integrity in a similar manner to the non-reducing disaccharides (Fu & Chen, 2011). Otherwise, Morgan *et al.* (2006) suggests that protein rather than sugars play the important role in glass formation. However, whether or not the significant protective effect of SM is due to the presence of the whey proteins remains still unexplored.

Moreover, the industrial pre-drying procedures require the homogenisation of the microorganisms with the selected protectants, which may cause damage to the cellular structures prior entering into the drying equipment (Fu & Chen, 2011). To avoid harsh processing conditions, thus allowing long-time interaction between cells and protectants, CPA-8 was mixed in a rotary shaker at 150 rev min^{-1} for 60 min at room temperature.

During a thermal drying process with elevated air temperature, the drying material generally undergoes a decrease in water content and an increase in the temperature of the product. Inlet air temperature was set at 65 ^{o}C, resulting in a maximal product temperature of 42 ^{o}C depending on the spraying rate, which ranged between 4 and 4.5 g min^{-1}. Parameters such as the airflow rate or the liquid feed rate, have been described to have an impact on the outlet temperature (Fu & Chen, 2011), which is the temperature that cells might have experienced inside the drier. Fluid-bed spray-drying is capable of drying cells at low temperatures. The temperature used to dry CPA-8 was not considered excessive, thus minimising the heat inactivation. It is also worth recalling the spore-form ability of CPA-8, which provides this bacterium with high degree of thermotolerance. However, dehydration inactivation needs to be still considered. The aim of dehydration the BCAs is to repress the metabolic activity by

the deprivation of water. Therefore, the outlet temperature is usually associated with the final moisture content of the dried products, immensely affecting the loss of microbial viability during storage.

The fluid-bed spray-dried CPA-8 products reported in this thesis research (previously developed in **Chapter IV** and subsequently optimised in **Chapter V**), contained moisture contents lower than 11 %. Moreover, a_w values between 0.33-0.36 were estimated. With regards to the pretreatment strategies used to improve CPA-8 survival during drying, it was found that the addition of the non-reducing disaccharide sucrose probably also exhibited a protective effect during storage. It could be due to the appropriate water binding capacities of the sucrose (Fu & Chen, 2011). It is though that the high viscosity of the sugar glasses retard molecular mobility and reaction rate, hence stabilising the biological system (Santivarangkna *et al.*, 2007). Nevertheless, as it was previously discussed in the section of CPA-8 ecophysiolgy responses (point 1.5), the pre-adaptation of the cells to stress by the accumulation of compatible solutes would help to alleviate the osmotic stress and stabilise essential macromolecules.

Other extrinsic parameter that must be considered in the formulation optimisation is the binder (agglutinant). It is crucial in the granulation process. Such granules are composed by CPA-8 (vegetative cells or endospores) and the substrate (carrier material). As it was shown in the figure 5, agglomeration affords products with large particle size and high porosity that provides an improved dispersion and dissolution with reduced compaction (Hemati *et al.*, 2003). Diverse binding mixtures allow to obtain particles with different application requirements based on particle size distribution, density, and surface chemistry (Strasser, 2008). Some frequently used binders include gelatine, starch, polyvinylpyrrolidone, and high concentrations of sugar (Srivastava & Mishra, 2010). CPA-8 was formulated using 3.5 g of pregelatinised potato starch. Due to their adhesive qualities, compared to HPMC (hydroxypropyl methylcellulose), this modified starch allowed to obtain the desired grade of agglomeration (**Chapter IV**).

Finally, it is worth mentioning the spherical carrier materials sucked into the product vessel during CPA-8 formulation. The sprayed droplets of CPA-8 cells mixed with the protectants (20 % sucrose plus 10 % SM) were brought into contact with the

repetitively passed carrier material and therefore, granulation occurred bit by bit. During the fluidification many factors such as, contact, spreading, coalescence, evaporation, and hardening occur almost simultaneously. The thickness of the dried particles is highly depending on the amount of carrier applied (Strasser, 2008). As it was previously mentioned, the bottom-spray position was used, thus creating a fluidisation pattern that prevents premature droplet evaporation and provides more uniform granulates (Strasser, 2008).

In this thesis (**Chapter V**), the two carrier materials used (300 g) consisted of easily degradable carbon sources: maltodextrin and potato starch (pea protein and pregelatinised potato starch were previously discarded in **Chapter IV**). When designing a distinct product formulation, the physical-chemical properties of the carrier and its compatibility with the active ingredient need to be considered as important factors influencing the functionality of the desired added-value properties of the product (Strasser, 2008). These two materials were chosen for enhancing powder recovery with no agglomerates an also for being low-cost commercial products. Once again, reduction of costs plays a crucial role, therefore expenses for different materials must be kept in mind. Nevertheless, they differ in the Dextrose Equivalent (DE) measure, which indicates the degree of polymerisation from starch to sugars. Maltodextrin is typically composed of an amount of reducing sugars between 3-20 % (whereas starch is close to zero) resulting in products with higher solubility (Shamekh *et al.*, 2002). That property could probably determine some differences observed between both CPA-8-formulated products once applied under laboratory or commercial conditions. However, it will be discussed in depth later**.**

The figure 8 illustrates the differences observed in solubility once both CPA-8 formulated products (using either, maltodextrin or potato starch as carrier material) were mixed with water. As it is shown, potato starch precipitates more than maltodextrin.

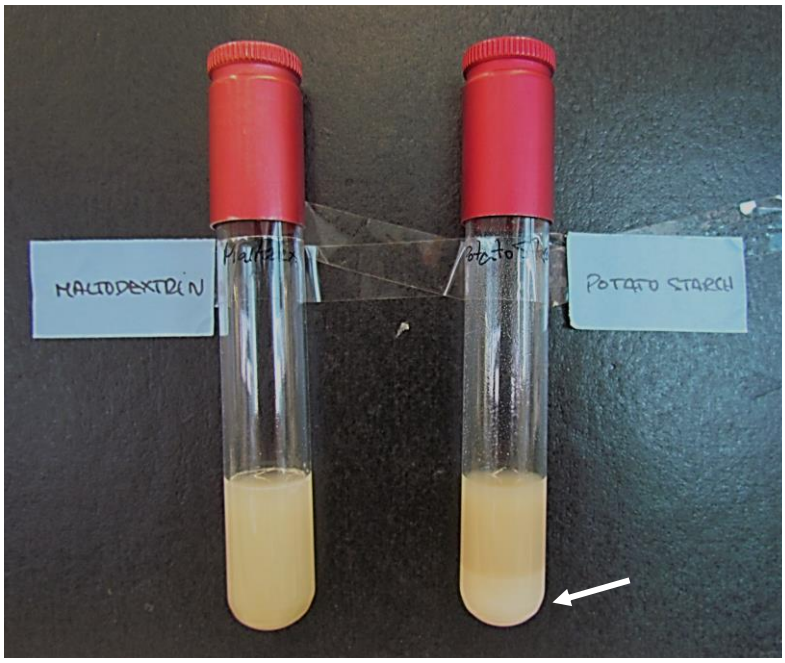

Figure 8. Differences in solubility between two CPA-8- formulated products including either, maltodextrin or potato starch as carrier material. Each dried CPA-8 formulate (1 g) was dissolved in 3 mL of water, shaken vigorously for 10 s and then allowed to rehydrate for 10 min in static.

As a commercial product, the stability of formulation during storage and the viability of cells upon dehydration are also an important criteria (Fu & Chen, 2011). In lights of this, studies focusing on the CPA-8 maintenance during storage at different temperatures have been carried out. Although it was demonstrated that CPA-8-formulated products could be kept under frozen conditions (**Chapter IV** describes the shelf-life of fluid-bed spray-dried products stored at -20 ºC for twelve months), optimised CPA-8-based products with final concentrations ranging from $7.8 \cdot 10^9$ to $1.2 \cdot 10^{10}$ CFU g^{-1} were obtained, extending the viability of CPA-8 for up to 15 months at 4 and 22 ºC. The capability of CPA-8 to survive under room temperatures also increases the potential of this BCA to be successfully commercialised, thus providing suitable product delivery. These data agree with the work conducted by Yánez-Mendizábal *et al.* (2012b), who described high stability of CPA-8 spray-dried products after storage at 22 ºC.

Another critical parameter is the rehydration process of the cells. Recovery of dried cultures is furthermore dependent on the rehydration conditions employed such as osmolality, pH, and nutritional energy source (Strasser, 2008). Yánez-Mendizábal *et al.* (2012b) investigated the capability of CPA-8 to be reconstituted in many different media. Likewise, in the present thesis, it was demonstrated that CPA-8 could be satisfactorily rehydrated with water (unpublished results), thus facilitating the large-scale handling of CPA-8-based products in the field.

Survival and shelf-life achieved for CPA-8 are satisfactorily enough for commercial application. Based on this, extra assays should be carried out to evaluate different packaging strategies to extend the shelf-life of CPA-8. This may involve careful selection of the packaging material to control gas exchange, prevent the loss or gain of moisture and avoid contamination of the product (Torres *et al.*, 2014; Usall *et al.*, 2016).

To complete the CPA-8 formulation strategy, different efficacy trials have been developed on fruit artificially inoculated with *Monilinia* spp. It was therefore demonstrated that the drying and storage processes did not affect CPA-8's efficacy, thus obtaining CPA-8-based products with retained biological activity. The figure 9 shows and example of the efficacy of both optimised CPA-8-formulated products, BA3 and BA4 (10^7 CFU mL^{-1}) against *M. laxa* on nectarines and *M. fructicola* on peaches.

a) *M. laxa*

untreated | CPA-8 formulated product BA3 | CPA-8 formulated product BA4

b) *M. fructicola*

untreated | CPA-8 formulated product BA3 | CPA-8 formulated product BA4

Figure 9. Efficacy of both optimised CPA-8-formulated products, BA3 and BA4 (10^7 CFU mL^{-1}) against *M. laxa* on nectarines (a) and *M. fructicola* on peaches (b).

3. DEFINITION OF THE CONTROL STRATEGY: CPA-8-BASED PRODUCTS

Formulation plays a significant role in determining the final efficacy of a BCA-based product. In this thesis, two optimised CPA-8-formulated products have been developed. Although they include the same proportion of protectants (sucrose and skimmed milk), they differ in the carrier material used in the fluidification process: maltodextrin (CPA-8-formulated product called BA3) and potato starch (CPA-8-formulated product called BA4). Henceforward, this nomenclature will be used to designate these two products, which have been largely studied and compared along the different assays regarding the definition of the BCA CPA-8 strategy.

3.1. Range of activity

One of the most important, but often underrated, limitations of the BCAs commercialisation is that they frequently present a restricted range of biocontrol activity. Farmers point out that such activity is mostly restricted to a specific host and pathogen. This limitation become even more critical for the postharvest BCAs, given the narrow size of the potential market (Usall *et al.*, 2016). In this thesis, the broad spectrum of action of CPA-8 against artificially infection with *Monilinia* spp. has been demonstrated on a wide range of stone fruit (**Chapter V**).

CPA-8-formulated products (BA3 and BA4) stored at either 4 or 22 ºC for ten months were tested against *M. laxa* and *M. fructicola* in artificially inoculated peaches, nectarines, flat peaches, and cherries. Regardless of the age of the formulation and temperature of storage, all formulations were as effective as fresh cells, exhibiting disease percentage reductions (compared to the control) ranging from 44.4 to 100 % and from 46.2 to 100 % in disease incidence (percentage of infected fruit) and severity (mean lesion diameter of brown rot), respectively. Moreover, the efficacy of these formulations (stored for 6 months) against the mentioned pathogens was also proved on artificially inoculated apricots and plums. In this case, the percentage of disease reduction (compared to the control) generally ranged from 45.0 to 95.0 % and from 45.5 to 100 % regarding disease incidence and severity, respectively. These data, once again demonstrated that drying and storage conditions (even at ambient temperature) did not

have any negative effect on the biocontrol efficacy of the CPA-8-based products. The figure 10 shows and example of the efficacy against *M. fructicola* of both optimised CPA-8-formulated products, BA3 and BA4 (10^7 CFU mL^{-1}) stored at 22 ºC for six (apricots and plums) and ten months (flat peaches and cherries).

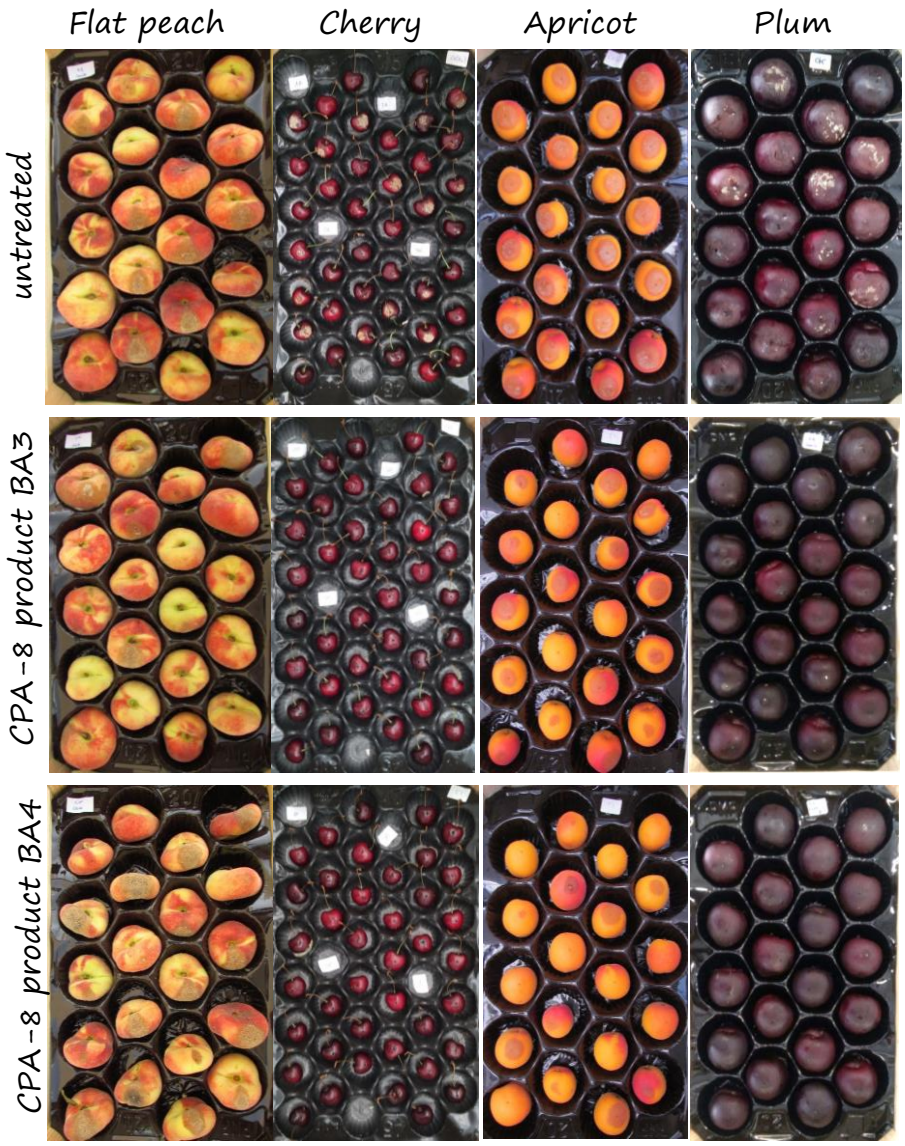

Figure 10. Efficacy against *M. fructicola* of both optimised CPA-8-formulated products, BA3 and BA4 (10^7 CFU mL^{-1}) stored at 22 ºC for six (apricots and plums) and ten months (flat peaches and cherries).

This allow to exploit the use of the two CPA-8-based products developed in large-scale experiments under field conditions. Moreover, CPA-8 biocontrol activity has been confirmed not only against *M. laxa* and *M. fructicola* in stone fruit but also against *B. cinerea* on apples (Yánez-Mendizábal *et al.*, 2011). As it was stated in **Chapter III**, VOCs emitted by CPA-8 could also reduce grey mould decay on cherries, thus extending its use to several diseases and crops.

3.2. Technical application thresholds: Environmental factors

Bacteria may survive and become more effectively established on the surface of fruit under naturally fluctuating environmental conditions as this could give BCAs a competitive advantage enabling more effective preemptive exclusion of pathogens from such niches (Teixidó *et al.*, 2006). This provide a method for improving consistency and efficacy of the antagonist in the field. Unfortunately, the study of the ecological fitness of antagonistic agents once applied in the field has received little attention as compared with other topics such as mechanism of action (Lahlali *et al.*, 2008; Norris *et al.*, 2002). The figure 11 schematically illustrates the main responses of antagonists to stress and some methods that may improve their tolerance to such circumstances.

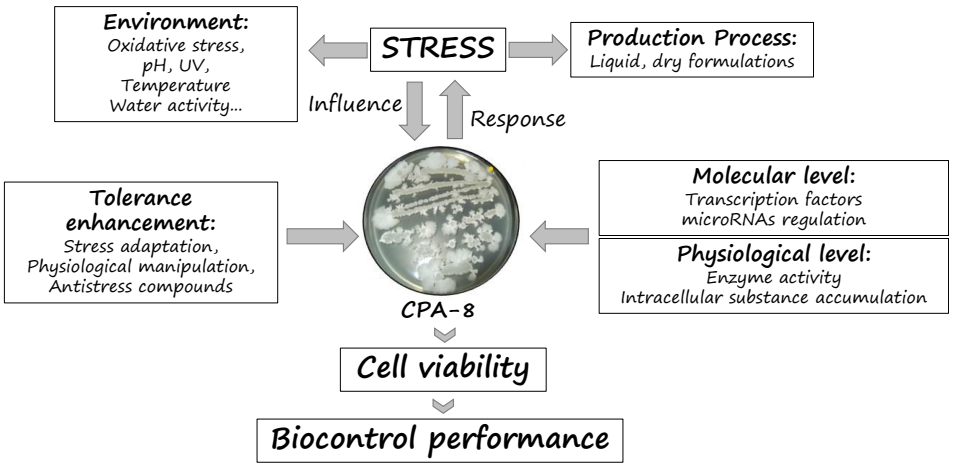

Figure 11. Diagram of responses of antagonists to stress and methods to improve their tolerance. Adapted from Sui *et al.* (2015).

In order to determine the technical application thresholds of the CPA-8-based products developed, the main factors which could affect the CPA-8 survival under field conditions: temperature, relative humidity (RH), and wash-off caused by simulated rainfall, have been studied (**Chapter VI**). BCAs have to withstand harsh and hostile conditions when applied to the fruit. For instance, desiccation could negatively impact their persistence and consequently their biocontrol efficacy. In addition, BCAs also have to resist sunlight, and in particular extreme temperatures and ultraviolet radiation, which have a strong bactericidal effect. Rain and wind may physically remove BCAs from the phyllosphere preventing the correct colonisation of the ecological niche and nullifying its control activity (Segarra *et al.*, 2015).

The formulation of the BCA may offer a solution to these problems. A formulated product is composed of the active ingredient (microorganism) and co-formulants, which must also endure the persistence and activity of the BCA at the target site (Burges & Jones, 1998).

As it has been reported in this work (**Chapter VI**), CPA-8 exhibited high degree of thermotolerance on nectarines and peaches exposed to both tested temperatures, 0 ^{o}C and 20 ^{o}C. Its viability remained practically unchanged regardless of the product applied, CPA-8-formulated products BA3 and BA4, reaching populations after storage over 5 $\cdot 10^{4}$ CFU cm^{-2} of fruit surface. While the optimum temperature of CPA-8 is close to 37 - 30 ^{o}C (see **Chapter II**), this study demonstrated that CPA-8 also tolerate exposure to 20 and to 0 ^{o}C, which simulates the temperature of the chambers conditioned for fruit storage. Moreover, the sensitivity of the CPA-8-based products to dry conditions on fruit was evaluated at three different RH values: 85 (suitable RH for the microorganism development), 60 (intermediate) and 40 % (average RH in the field in the summer time). Although bacteria are generally very sensitive to the absence of free water in the phyllosphere (Köhl & Fokkema, 1998), CPA-8 populations were largely maintained, obtaining more than 10^{4} CFU cm^{-2} of fruit surface.

Finally, the effect of the rain upon the population dynamics of CPA-8 on the surface of nectarines and peaches treated with both formulated products was measured. Factors such as the dilution, redistribution, physical removal and extraction from the fruit tissue probably had the greatest effect since most losses were observed after 20 mm of rain exposure. Although higher volume of rain caused little additional

CPA-8 removal, it was hardly detected. However, wash-off caused a decrease in CPA- 8 populations as much as 1 log unit, obtaining fruit covered with $6.01 \cdot 10^3$ - $1.53 \cdot 10^4$ CFU cm^{-2}. Additionally, what happened after letting the CPA-8 cells be established on the fruit prior rainfall exposure was studied. Depending on the properties of each product applied, different establishment times were needed to achieve a minimum reduction in CPA-8 populations of -0.22 log units (CFU cm^{-2}). The positive effect of an establishment time prior exposure to rainfall was previously reported by Calvo-Garrido *et al.* (2014), who highlighted the capability of colonisation on many BCAs as an strategic factor compared to chemical applications.

The capability of CPA-8 to tolerate different environmental conditions has been widely demonstrated. The establishment of bacterial populations on plant surfaces is a critical phase in disease control. Having a minimal antagonistic population level on fruit surface is crucial in order to guarantee competition with pathogens (Cañamás *et al.*, 2008; Ippolito & Nigro, 2000). The presence of CPA-8 over the fruit after exposure to hostile environmental situations was considered enough to ensure biocontrol efficacy (>10^4 CFU cm^{-2}). However, noteworthy differences were observed between the two CPA-8-based products applied. Such differences were mainly based on the composition of the carrier used in the formulation process: maltodextrin (product BA3) or potato starch (product BA4). To sum up what has been already stated in **Chapter VI**, the higher degree of solubility of the maltodextrin probably made of the CPA-8-based product BA3 the one most easily removed from the fruit surface, being even more evidenced after rain exposure. Moreover, the shape and composition of the fruit surface are also of practical importance. Peaches (whose peel is rougher than the nectarines), retained better the product applied thus facilitating the maintenance of the microorganism on the surface.

In order to improve the adherence and persistence of the microorganism in the foliage after heavy events of wind and rain, Cañamás *et al.* (2008) and Hunsche *et al.* (2011) suggested that an adequate use of adjuvants may improve the overall coverture of the fruit by the applied product and thereby ensuring its biological efficacy. A step toward obtaining the formulation goal (aid biomass delivery, target coverage, and target adhesion) is to be aware of the physical and chemical environment of the application target because this knowledge will dictate the choice of a wetting agent

(surfactant) and/or an sticker that should be added to the applied products (Schisler *et al.*, 2004).

Preliminary tests were done on apples mixing the CPA-8-based products with three commercial wetting agents or emulsifiers: Li 700®, Nu-Film-P® or Food Coat (unpublished results). The effect of the combined application compared to those applications containing just the CPA-8 formulation was analysed after 0 and 24 h of application with and without exposure to simulated rainfall. Such effect was compared by estimating the population dynamics of CPA-8 (CFU cm^{-2}) over the fruit. However, consistent results have not been yet achieved. It seems that both CPA-8-formulated products, BA3 and BA4, enhanced enough the fruit coverage obtained by the CPA-8 fresh cells (without formulating). Nevertheless, further research would be needed in order to accomplish this issue. Moreover, extra studies should be considered to establish the impact of other stresses caused by environmental factors such as solar radiation (UV light), wind or oxidative stress associated with controlled-atmosphere storage.

Eventually, and as a requisite step before jumping into full-testing in the orchard, the CPA-8 compatibility with the standard cultural and crop protection practices is another point that had to be considered. It is necessary to know their compatibility with other sanitizers such as, waxes or other chemicals, which are standardly applied to the crop in greenhouses or in the field (e.g. against non-target diseases), and in packinghouses (Usall *et al.*, 2016). The compatibility of CPA-8 with all the tested commercial products (table 3) commonly applied in the mainly producer countries of stone fruit in Europe (Spain, Italy, France, and Belgium), has been demonstrated (unpublished results).

Table 3. Chemical compounds used in the CPA-8 compatibility tests.

Comercial Product	Active ingredient	Comercial Product	Active ingredient
Alkir®	Emulsifier/surfactant	Karate Zeon®	Lambda Cyhalotrin
Apache® Plus 535 SC	Trifloxystrobin/ Cyproconazole	LI-700®	Emulsifier/surfactant
Arius®	Quinoxyfen	Luna® Devotion	Fluopyram/ Triadimenol
Atemi® 10 WG	Cyproconazole	Luna Experience	Fluopyram/ Tebuconazole
Audace®	Deltamethrin	Luna Privilege	Fluopyram
Bellis®	F-500®/Foscalid	Merpan® 80 WG	Carptan
Bravo® 50	Chlorthalonil	Movento® Gold	Spirotetramat
BulldocK® 2.5 SC	Betaciflutrin	Naturalis-L®	*Beauveria bassiana*
Calypso®	Thiacloprid	Nu-Film-P®	Emulsifier/surfactant
Ceremonia® 25EC	Difenoconazole	Ossiclor® 50 WG	Dicopper chloride trihydroxide
Chorus®	Cyprodinil	Plenum®	Pymetrozine
Confidente® 20 LS	Imidacloprid	Prolectus®	Fenpirazamine
Coragen®	Rynaxypyr®	Proteus® O-Teq	Thiacloprid/ Deltamethrin
Decis Protech®	Deltamethrin	Rovral® Aquaflo	Iprodione
Dursban® 75 WG	Chlorpyrifos	Signum®	Boscalid/ Pyraclostrobin
Expedient® 10 EC	Pyriproxyfen	Spintor® 480 SC	Spinosad
Flint®	Trifloxystrobin	Steward®	Indoxacarb
Folicur® 25 WG	Tebuconazole	Surfactante DP	Emulsifier/surfactant
Food Coat	Emulsifier/surfactant	Switch®	Cyprodinil/ Fludioxonil
Fury® 100 EW	Cypermethrin	Syllit® Flow	Dodine
Geoxe®	Fludioxonil	Tasis® 12.5 EC	Myclobutanil
Imidan® WP	Phosmet	Teppeki®	Flonicamid
Impala®	Fenbuconazole	Thiovit Jet®	Sulphur
Insegar® 25 WG	Fenoxycarb	Thiram® 80 WG	Thiram

The approaches presented above can be used to support the development of ecologically competent CPA-8-based products, thus allowing the implementation of such microorganism into brown rot control strategies.

3.3. Commercial trials

The bottom line of biocontrol is whether it works under commercial conditions. The step-wise screening of microorganisms for commercial use in biological control (already exposed in the theoretical framework of this thesis) requires a set of full field experiments that consider complete crop protection schedules (Köhl *et al.*, 2011). One of the major hurdles in the development of postharvest BCAs is their inability to control previously established infections, such as field latent infections. Therefore,

preharvest applications of microbial antagonists become of practical importance to effectively control postharvest decay of fruit (Ippolito & Nigro, 2000; Moretto *et al.*, 2014).

Spadaro *et al.* (2014) distinguish two approaches in applying BCAs in the field to control postharvest decays. In the first one, antagonists are applied just before harvest so wounds inflicted during harvest can be colonised by the antagonist prior infection with the pathogen. Secondly, antagonists are applied through fruit development. Therefore, latent infections originated as early as bloom time could be reduced.

As part of a well-designed disease program, CPA-8-based products were applied in four peach commercial orchards located in Lleida (Catalonia, Spain) over two growing seasons, 2014 and 2015 (**Chapter VII**). For the CPA-8 efficacy tests, evaluated at harvest and postharvest time, treatments consisted of CPA-8 fresh cells (BA), non-optimised CPA-8-formulated products (BA1 and BA2 developed in **Chapter IV**) –applied in 2014 season-, and those optimised CPA-8-formulated products (BA3 and BA4), developed in **Chapter V** and largely studied thereafter –applied in 2015 season-. Such efficacy was compared with two control treatments: one based on chemical applications and other one based on non-treated trees. All treatments were preharvest applied four times following application schedule for controlling brown rot: 30, 14, 7 and 3 days approximately before harvest.

As it was described, different degree of biocontrol activity was obtained mainly due to the inoculum pressure, which is greatly depending on the meteorological conditions. Gell *et al.* (2008) demonstrated that temperature and wetness were the two most important factors that contribute to the incidence of latent infections caused by *Monilinia* spp. in Spanish peach orchards, causing approximately 90 % of brown rot decay**.** When the presence of *Monilinia* spp. in the field was higher than 50 % (2014 season and the late ripening variety of 2015 season), brown rot disease could not be controlled at harvest time, not even when fruit was treated with chemical applications. It was not the case detected at postharvest (5-7 days of shelf-life storage at 20 ^{o}C and 85 % RH), in which despite the high presence of inoculum (>78 %), the chemicals applied successfully decreased brown rot decay. Otherwise, when *Monilinia* spp. incidence was in the range of the standard levels recorded in the area (Orchard03, middle season cultivar

in 2015), treatments based on CPA-8 formulations proved to be efficacious. Such standard levels have been considered over four growing seasons (2013-2016) including 26 field trials comprising early and late varieties. These data revealed that 90 % of the cases showed disease incidence at harvest lower than 10 %. Regarding postharvest studies, the percentage of trials which presented <30 % of *Monilinia* spp. incidence raised the 77 % (Casals, personal communication). Therefore, results obtained from the Orchard03 could be included into these standard ranges. At harvest (disease pressure of 5.3 %), BA3 and BA4 treatments significantly reduced *Monilinia* spp. brown rot compared to the untreated trees (54.7-64.1 % disease reduction). However, the effectiveness in decay reduction by these treatments was inferior compared to the one obtained by the synthetic fungicides applied. Otherwise, at postharvest (disease pressure of 17.3 %) while both CPA-8-based products (BA3 and BA4) were also effective in reducing brown rot decay, the BA4 treatment significantly reduced *Monilinia* spp. (50.3 % compared to the untreated control) even statistically similar to chemical applications.

The figure 12 below illustrates some phases along the field trials conducted in Lleida area. The treatment application, the evaluation of the results at harvest, and the shelf-life storage for postharvest analysis are shown.

Figure 12. Different phases along the field trials conducted in Lleida area: the treatment application, the evaluation of the results at harvest, and the shelf-life storage for postharvest analysis.

The CPA-8-based product called BA4, as it has been already mentioned, presents different composition regarding the material used in the drying process. Although further studies should be conducted in order to better clarify this issue, potato starch appears to be an essential component in the formulation of CPA-8, rather than maltodextrin. In order to understand why the BA4 product seems to be more effective, extra studies based on whether *Monilinia* could take more profit from the maltodextrin (which is a polysaccharide produced from the partial hydrolysis of the starch) or the CPA-8 endospore activation procedure is characteristic of each formulation, are now the next step research.

It has also been demonstrated the capability of CPA-8 to survive over the fruit surface since the first treatment application in the field until the postharvest shelf-life.

It is crucial in order to accomplish preharvest application requirements, thus demonstrating that CPA-8 has the capability to withstand with variable and frequently hostile conditions such as those discussed before. Moreover, specific analysis methods are necessary not only to control the quality of the BCA applied but also to study its traceability, residue analysis, and environmental impact (Montesinos, 2003).

The **Annex I** of this thesis includes a detailed investigation into the environmental fate and behaviour of CPA-8 after preharvest application in a peach orchard.

The dispersion of CPA-8 was evaluated using water-sensitive papers (WSPs), obtaining a complete area coverage on leaves from treated trees and less than 1 % coverage in leaves from those non-treated. In addition, the persistence of CPA-8 on fruit, leaves, and weed was evaluated by using ten-fold dilutions or contact (Rodac plates) methods. The results revealed that CPA-8 was largely maintained on fruit from treated trees. Its presence on leaves and weeds, however, was slightly decreased 21 days after treatment. Regarding the non-treated trees, CPA-8 on leaves could be even detected 180 days after treatment and on weed, the population of CPA-8 was dependent of the distance to the treated trees. Moreover, CPA-8 persisted on inert materials such as, clothes and gloves worn by handlers, and on the plastic harvesting boxes used. By using the molecular maker from *RBAM 007760* gene developed in **Chapter I**, more than 99 % of the samples with morphology similar to CPA-8 were confirmed by PCR. This work demonstrated a good distribution, persistence, and adaptation of CPA-8 in the field and postharvest conditions, thus providing crucial information not only to design more precise programs for disease control but also to accomplish registering purposes.

The figure 13 shows, in a graphical way, the methodologies used to detect de dispersion and persistence of CPA-8 in the field.

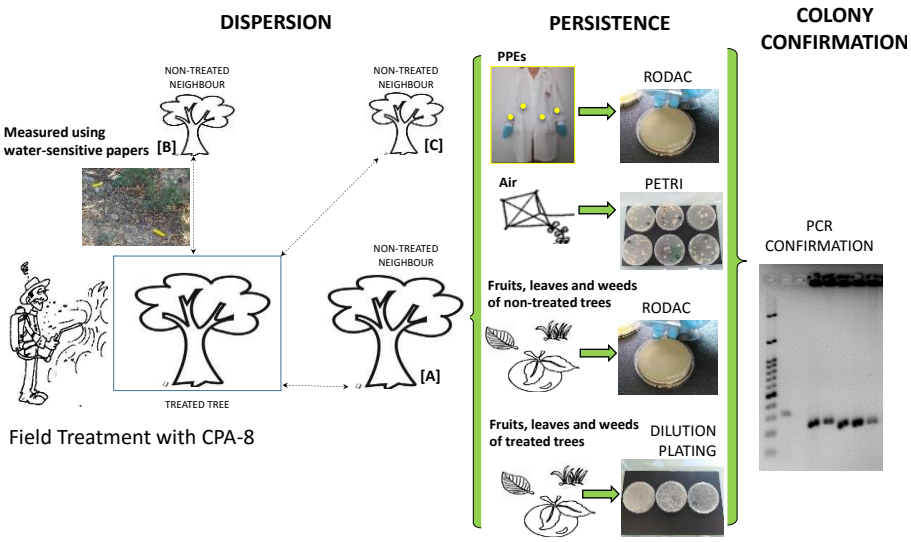

Figure 13. Graphical abstract of the methodologies applied to study the CPA-8 environmental fate and behaviour after preharvest application in a peach orchard (Vilanova *et al*., unpublished results, Annex 1).

As part of a BCA-based strategy, the application of mixtures of different microbial antagonists has certain advantages: (i) widening the spectrum of microbial activity resulting in the control of two or more diseases, (ii) increasing the effectiveness under different situations such as, cultivars, maturity stages, and locations; and (iii) enhancing the efficiency and reliability of biocontrol allowing a reduction in the application times and treatment costs (Spadaro *et al*., 2014). However, despite such potential benefits, the combined use of CPA-8 with *Penicillium frequentans* 909, did not improve the CPA-8 efficacy (used alone) in the control of brown rot disease (**Chapter VII**). Applications of *P. frequentans* formulations at preharvest time had resulted in a decrease of pathogen inoculum density, thus resulting an effective antagonist of *Monilinia* spp. (Guijarro *et al*., 2007; Guijarro *et al*., 2008). Such efficacy has been confirmed in the efficacy trials here conducted (**Chapter VII**), in which *P. frequentans* treatments successfully controlled *Monilinia* spp. incidence at postharvest time, when the disease pressure was close to the standards levels already described (17 %).

Similarly, by combining different environment-friendly strategies, not only in the orchard but also along the packing line, the efficacy of the CPA-8-based products here developed can be enhanced. The combination of field and postharvest applications has great potential in achieving effective control of postharvest decays (Janisiewicz & Korsten, 2002; Lahlali *et al.*, 2008). In this context, the study of CPA-8 combined with physical and chemical methods in the control of postharvest fruit diseases would give a substantial boost, suggesting new possibilities for fruit loss reduction (Mari *et al.*, 2014; Spadaro *et al.*, 2014).

This thesis also provides information regarding the optimised dose and timing of application of CPA-8 (**Chapter VII**). Such factors must be determined to ensure that the BCA is targeted effectively at the growth stage that the pest is most susceptible (Hynes & Boyetchko, 2006). The established CPA-8 dose of treatment (10^7 CFU mL^{-1}) is easily achievable (around ten times lower than those applied for the commercialised Serenade Max, Bayer CropScience, Germany), thus faciliting the biomass production and formulation procedures. Biontrol is, fundamentally, applied ecology (Andrews, 1992). More specifically, the goal is to manage a microbial population to favour the antagonistic function and disfavour the pathogen. Predicting the result of the host-BCA-pest interaction under a variety of environmental conditions including time before or after rainfall event, sunlight, and wind will help in reducing field performance losses (Hynes & Boyetchko, 2006).

Recapitulating, the most important criterion for the success of a BCA product is whether or not it performs effectively under commercial conditions, providing an acceptable and consistent level of control of the target disease (Fravel *et al.*, 1999). As part of the last phase of the commercial development process, both CPA-8-based products here developed need to be tested now on targeted crops at different locations, even different countries.

Last season (from July to September of 2016), the CPA-8 control strategy (defined in **Chapter VII**), was validated in four different stone fruit producing areas in Europe: Belgium (by PCFRUIT: two fields of sweet cherries 'Lapins' and 'Sweetheart' varieties, one field each); France (by OPENNATUR: on peaches 'Fidelia' and nectarines 'Tourmaline', one field each); Italy (by BIOGARD: on peaches 'Corindom' and nectarines 'Morsiani 90', one field each); and Spain (by

OPENNATUR and IRTA: on peaches 'Tardibelle' and nectarines 'Red Jim', one field each).

The protocols used to perform the above mentioned trials were written taking into account the methodology described in **Chapter VII**. Such trials included: an untreated control: no treatments; a chemical strategy with the active ingredients most commonly used in each country; two formulations of the BCA *P. frequentans* Pf 909 (PF1 and PF2 provided by BCBS, Bayer CropScience Biologics GmbH, Malchow, Germany); and two formulations of the BCA CPA-8 (the optimised CPA-8-based products BA3 and BA4 here described). All the treatments were applied four times: 30, 14, 7, and 3 days approximately before harvest (except nectarines in France that could not receive the 4th application due to the fact that it was necessary to advance the harvest at last moment by maturity reasons). As before, the efficacy trials consisted of estimating *Monilinia* spp. incidence at harvest and postharvest time (after 7 days of cold storage (0 ºC) plus 5 days at 20 ºC and 85 % RH).

Although out of this thesis, but part of the BIOCOMES European Project (unpublished results), the most important findings are highlighted below. Three different scenarios could be distinguished depending on the disease pressure in the field:

- Scenario 1: There was not inoculum in the field. This was the case of Spain (Lleida), in which 2016 season was drastically dry and no infections by *Monilinia* spp. were found in the orchards (no disease incidence at harvest and < 4 % at postharvest time). Therefore, no differences between treatments were observed since treatment applications were not necessary.
- Scenario 2: There was a high inoculum pressure and extremely adverse meteorological conditions. It was called an uncontrolable disease level. This was the case of Belgium, in which the production area was affected by an important hailstorm that destroyed the cherry production and make fruit uncommercial. In this case, treatments would had not been worthwhile. The results showed that not even chemical applications were able to reduce brown rot decay at harvest. In postharvest results, despite the high pressure of inoculum, two treatments reduced the incidence of brown rot (chemicals and PF1).

- Scenario 3: There were inoculum and meteorological conditions in which disease pressure is worthwhile and possible to be controled. It was called a controlable disease level. This was the case of France and Italy. It was also the case previously defined in the middle season variety of 2015 season (**Chapter VII**). In France, not differences were observed between treatments, including the chemical, to control brown rot at harvest due to the low *Monilinia* spp. incidence registered. At postharvest, however, satisfactory results were observed among the BCA treatments. In nectarines, treatments BA3, PF1 and PF2 behaved similar to the chemicals, although just the chemical could reduce the disease (compared to the control). Otherwise, in peaches all treatmenats significantly reduced *Monilinia* spp. incidence compared to the control, being BA4 and PF2 as much efficacious as chemicals. In the case of Italy, nectarines and peaches revealed similar results. As figure 14 demonstrates, in peaches at harvest, all BCAs evaluated showed the same level of efficacy as the chemical treatment and significantly reduced the disease incidence compared to the untreated control. At postharvest, almost all treatments evaluated (except BA3) significantly controlled the disease, although the disease incidence in the chemical treatment was significantly lower. Once again, the CPA-8-based product BA4 performed better than BA3.

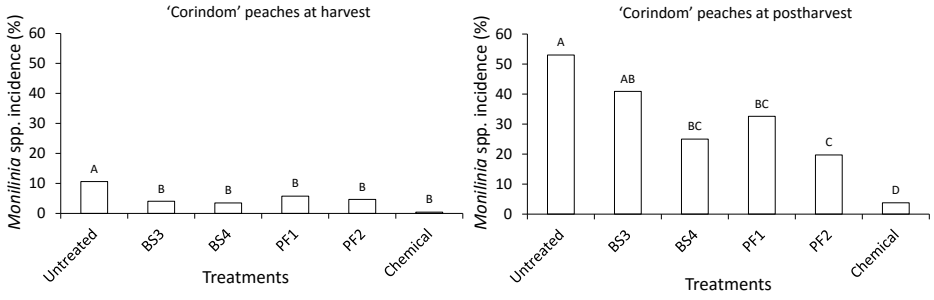

Figure 14. Results of field trials in 'Corindom' peaches in Italy, BIOGARD 2016 season. Different letters indicate significant differences ($P<0.05$) according to the LSD test.

Such results, including the findings obtained in **Chapter VII**, leaded us to conclude that within the standard levels of inoculum pressure habitually registered in the orchard (scenario 3); when the presence of the pathogen is medium-high, CPA- 8 is effective though less than the chemicals. Otherwise, under inoculum pressure medium-low, CPA-8 performs similar to chemicals.

However, to complete the validation strategy of the BCA CPA-8, the results above discussed will be repeated in further trials around European stone producing areas during the next growing season (2017 season).

The success of implementing biocontrol or integrated disease management systems will largely depend on the product knowledge and a thorough understanding of the complexity of the disease in the pre- and postharvest environment (Spadaro *et al.*, 2014). Since outbreaks of brown rot in stone fruit are dependent on the prevailing environmental conditions, additional work is now needed to successfully develop a predictive model of the disease expression for each characteristic region and cultivar (Gell *et al.*, 2008; Villarino *et al.*, 2012). It is important to understand the seasonal pattern of fruit susceptibility to infection in order to develop a more accurate strategy for brown rot control (Larena *et al.*, 2005). IRTA (unpublished results) recently developed a predictive model for *Monilinia* spp. disease spread in the orchard. Preliminary assays based on CPA-8-formulated products applications revealed encouraging results in which such model really works well. An accurate prediction of the BCA treatment timing involves a considerable reduction in both, manufacturing and labour costs.

The use of BCAs as an alternative to the synthetic chemical fungicides currently applied to control postharvest pathogens has many constraints and obstacles that make it difficult to implement their use as a practical control strategy (Spadaro *et al.*, 2014). However**,** the advances made with the two CPA-8 products developed have been far discussed in this thesis research. Although with limitations, they represent promising possibilities.

Scientists, growers and consumers alike must accept the fact that BCAs are usually not as effective as pesticides. The success of biocontrol greatly depends on convincing the consumer to prefer quality and not just outward appearance (Spadaro

et al., 2014). Biocontrol should be viewed more and more as an important component of an integrated disease management scheme for a significant and permanent reduction of pesticide use. The science and practice of biocontrol is still in its infancy compared to the development of synthetic chemical fungicides. However, the progress made in this area during the past decades, has been remarkable (Spadaro *et al.*, 2014). Gradual removal of the major regulatory barriers for BCAs is encouraging. Consequently, new antagonists under development, such as the CPA-8-based products here presented, further testify the currently increasing interest in biocontrol of postharvest diseases.

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CONCLUSIONS

Conclusions

After thoroughly detailing the content of this Ph.D. thesis research (Chapters I-VII), let us proceed to come up with firm statements about the findings obtained:

❖ **Characterisation of CPA-8:**

- Two approaches to successfully identify *Bacillus amyloliquefaciens* CPA-8 within a large number of *Bacillus* related strains have been developed: a semi-specific SCAR marker (SCAR-4) and a strain-specific genomic marker related to adaptative DNA sequences (*RBAM 007760* F2/R2).

- *B. amyloliquefaciens* CPA-8 has a wide tolerance to different pH-temperature and water activity (a_w)-temperature profiles which enable this strain to actively grow under a wide range of environmental conditions. CPA-8 grew better under warmer temperatures and neutral pH (37 ^{o}C and pH 7) and the type of solute used to modify a_w greatly influenced the minimum a_w at which the CPA-8 was able to grow (CPA-8 could not grow at a_w lower than 0.950 at optimal temperatures and using glycerol to modify the medium).

- *B. amyloliquefaciens* CPA-8 was susceptible to gentamicin, ampicillin and nalidixic acid and also to chloramphenicol and streptomycin at concentrations higher than 80 and 200 ppm, respectively. This strain manifested resistance to hygromicyn.

- The absence of *Bacillus cereus* enterotoxins (HBL and NHE) in *B. amyloliquefaciens* CPA-8 has been studied by PCR-technique. CPA-8 amplified for *nheA* gene, suggesting that it is not enough for the toxicity expression.

- The antifungal effect of the volatile organic compounds (VOCs) emitted by *B. amyloliquefaciens* CPA-8 to reduce cherry rot caused by *Monilinia laxa*, *Monilinia fructicola* and *Botrytis cinerea* has been evidenced, thus suggesting that lipopeptide production was not the only mechanism of action of this strain. Main VOCs were identified as 1,3 pentadiene, acetoin (3-hydroxy-2-butanone), thiophene, and ethylacetate; being the pure compound thiophene the most effective one with EC_{50} values lower than 6.67 µL mL^{-1} headspace (although it did not manifest any effect under *in vivo* conditions).

❖ **Production and formulation of CPA-8:**

- Low-cost culture medium based on the isolated soy protein PROSTAR 510A at 20 g L^{-1} provided the best growth curves for *B. amyloliquefaciens* CPA-8 (10^9 CFU mL^{-1} after 72 h of growing), compared to the boiled extract from Defatted Soy Flour (DSF) and protein PROSTAR 510A at 10 g L^{-1}, thus avoiding the hurdles caused by the raw DSF medium (microbial contamination and low solubility).

- *B. amyloliquefaciens* CPA-8 endospores significantly increased from 24 to 72 h of culture (medium containing protein PROSTAR 510A at 20 g L^{-1}), suggesting that the ability of this strain to survive heat incubation is highly related with the age of the culture. The high concentration of endospores obtained (>10^8 CFU mL^{-1}) is crucial to successfully survive the formulation process and the extreme conditions occurred in the orchard.

- Three different approaches were carried out to successfully formulate *B. amyloliquefaciens* CPA-8: liquid, freeze-drying and fluid-bed spray-drying. However, due to its less damage factors, good control over residual moisture content and water activity, and suitable powder flowability and particle size, fluid-bed spray-drying appeared to be the most suitable technology for CPA-8 formulation.

- Two *B. amyloliquefaciens* CPA-8-formulated products have been optimised by fluid-bed spray-drying using pregelatinised potato starch as binder and 20 % sucrose plus 10 % skimmed milk as protecting agents combined with either maltodextrin (CPA-8-formulated product BA3) or potato starch (CPA-8-formulated product BA4) as carrier material. Both products allowed to obtain an adequate survival of CPA-8 ($7.8 \cdot 10^9$ -$1.2 \cdot 10^{10}$ CFU g^{-1}), extending its shelf-life up to fifteen months at 4 and at 22 ºC.

Conclusions

❖ **Definition of the control strategy of CPA-8-based products:**

- The broad spectrum of action of the two optimised *B. amyloliquefaciens* CPA-8-based products (BA3 and BA4) against artificially infection with *M. laxa and M. fructicola* has been demonstrated in a wide range of stone fruit: nectarines, peaches, flat peaches, cherries, apricots, and plums.

- The persistence of the two optimised *B. amyloliquefaciens* CPA-8-based products (BA3 and BA4) applied on nectarines and peaches was evaluated after exposure to hostile environmental conditions of temperature, relative humidity, and wash-off caused by simulated rainfall. Although rain resulted to be the most constricting factor, antagonist populations generally higher than 10^4 CFU cm^{-2} remained on the fruit surface, thus ensuring high treatment coverture and therefore, efficacy. The higher degree of solubility of the maltodextrin made of the BA3 the product most easily removed from the fruit surface after rainfall exposure. Moreover, the shape and composition of the fruit surface were also of practical importance providing peaches (whose peel is rougher than the nectarines) with better product maintenance.

- The two optimised *B. amyloliquefaciens* CPA-8- based products (BA3 and BA4) were able to reduce *Monilinia* spp. incidence in commercial trials as long as the disease pressure was in the range of the standard levels recorded in the area, thus confirming the suitability of this strain as an alternative or complementary strategy to control brown rot decay in stone fruit.

Next Approaches

Next Approaches

While conducting the experiments of this thesis, the findings obtained lead us to ask ourselves for many questions that still remain unsolved. Therefore, I thought that it would be very interesting to outline what comes next. Although some issues have been already highlighted in the general discussion, some examples regarding which direction should take the future research are pointed below:

❖ **Characterisation of CPA-8:**

- It would be possible to obtain a qPCR marker (based on those molecular marker already developed) to not only identify but also quantify *Bacillus amyloliquefaciens* CPA-8 once applied in large-scale experiments at preharvest and postharvest conditions?

- Which endogenous compounds *B. amyloliquefaciens* CPA-8 accumulates during the production process? Such compounds may provide better environmental responses and therefore increase its biocontrol potential. May the growth conditions be manipulated in order to enhance this beneficial production?

- Has *B. amyloliquefaciens* CPA-8 any other mechanism of action different to those already described? It would be of practical interest to thus leading to a more efficient use of biocontrol strategies.

❖ **Production and formulation of CPA-8:**

- Which packaging and storage strategies (packaging materials to control gas exchange, prevent the loss or gain of moisture and avoid contamination) are the best to extend the shelf-life of the *B. amyloliquefaciens* CPA-8-based products? Regarding this point, trials on both CPA-based products (BA3 and BA4) are currently being carried out.

- Potato starch appears to be an essential component in the formulation of *B. amyloliquefaciens* CPA-8. Why it performs better than maltodextrin? It is because maltodextrin is more soluble? Can the pathogen (*Monilinia* spp.) take

profit from the more hydrolysed components of maltodextrin in detriment of the antagonist? Have maltodextrin and potato starch different effect on the CPA- 8 endospore dormancy/activation under specific situations?

❖ **Definition of the control strategy of CPA-8-based products:**

- May *B. amyloliquefaciens* CPA-8 survive under stresses caused by environmental factors such as, solar radiation (UV light), wind or oxidative stress associated with controlled-atmosphere storage?

- As a part of a well-designed program for the brown rot control, the validation of the CPA-8 strategy needs to be confirmed in the next growing season (2017). How the *B. amyloliquefaciens* CPA-8-based products behave against *Monilinia* spp. once applied in peach orchards from different producing areas?

- Which would be the effect on the control of brown rot decays of combining different environment-friendly strategies based on the *B. amyloliquefaciens* CPA- 8-based products and other physical and chemical methods, not only in the orchard but also along the packing line?

ANNEXES

Annex I

Environmental fate and behaviour of the biocontrol agent *Bacillus amyloliquefaciens* CPA-8 after preharvest application to stone fruit

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ABSTRACT

Bacillus amyloliquefaciens strain CPA-8 has been described as an effective biocontrol agent to control brown rot in stone fruit in both, preharvest and postharvest applications. However, information about the environmental fate and behaviour of this strain under field conditions is not available. In this work, dispersion of CPA-8 application was evaluated using water-sensitive papers. While a complete area coverage was obtained in leaves from treated trees, less than 1 % coverage was observed in leaves from non-treated trees. Moreover, the persistence of CPA-8 on fruit, leaves, and weed was also evaluated by using plating or contact methods and PCR confirmation. The presence of CPA-8 on fruit from treated trees was maintained during preharvest and postharvest conditions. However, a significant decrease on leaves and weed was observed 21 days after treatment. On non-treated trees, the presence of CPA-8 on leaves was detected until 180 days after treatment and on weed, the population of CPA-8 was dependent on the distance to the treated tree. High persistence of CPA-8 was detected on inert materials such as, clothes and gloves worn by handlers and on harvesting plastic boxes. Finally, more than 99 % of the samples with CPA-8 morphology were confirmed as CPA-8 using PCR. This work demonstrated a good distribution, persistence and adaptation of the CPA-8 strain to field and postharvest conditions. Monitoring dispersion and persistence is an excellent tool to decide the calendar of application and an interesting information for registering issues.

Keywords: Peach; biological control agent; antagonist; persistence; population dynamics; PCR.

INTRODUCTION

Monilinia spp. are the causal agents of brown rot in stone fruit and are responsible for economic losses during preharvest, but especially during postharvest. During favourable climatic conditions for disease development, these losses can reach up to 80 % for more susceptible late-ripening varieties (Usall *et al.*, 2015). Currently, the primary strategy to control this disease in stone fruit is based on spray programmes in the field using chemical fungicides combined with adequate cultural practices, since fungicide applications are not allowed or rarely occur (Sisquella *et al.*, 2014). Moreover, several currently used preharvest fungicides will have limited availability in the coming decade due to health and environmental reasons, leading to serious challenges for growers to produce healthy fruit in a sustainable way in the near future. These concerns, occurring at the same time that the appearance of fungicide-resistant strains, have promoted the development of alternative methods to synthetic fungicides. Biological control using microbial antagonists is considered the most promising strategy to replace the use of chemical fungicides, and its efficiency has been demonstrated in a large number of studies over the last 20 years (Wisniewski *et al.*, 2016).

Many species of the genus *Bacillus* have been reported as biological control agents (BCAs) with an effective broad spectrum activity against different plant diseases caused by soil borne (Chowdhury *et al.*, 2013; Pane *et al.*, 2012; Pastrana *et al.*, 2016), foliar (Lee & Ryu, 2016; Romero *et al.*, 2007), and postharvest pathogens (Casals *et al.*, 2010; Gao *et al.*, 2016; Hong *et al.*, 2014; Pusey & Wilson, 1984). In particular, *Bacillus amyloliquefaciens* strain CPA-8 (formerly *B. subtilis*), which was isolated from the surface of a nectarine, was demonstrated to be effective in controlling brown rot caused by *Monilinia* spp. alone or in combination with other postharvest treatments (Casals *et al.*, 2012; Yánez-Mendizábal *et al.*, 2011a). Moreover, recent studies revealed that applications of *B. amyloliquefaciens* during the preharvest of peaches and

nectarines substantially reduced brown rot decay during postharvest (Gotor-Vila *et al.*, unpublished results).

One of the main difficulties in commercialising a BCA is the registration procedure established by the European Commission regulation 1107/2009 (Soto-Muñoz *et al.*, 2015). This guideline concerns the commercialisation of plant protection products. Particularly, the Annex II section establishes the procedures and criteria for the approval of active substances in which the fate, behaviour, and persistence of the product in the intended environment are considered. Compliance with this regulation requires the development of monitoring methods that allow the precise identification and quantification of the microorganisms at the strain level (Alabouvette & Cordier, 2011) since applied BCAs usually belong to the same species as members of the microbiota (Lindow & Brandl, 2003).

Traditional methods for monitoring BCAs in the environment have been primarily based on dilution plating methodologies using selective or semi-selective culture media (Narayanasamy, 2013). However, this methodology is arduous because it requires a high level of expertise to differentiate among other morphologically similar species. These limitations have been solved using more specific methodologies based on DNA amplification with strain specific primers.

Many studies have used DNA-based techniques to identify the applied BCA from the non-target microbiota (Holmberg *et al.*, 2009; Nunes *et al.*, 2008; Soto-Muñoz *et al.*, 2014; Villarino *et al.*, 2016). With regards to *B. amyloliquefaciens* CPA-8, two different molecular markers were developed for its detection and identification (Gotor-Vila *et al.*, 2016). The first is based on a SCAR marker, in which a semi-specific fragment is amplified for CPA-8 and other 12 *Bacillus* strains that could be morphologically distinguished. In the second approach, a strain-specific genomic marker for CPA-8 was obtained from the *RBAM 007760* gene, which is primarily involved in bacterial surface adhesion.

With this in mind, we established an easy and reliable methodology to study the fate and behaviour of the CPA-8 strain after its application to flat peaches under field conditions and during postharvest storage using a combination of conventional and PCR methodologies. Specifically, the objectives of this study were (a) to evaluate the dispersion of the preharvest treatment, (b) to determine the air distribution of the CPA-8 cells, (c) to quantify the population of the CPA-8 strain on the surface of peaches, leaves and weed from the treated and non-treated neighbour trees, (d) to determine the persistence of the CPA-8 strain on the personal protective equipment and motorised sprayer, and (e) to quantify the population of the CPA-8 strain on fruit during postharvest storage. Moreover, colonies phenotypically similar to *Bacillus* spp. were identified using the PCR technique.

MATERIALS AND METHODS

Biological control agent

The *B. amyloliquefaciens* strain CPA-8 (Gotor-Vila *et al.*, 2016) was obtained from the culture collection of the IRTA Centre (Lleida, Catalonia, Spain). This strain was initially isolated from the surface of a nectarine in an experimental orchard in Lleida (Catalonia, Spain) and was selected for its efficacy in controlling brown rot disease (Casals *et al.*, 2010).

Bacteria were cultured and formulated by fluid-bed spray-drying according to the work reported by Gotor-Vila *et al.*, (2017). CPA-8 was cultured in 2-L laboratory scale bioreactors (BioFlo/CelliGen 115, Eppendorf, New Brunswick, Canada) containing optimised growth medium containing 20 g L^{-1} of the protein PROSTAR 510A (Brenntag Química, S.A.U., Barcelona, Catalonia, Spain). Then, CPA-8 cells were harvested by centrifugation at 9820 *g* for 12 min at 10 °C in an Avanti J-20 XP centrifuge (Beckman Coulter, CA, USA) and resuspended approximately at 10^{10} CFU mL^{-1} in the same CPA-8 culture supernatant to include the antifungal lipopeptides synthesised by the bacteria during growth. Then, protecting agents (20 % sucrose and 10 % skim milk) were added to the cell solution, mixed with 3.5 g of pregelatinised potato starch as binder and fluidised with 300 g of maltodextrin as carrier material. A dried product was obtained by

using a fluid-bed spray-dryer (HüttlinGmbH, Bosch Packaging Technology Company, Schopfheim, Germany).

Preharvest treatments in the field

Field experiments were carried out in a flat peaches cv. '796' commercial orchard located in Malpartit (Alpicat, Catalonia, Spain). The BCA CPA-8 was sprayed onto flat peaches 3 days before harvest, on the 7th of July, 2016. A portable weather station was located inside the orchard for measuring temperature, relative humidity, wetness, and rate of precipitation, and was connected to a data logger for data registration.

To prepare an adequate CPA-8 concentration for the treatment, 40 g of dried cells ($9 \cdot 10^9$ CFU g^{-1}) were dissolved into 30 L of water to obtain a final adjusted concentration of 10^7 CFU mL^{-1}. Each tree was sprayed for 20 s (approximately 3 L) using a motorised sprayer (Benza P30P 2R, Ordes, Spain) at 15 bars of pressure.

The experimental design for each replicate consisted of 1 treated (T) and 3 non-treated trees (A, same row neighbour; B, same column in the next row neighbour; C, diagonal in the next row neighbour) and 3 replicates were evaluated (Fig. 1A). Each replicate was separated by 3 non-treated trees. Both treated and non-treated trees were used to monitor the dispersion, environmental Distribution, and the persistence of the CPA-8 preharvest treatment under field conditions. The sampling times were at 0 h (after letting the treatment dry) and at 1, 3 (fruit harvest for postharvest storage), 7, 14, 21, 28, 45, 60, 90, 120, 150 and 180 days after treatment. The last sampling time was in December when the leaves had already fallen.

Monitoring the dispersion and the aerial distribution of the CPA-8 preharvest treatment in the field

The dispersion of the CPA-8 treatment was monitored on the leaves and on the orchard floor using water-sensitive papers (WSPs, Singenta, Madrid, Spain) (Fig. 1B). The WSPs were positioned on the leaves of the outside and inside canopies of the treated and non-treated trees using a doubled-sided adhesive tape. The WSPs on the ground were attached to a petri plate using doubled-sided adhesive tape and were

placed around the treated (1 WSP in each vertex of the square of the treated tree, outside of the canopy) and non-treated trees (1 WSP in line with the treated tree, outside of the canopy, with another located at medium the distance between the canopy of the treated and non-treated trees). Once the WPSs were completely dried, they were stored in a plastic bag until further analysis. WSPs were scanned with a 600 dpi imaging resolution, and the percentage of the covered surface was estimated using the ImageJ Software (ImageJ, US National Institutes of Health, https://imagej.nih.gov/ij/) in combination with the macro developed by Gil *et al.* (2016).

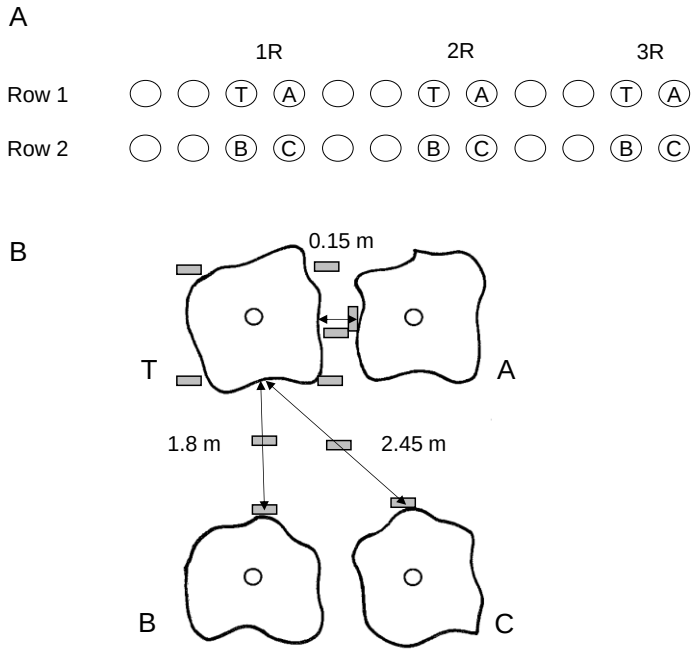

Figure 1. Experimental design of the CPA-8 treatment in the field. (A) The experimental design for each replicate consisted of 1 treated (T) and 3 non-treated trees (A, same row neighbour; B, same column in the next row neighbour; C, diagonal in the next row neighbour). A total of 3 replicates were evaluated, each one separated by 3 non-treated trees. (B) The dispersion of the CPA-8 treatment was monitored on the orchard floor using water-sensitive papers (WSPs) distributed around the canopy of the treated tree (4 WSPs), outside of the canopy of the non-treated trees (0.15, 1.8 and 2.45 m distance to non-treated trees A, B and C, respectively) and at medium distance between the canopy of the treated and non-treated trees. The same distribution was used to monitor the aerial conidia distribution.

The aerial distribution and persistence were evaluated by gravimetry in the same points where the floor WSPs were located. Petri plates containing NYDA medium were left opened for 2 min. Plates were incubated at 30 °C for 24 h, and 10 % of the total colonies from each plate that were phenotypically similar to *Bacillus* spp. were selected for PCR identification.

Population dynamics of the CPA-8 preharvest treatment in treated trees

The population dynamics of the CPA-8 treatment was quantified in different plant tissues of the treated trees, including the fruit, leaves and weed. For the fruit, 5 fruits per replicate were harvested, and 8 pieces of peach peel (16 mm diameter) were removed with a cork borer from each fruit. These 40 pieces were dipped into 20 mL of phosphate buffer solution (0.05 mol L^{-1} potassium phosphate, pH 6.5) in a sterile filter bag and were homogenised in a Stomacher (Seward, London, UK) set at 12 strokes s^{-1} for 90 s. For the leaves, 10 leaves were harvested, and two pieces from each (19.6 mm diameter) were removed with a cork borer. These 20 pieces were dipped into 10 mL of phosphate buffer solution and homogenised in a Stomacher. For the weed, 1 gram of weed was collected from two different sample points, dipped into 10 mL of phosphate buffer solution and homogenised in a Stomacher. For all plant tissues, serial ten-fold dilutions of suspension samples were made thrice by dilution plating on NYDA media, which were incubated as described above. The results were expressed as CFU cm^{-2} or CFU g^{-1} depending on the sample material. Two colonies were selected from each dilution plate for PCR confirmation.

Persistence of the CPA-8 preharvest treatment in non-treated trees

The persistence of the CPA-8 strain in non-treated trees (A, B and C) was evaluated in different plant tissues, including the fruit, leaves, and weed. The sampling method used to detect the CPA-8 strain in fruit and leaves was the contact plating method using Rodac (replicate organism direct agar contact) plates containing NYDA medium. For each sampling time and replicate, three Rodac plates were used for each tissue sampled. The colonies were incubated for enumeration and then were selected as follows: for Rodac plates, if the plate contained more than 10 colonies that were morphologically similar to *Bacillus* spp., 40 % were selected for

PCR confirmation. Otherwise, if the plate contained less than 10 colonies, all the colonies were confirmed by PCR.

The sampling method used to detect the CPA-8 strain in weed from the non-treated trees was dilution plating, following the same protocol described previously for weed of the treated trees. The plates were incubated for colony enumeration and were selected as described above for dilution plating.

Population dynamics of the CPA-8 during postharvest storage

The population dynamics of the CPA-8 strain on the surface of flat peaches was quantified during postharvest storage (0 °C and 85 % relative humidity (RH)) at 7, 15, 21, 28 and 45 days after harvest. Moreover, the CPA-8 population was also quantified after simulating the shelf-life period during the commercialisation process (20 ^{o}C and 85 % RH for 5 days after 28 and 45 days of cold storage). The population dynamics of CPA-8 on the fruit surfaces was determined by dilution plating, which, in addition to the number of colonies selected for confirmation by PCR, was done as previously described.

Moreover, the persistence of the CPA-8 strain in the plastic boxes used to store fruit after harvest was also evaluated after 7, 15, 21, 28 and 45 days of storage at 0 °C and 85 % RH using Rodac plates as described above. At the end of the postharvest storage, the plastic boxes were cleaned by immersion for 30 s in a 5 % soap water solution and were disinfected for 1 min in a 2 % sodium hypochlorite water solution. Four days later, the same protocol was repeated but increasing the percentage of sodium hypochlorite up to 10 %. Once the plastic boxes were air dried, the persistence of the CPA-8 was evaluated using the contact plating methodology. The colonies were incubated for enumeration and selected as previously described for contact plating methodology.

Persistence of the CPA-8 preharvest treatment in the personal protective equipments (PPE) and in the motorised sprayer

The persistence and survival of the CPA-8 strain in the PPE of the treatment applicator was estimated on gloves and on the working clothes using the contact

plating method with Rodac plates containing NYDA medium. Both right- and left-hand gloves were sampled, and the working clothes were sampled from both arms and legs, using two plates for the stomach and two plates for the back, totalling twelve Rodac plates for each sampling time. In the case of the motorised sprayer, five Rodac plates were used to detect the persistence of CPA-8 before and after treatment, and after cleaning the sprayer with water. The plates were incubated for colony enumeration as previously described for the contact planting methodology.

Colony confirmation by PCR

DNA from CPA-8 was extracted using physical methods. Colony samples (2-5 mg) were introduced into a tube and were heated in the microwave at maximum voltage for 1 min and then cooled on ice for 5 min. Samples were diluted with 20 µL of molecular grade water (Fisher Scientific, Madrid, Spain).

To identify the CPA-8 strain, the primer pair RBAM007760F (GTACCGATTGCAACAGGTTTAGATG) and RBAM007760R (CTGTTGCCCCG GTTCGTC) described by Gotor-Vila *et al.* (2016) was used to amplify a 265 bp fragment. Each reaction had a final volume of 12.5 µL and contained 1x Kapa 2G Robust Hot Start Ready Mix (Kapa Biosystems Inc., Wilmington, MA, USA), 500 nmol L^{-1} of each primer and 2 µL of extracted DNA. The amplification was carried out in a GeneAmp PCR System 2700 (Applied biosystems, Madrid, Spain) thermal cycler. The cycling conditions were as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 65 °C for 45 s and 72 °C for 30 s, followed by a final extension step of 72 °C for 7 min. Two negative controls were used, one without DNA and the other with *Bacillus subtillis* (Serenade, Bayer CropScience AG, Leverkusen, Germany), and one positive control with CPA-8. The products amplified by PCR were analysed by electrophoresis on a 1.2 % agarose gel using a 100 bp DNA ladder (Nippon Genetics Europe, Dueren, Germany).

Statistical analysis

All data were analysed for significant differences using analysis of variance (ANOVA) with JMP V.8 (SAS Institute Inc.). Statistical significance was defined as

$P<0.05$. When the analysis was statistically significant, t-test for two sample comparison or Tukey's test for the separation of means was performed.

RESULTS

Meteorological data

Meteorological data comprising the relative humidity (RH), temperature (T), wetness (W) and rainfall (R) are shown in Figure 2 for the experimental period from 04/07/16 to 12/12/16. Specifically, July, August, and September were characterised by a very warm and dry period (T mean = 24.4 °C, T max = 39.3 °C, RH mean = 57.1 % and RH min = 22.9 %). Moreover, during these three months, it only rained four times, with a rainfall accumulation of 15 mm and a wetness accumulation of 16.6 h after raining. October was a warm month (T mean = 16.2 °C, T max = 30.6 °C, RH mean = 77.9 % and RH min = 33.7 %), and during that time it rained five times with a rainfall of 31 mm and a subsequent wetness accumulation of 79.7 hours after raining. November and December were characterised by cold temperatures and high relative humidity (T mean = 8.3 ºC, T min = 0.5 °C, RH mean = 86.2 % and RH min = 48.1 %). This high RH was accompanied with a high wetness accumulation (a total of 384 h during 1.5 months) mainly due to the presence of fog rather than rain accumulation (54 mm).

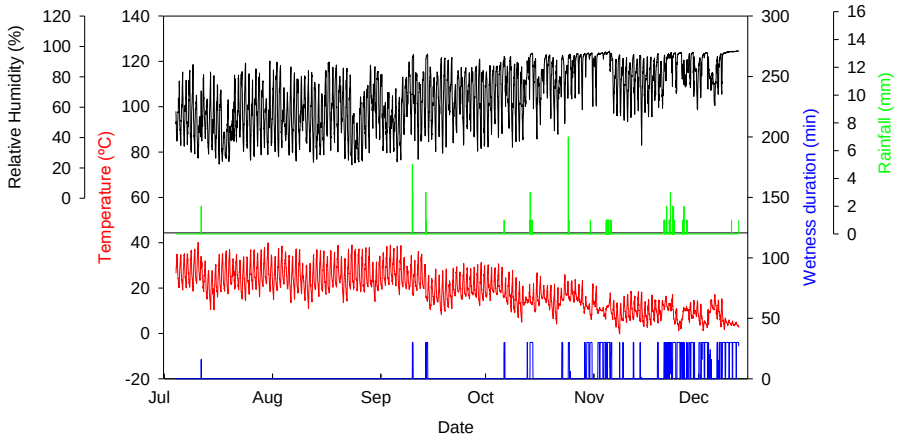

Figure 2. Meteorological data from the commercial orchard of flat peaches located at Malpartit (Catalonia, Spain). Temperature (ºC, red), relative humidity (%, black), wetness duration (min, blue) and rainfall (mm, green) were registered since the day of the treatment (04/07/2016) until the fall of the leaves (180 days after treatment, 12/12/2016) every 30 min.

Assessment of spray dispersion using water-sensitive papers

The spray dispersion of the CPA-8 treatment was measured using WSPs located on the leaves (Fig. 3A) and on the ground (Fig. 3B). Significant differences were observed between the area covered from the papers located on the leaves from treated trees (T) and on the leaves from the non-treated trees (A, B or C). While the application of the sprayed CPA-8 strain covered 100 % of the surface of WSPs that were inside and outside of the canopy leaves of the treated trees, less than 1 % of the area was covered in WSPs from the canopy leaves of the non-treated trees.

The dispersion of the CPA-8 treatment on the ground was represented as the relationship between the distance to the canopy of the treated and non-treated trees and the coverage area of the WSPs (Fig. 3B). It was observed that a larger area of the papers was covered for WSPs located at distances shorter than 0.5 m, corresponding to the ground WSPs around the treated trees and the non-treated tree A. The WSPs located at a medium distance between the non-treated trees B and C showed a medium area of coverage (in a range of 1 to 38 %) while the WSPs located in the most distant area (2.45 m) showed less than 5 % of area coverage.

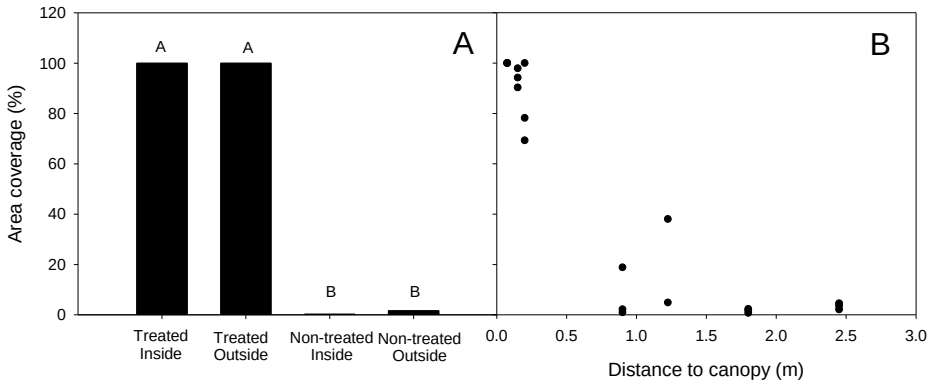

Figure 3. Percentage area coverage with CPA-8 spray treatment obtained from the analysis of water sensitive papers (WSPs) using Image J Program. The WSPs were located (A) on the leaves of the outside and inside canopy of treated and non-treated trees and (B) on the ground around the treated and non-treated trees at different distances. Different letters indicate significant differences using Tukey's test ($P < 0.05$).

Aerial distribution of the CPA-8 strain at different distances to the canopy

The aerial distribution of the CPA-8 strain was studied in relation to the application distance to the canopy and the sampling day after treatment (Fig. 4). Just after the application of the treatment (0 d), it was observed that CPA-8 was distributed randomly around both the treated and non-treated trees, even at the farthest application distance to the canopy (non-treated tree C = 2.45 m). The general pattern observed over the next sampling days was similar, with a higher colony accumulation (the sum of all colonies ranged from 77 to 160 colonies plate^{-1}) at distances shorter than 0.5 m, corresponding to the area around the treated trees and the non-treated tree A. At greater distances, CPA-8 colonies were also detected. In contrast, after 90 days of treatment it was observed a decrease in the number of aerial colonies at all checked distances. At distances shorter than 0.5 m, a decrease in the number of aerial colonies was observed, from 28 colonies plate^{-1} to just 1 colony plate^{-1} after 90 and 180 days after treatment, respectively. At distances greater than 0.5 m, the decrease was from 22 colonies plate^{-1} (90 days after treatment) to 6 colonies plate^{-1} (150 days after treatment) (data not shown), and none was observed 180 days after treatment. It was remarkable that on the last sampling day (180 days after

treatment) only one colony of CPA-8 was detected. Figure 5A and 5B show the phenotypic morphologies of CPA-8 colonies detected from air samples of treated and non-treated trees, respectively, and Figure 5C shows the PCR confirmation of these samples.

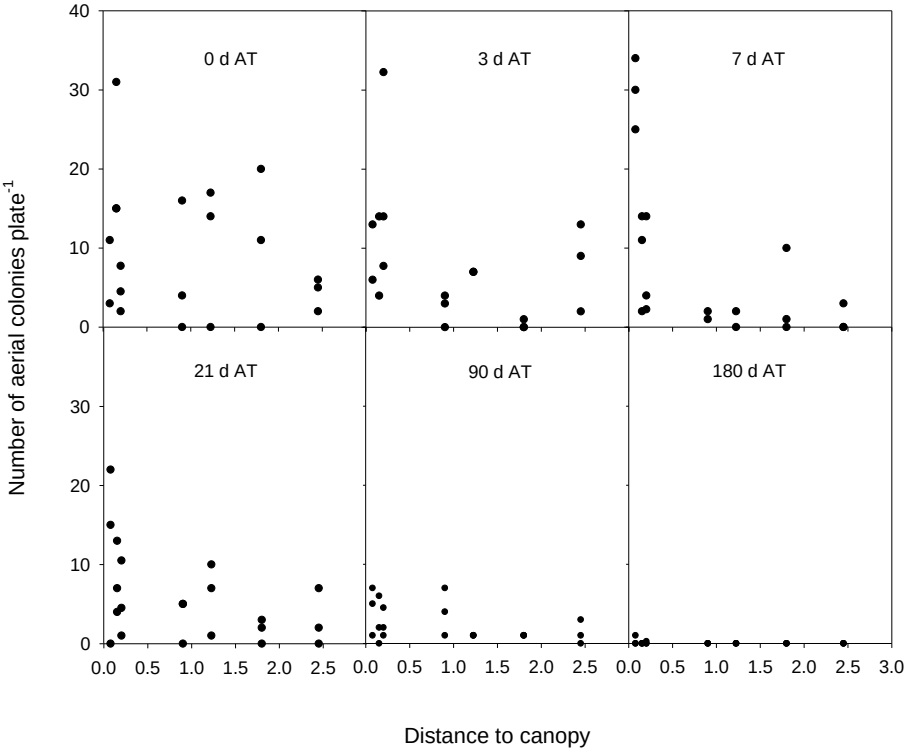

Figure 4. Quantification of aerial colonies of the CPA-8 in relation to the distance to the canopy of the treated and non-treated trees at different times after treatment (AT). Colonies were quantified using the gravimetric methodology and colonies morphologically similar to *Bacillus amyloliquefaciens* were selected for CPA-8 confirmation.

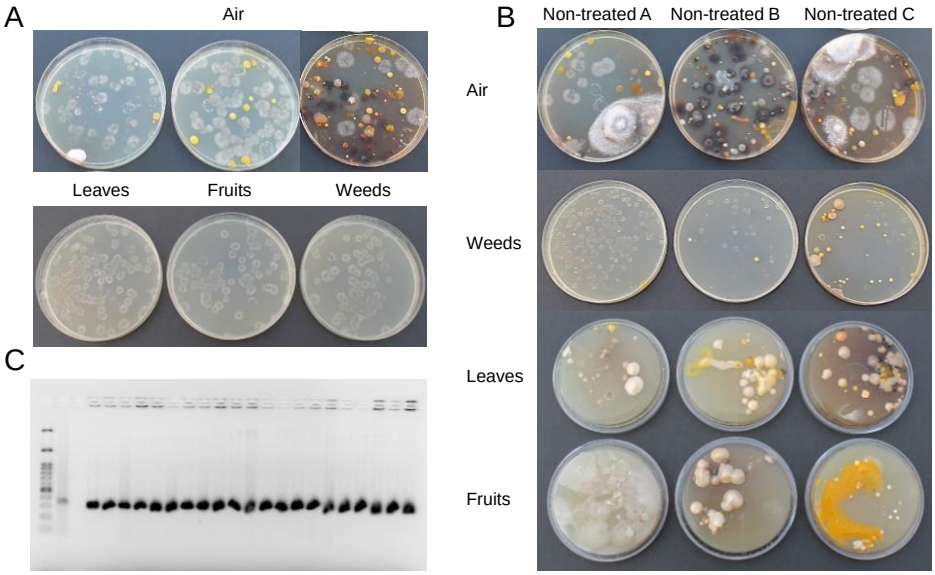

Figure 5. Plate sampling from air, leaves, fruit, and weed from (1) treated and (2) non-treated trees that showed the presence of colonies morphologically similar to *Bacillus amyloliquefaciens*. (3) Example of sample confirmation as CPA-8 by polymerase chain reaction (PCR).

Persistence of the CPA-8 strain on the treated trees

The population dynamics of the CPA-8 strain on fruit, leaves, and weed of the treated trees were measured by dilution plating and confirmed by PCR (Figs. 6 and 7). Just after the preharvest application of the CPA-8 treatment, the population level of the bacteria on fruit surfaces was 4.13 $\log_{10}$ CFU cm^{-2} and remained stable for 3 and 45 days under field or postharvest conditions, respectively (Fig. 6). At postharvest, no statistical differences were observed between the population levels of CPA-8 after 28 days at cold storage and 28 days at cold storage with a shelf-life period of 5 days. However, a lower population of the CPA-8 strain was observed at 45 days at cold storage conditions (4.00 $\log_{10}$ CFU cm^{-2}) in comparison to 45 days at cold storage with a shelf-life period of 5 days (4.24 $\log_{10}$ CFU cm^{-2}).

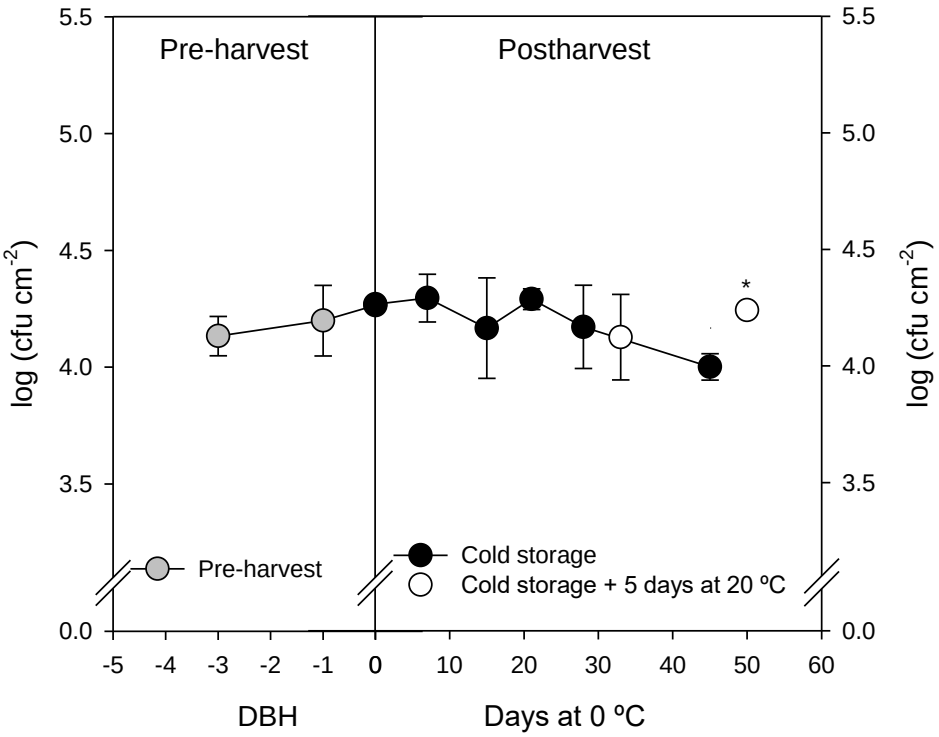

Figure 6. Population dynamics of CPA-8 on flat peaches surface quantified by dilution plating and confirmed by PCR. The CPA-8 treatment was sprayed 3 days before harvest (DBH). After harvest, fruit was stored at cold conditions (0 ºC and 85 % RH) for 7, 15, 21, 28 and 45 days. After 28 and 45 days of cold storage, fruit was exposed to 20 ºC and 85 RH for 5 days, simulating shelf-life conditions. Each point represents the mean of three biological replicates and error bars represent the standard deviation of three biological replicates. * indicates significant differences according to t-test ($P < 0.05$).

The initial population of CPA-8 on leaves from the treated trees was 4.43 $\log_{10}$ CFU cm^{-2}, remaining stable until 21 days after treatment (Fig. 7). At this time, the population levels of the CPA-8 strain progressively decreased until 120 days after treatment (1.88 $\log_{10}$ CFU cm^{-2}) and remained stable until the last sampling time after 180 days of the treatment (1.68 $\log_{10}$ CFU cm^{-2}).

In the case of the weed, the initial population of CPA-8 was 6.26 $\log_{10}$ CFU g^{-1} fresh weight (FW) and remained stable until 21 days after treatment (5.53 $\log_{10}$ CFU g^{-1} FW) (Fig. 7). Afterwards, the CPA-8 population decreased until 3.2 $\log_{10}$ CFU g^{-1} FW, detected at 90 days after treatment. It is interesting to note the differences observed in the population levels among biological replications. This could be due to

the presence of weed at the beginning of the trials and the growth of new weed over the time-course of the sampling.

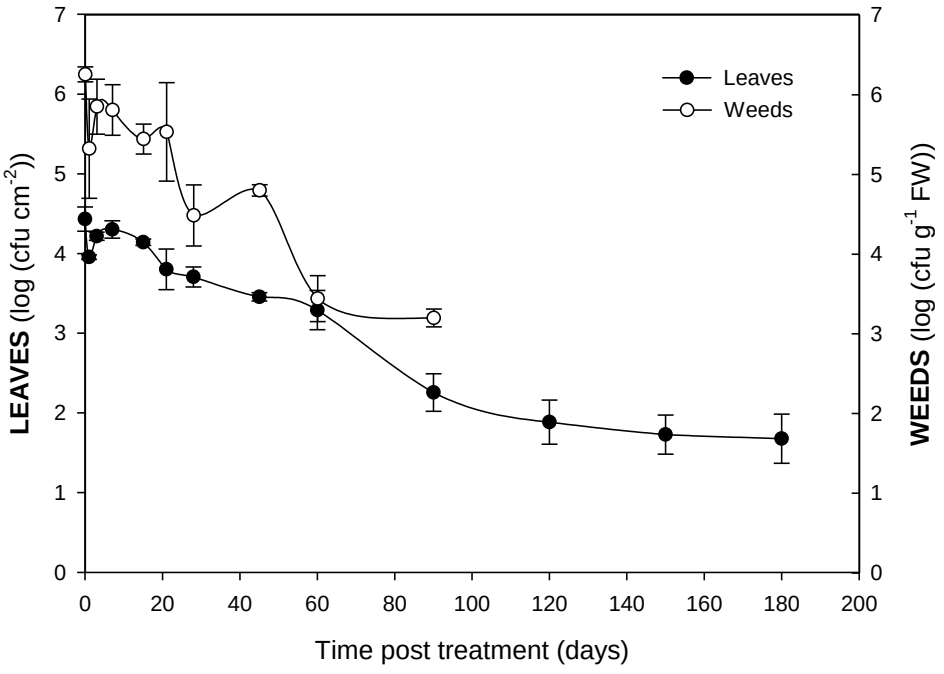

Figure 7. Population dynamics of CPA-8 on leaves (log CFU cm^{-2}) and weed (log CFU g^{-1} fresh weight) of the treated trees quantified by dilution plating and confirmed by PCR at different sampling times. Each point represents the mean of three biological replicates and error bars represent the standard deviation of three biological replicates.

Figure 5A shows the phenotypic morphologies of CPA-8 colonies obtained from the leaves, fruit and weed of treated-trees, and Figure 5C shows the PCR confirmation of these samples.

Persistence of the CPA-8 strain on non-treated trees

The persistence of the CPA-8 strain in the non-treated trees was evaluated using printings on fruit and leaves and by using dilution plating methodology for weed (Table 1 and Fig. 8). In fruit, after just 0 and 1 day of CPA-8 application, 89 % of Rodac plates showed colonies that were phenotypically similar to CPA-8, which could be mostly confirmed (100 and 87 %, respectively) (Table 1). After 3, 7 and 15 days of

treatment, 100 % of sampled plates showed colonies that were phenotypically similar to CPA-8, and they were all confirmed as well. The persistence of CPA-8 on the surfaces of fruit under field conditions was probably longer than 15 days, however, no more sampling times were analysed due to the over-maturity of the fruit.

Table 1. Sampling plates from fruit and leaves of the non-treated area that showed similar morphology to CPA-8 and that were confirmed as CPA-8 using polymerase chain reaction (PCR).

Plant material	Days after treatment (d)	Morphologically similar to CPA-8		CPA-8 confirmed by PCR	
		Plate ratio[a]	Percentage (%)	Plate ratio[b]	Percentage (%)
Fruits (n = 9)	0	8/9	89	8/8	100
	1	8/9	89	7/8	87
	3 (harvest)	9/9	100	9/9	100
	7	9/9	100	9/9	100
	15	9/9	100	9/9	100
Leaves (n = 9)	0	9/9	100	9/9	100
	1	7/9	78	7/7	100
	3 (harvest)	9/9	100	9/9	100
	7	9/9	100	9/9	100
	15	9/9	100	8/9	89
	21	9/9	100	9/9	100
	28	9/9	100	9/9	100
	45	9/9	100	9/9	100
	60	9/9	100	9/9	100
	90	9/9	100	9/9	100
	120	6/9	67	6/6	100
	150	7/9	78	7/7	100

[a] Ratio of plate number with similar morphology to CPA-8 in relation to the total number of sampled plates.
[b] Ratio of plate number confirmed as CPA-8 using PCR methodology in relation to the total number of sampled plates.

In leaves sampled up until 90 days after treatment, a general pattern was observed in which most of the Rodac plates yielded colonies that were morphologically similar to CPA-8, and they were all confirmed as the CPA-8 strain (Table 1). However, after 120 days, a decrease in the number of plates containing colonies similar to CPA-8 was observed, which was even more pronounced 180 days after the CPA-8 treatment (when only one of the plates showed the presence of colonies that were morphologically similar to CPA-8). It is noteworthy that all the colonies that were morphologically similar to CPA-8 were confirmed except one, which was sampled 15 days after the CPA-8 application

Figure 8 shows the population dynamics of the CPA-8 strain on weed from non-treated trees being differentiated from the non-treated trees A, B and C. The same

tendency in the population levels during the time course of the different non-treated trees was observed. The population levels decreased 1 day after treatment, increased between 3 and 7 days after treatment and finally start falling again until being maintained during the last sampling times (15-28 days after treatment). However, probably due to the important differences between the biological replicates, no statistical differences in the population levels during the time course were observed. The initial population levels detected in the weed of the non-treated tree A were higher (6.10 CFU g^{-1} FW) than those detected in the non-treated trees B and C (3.96 and 3.84 CFU g^{-1} FW, respectively). During the first sampling times (1-7 days after treatment) the population levels detected in the weed of the non-treated tree A were also higher than those detected in B and C.

Figure 5B shows the phenotypic morphologies of the CPA-8 colonies detected from leaves, fruit and weed of treated-trees, and Figure 5C shows the PCR confirmation of these samples.

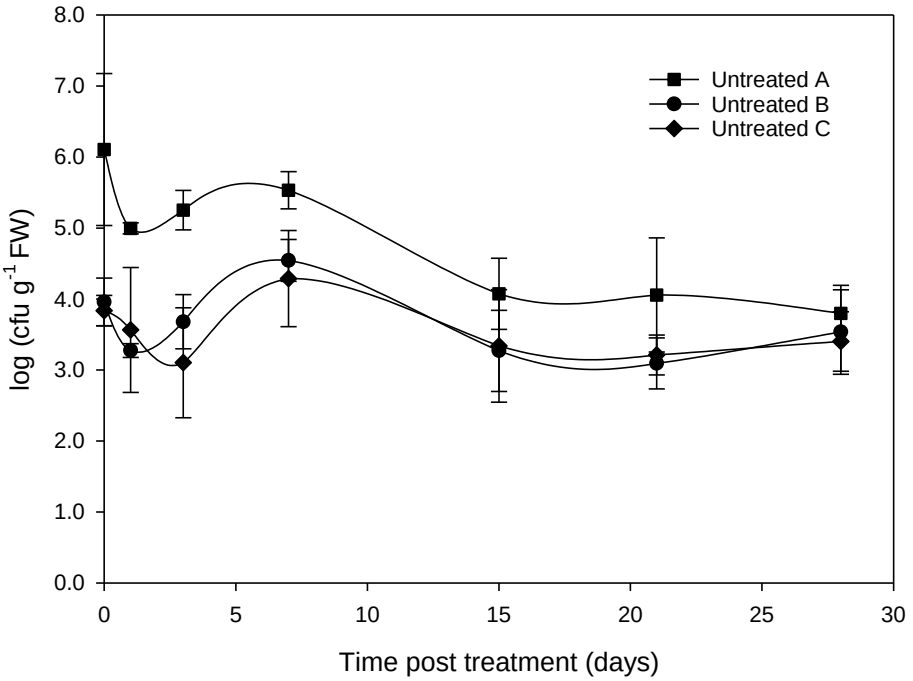

Figure 8. Population dynamics of the CPA-8 on weed of non-treated trees (A, B and C) quantified by dilution plating and confirmed by PCR at different sampling times. Each point represents the mean of three biological replicates and error bars represent the standard deviation of three biological replicates.

Persistence of the CPA-8 strain on the personal protective equipment, on the motorised sprayer and postharvest plastic boxes

On the gloves and clothes worn by handlers, the CPA-8 strain was detected and confirmed on all the plates from the day of the treatment until the last sampling time (180 days after treatment) (data not shown). In the case of the motorised sprayer, CPA-8 was not detected before the treatment (data not shown). However, once CPA- 8 was applied, it was detected and confirmed for all sampled plates. Similar results were obtained after washing the motorised sprayer with water.

The CPA-8 strain was also detected and confirmed in all sampled plates from the plastic boxes that contained the fruit during postharvest storage (7, 15, 21, 28 and 45 days at 0 ºC and 85 % RH after treatment) (data not shown). Furthermore, while the disinfection of the plastic boxes with 2 % sodium hypochlorite did not affect the

presence of CPA-8, the re-disinfection with 10 % sodium hypochlorite partially eliminated the presence of CPA-8 colonies in 66.7 % of the total sampling plates. Seven days after the re-disinfection, CPA-8 was not detected in any plastic boxes (data not shown).

DISCUSSION

The efficacy of the preharvest and postharvest application of the BCA *Bacillus amyloliquefaciens* CPA-8 to control brown rot in stone fruit has already been demonstrated (Gotor-Vila *et al*., unpublished results; Yánez-mendizábal et al., 2001). However, to assure its efficacy after field treatment, it is necessary to know the ecological fitness of this bacterium, which is determined by the physical and microbiological environment (Pujol *et al*., 2007). Monitoring the ecological fitness is necessary for registration procedures since it is required to determinate its traceability, persistence and environmental impact (Montesinos, 2003). Different methodologies have been used to assess the ecological fitness of BCAs. Taking advantage of the PCR technique developed by Gotor-Vila *et al*.(2016) it was possible to distinguish CPA-8 from the non-target microbiota.

In this study, more than 1800 samples that were phenotypically similar to CPA-8 were analysed, and only 10 of them gave a negative result by PCR confirmation. These results demonstrated that the plate counting methodology was suitable for monitoring the CPA-8 strain primarily due to the characteristic colony morphology, thus making it difficult to confuse within the other microbiota. No colonies phenotypically similar to CPA-8 strain were detected in the orchard before CPA-8 application. Moreover, the fast growth of CPA-8 allowed incubation times of 24 h. In contrast to the results obtained in this study, in the monitoring of the BCA *Pantoea agglomerans* CPA-2 strain in a citrus fruit orchard, the use of the PCR technique was practically indispensable, since the target microorganism was phenotypically similar to other species in the field, specifically *Pantoea ananatis* (Soto-Muñoz *et al*., 2015).

The dispersion of the treatment is one of the most important factors to take into account, since a correct spray can improve the accuracy and the efficiency of the

treatment, thus achieving a more uniform spray deposition and avoiding loss of product and environmental contamination (Marçal & Cunha, 2008). For this reason, it is also important to monitor the spray treatment in treated and non-treated areas. In this study, the sprayed area of the WSPs was 100 % when they were located on leaves outside and inside of the canopy of treated trees, while less than 1 % of the covered area was detected on leaves of non-treated trees. These results agreed with those obtained by Soto-Muñoz et al (2015) in a sprayer application of the BCA *P. agglomerans* CPA-2 in a citrus fruit orchard, despite the different training systems. However, both results are quite different from that obtained when using a chemical fungicide to control fruit diseases caused by species such as *Monilinia* spp. Syngenta Crop Protection AG (Basel, Switzerland) recommends at least 50-70 droplets cm^{-2} (Zhu *et al.*, 2011) and that a total coverage area of approximately 15 % should be enough for satisfactory fungicide applications (Deveau, 2016). These important differences in area coverage observed between the biocontrol applications and chemical treatments could be related with the mode of action of each product. While most chemical fungicides are systemic, *B. amyloliquefaciens* CPA-8, as other BCAs, needs a high population and viability to effectively colonise the fruit surface (Gotor-Vila *et al*, unpublished results). In the case of the WSPs located on the floor, an inverse relationship was observed between the distance to the canopy and the area of coverage, as well as in the number of air colonies at different sampled times. Only just after the treatment was a high density of aerial colonies observed around all the treated and non-treated areas.

The population dynamics of the CPA-8 strain were evaluated at different time courses on the fruit, leaves, and weed of treated trees to observe the presence and persistence of this BCA on different plant tissues, and to evaluate its adaptation to field conditions. This study demonstrated that CPA-8 was able to maintain the same population level for 3 days under field conditions and for 45 days at postharvest storage conditions. Previous studies with the BCA *P. agglomerans* CPA-2 on citrus demonstrated that the bacterial population remained stable after preharvest applications at optimal levels for 20 days under field conditions (Cañamás *et al.*, 2008). In addition, when fruit from inside and outside of the canopy were analysed separately, a higher bacterial population stability was observed on fruit from the

inside part (Soto-Muñoz *et al.*, 2015). The authors attributed these results to the decreased exposure of the fruit to environmental factors, such as UV radiation, wind, humidity, and high temperature. In this study, the field conditions recorded during the trials were very warm and dry, achieving maximum temperatures of 40 °C and a minimum RH of 22 %, characteristics from a cold semi-arid climate as classified by Köppen (Peel *et al.*, 2007). Gotor-Vila *et al.*, (unpublished results) demonstrated that CPA-8 is a microorganism that is highly adapted to high temperatures under *in vitro* conditions, with stable viability on stone fruit surface exposed to 0 and 20 ^{o}C and different RH values (85, 60 and 40 %). However, the population levels of the yeast *Candida sake* on treated grapes decreased when they were exposed to high temperatures (40 and 45 °C) and 30 % RH. In the case of *Candida oleophila* strain O on the surface of apples under postharvest conditions (low temperature and ultra-low oxygen concentrations) the viability was maintained over 60 days (Massart *et al.*, 2005). The BCA *Penicillium oxalicum* isolate 121 is adapted to dry and warm conditions, having an optimal growth range between 15 and 30 °C, but it was not able to grow under 4 °C or above 35 °C (Pascual *et al.*, 1997). Overall, depending on the microorganism (bacteria, yeast or fungi) these environmental factors could affect their viability to a greater or lesser degree. This is the reason why many studies have been conducted based on the adaptation of the microorganisms to improve their efficacy (Droby *et al.*, 2016).

In the phyllosphere and in the weed, the population levels of CPA-8 showed a high dependence on environmental conditions. This study determined that population levels of CPA-8 remained stable until 21 days after treatment and then started falling. Despite the leaf surface being characterised by low nutrient availability and high exposure to environmental conditions (Lindow & Brandl, 2003), CPA-8 maintained a stable population level of 1.68 $\log_{10}$ CFU cm^{-2} until the fall of the leaves, 180 days after treatment. Some authors have correlated this decrease to the stressful environmental conditions that stimulate the entry in a viable, but unculturable bacterial state (Pujol *et al.*, 2007). In this study, the decrease in bacterial populations on leaves could be related to the rainfall events accompanied with lower temperatures. Some authors demonstrated that the first 20 mm of rain considerably

reduced the population of the BCAs from the fruit surface (Calvo-Garrido *et. al*, 2014b; Gotor-Vila *et al.*, unpublished results). Similar tendencies were observed in the reduction of chemical treatments on grapes and vine leaves due to rainfall (Cabras *et al.*, 2001). However, in blossoms a high and stable population of the bacteria *Pseudomonas fluorescens* EPS62e was observed, and it was even able to increase in population during the first days after treatment (Pujol *et al.*, 2006; Pujol *et al.*, 2007). It is interesting to note that the weather conditions in both Mediterranean and Atlantic climates during spring were not as extreme as in the summer and can boost the colonisation of flowers to achieve an efficient control of fire blight.

Although the sprayed treatment seemed not to be dispersed on the leaves from the non-treated trees, CPA-8 was detected on both leaves and fruit over all analysed sampling times. While small drops of the treatment were not detected in WSPs at long application distances (Salyani *et al.*, 2013), CPA-8 could be most likely be detected due to the small size of the bacteria. These results indicate the high dispersion of the CPA-8 strain in the field and its efficient colonisation and adaptation on fruit and leaf surfaces. In the case of the weed, higher population levels of CPA-8 were observed in the non-treated tree A compared to B or C. Moreover, the population levels detected in the non-treated tree A were quite similar to those detected in the weed around the treated tree. These results are consistent because the non-treated tree A is closely located to the treated tree. The differences between the population levels detected in the weed of the non-treated tree A were approximately 100-fold higher than the ones detected in the non-treated trees B or C. Other authors also detected differences of approximately 10-100-fold in the BCA population levels between the treated and non-treated trees, probably because BCAs better colonised the niches compared to the indigenous microbiota (Johnson *et al.*, 2000; Lindow & Brandl, 2003; Pujol *et al.*, 2007). Pujol *et al.* (2007) even detected the BCA in non-treated flowers 15-35 m far away from the treated trees.

The persistence of the CPA-8 strain in inert materials, such as PPE (clothes and gloves) was detected until 180 days after treatment. It is necessary to remark that these PPE were conveniently stored indoors. In the case of the BCA *P. agglomerans* CPA-2, Soto-Muñoz *et al.* (2015) found that the persistence on working clothes was less than 7 days. It is known that *Bacillus* endospores are extremely persistent, being viable in soils for a long time (Nicholson *et al.*, 2000). Wood *et al.* (2015) showed that

Bacillus subtilis was found on glass, wood, concrete, and topsoil up to 56 days after inoculation. However, the viability was reduced when these materials (except topsoil) were exposed to UV radiation. In the case of the postharvest pathogen *Monilinia fructicola,* the conidia viability on inert surfaces was higher at cold storage temperatures (0 °C) than at 20 °C (Bernat *et al*., unpublished results). However, despite the high persistence of CPA-8, it was also found that the disinfection of postharvest plastic boxes with a 10 % of bleach solution just for one minute significantly reduced the viability of this microorganism.

In conclusion, this work revealed the good adaptation of the BCA CPA-8 to field and postharvest conditions for optimal management of *Monilinia* spp. Moreover, the ability of CPA-8 to spread from treated to non-treated trees was also demonstrated, thus allowing a better homogeneity of the treatment and colonisation of the habitat. This knowledge could be used in the near future to not only to improve the BCA-based products applied but also to design more detailed protocols for the control of disease (permitting better adjustment of the treatment doses and calendar applications). Finally, crucial information for BCA registration purposes has been obtained.

Acknowledgments

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Annex I

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