Universitat de Lleida

Elucidating the plant growth-promoting effects of three microorganisms on deciduous fruit tree plants using *in vitro* culture conditions

Daniel Cantabella Velázquez

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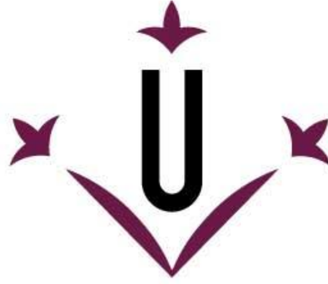

Ph.D. THESIS RESEARCH

Elucidating the plant growth-promoting effects of three microorganisms on deciduous fruit tree plants using *in vitro* culture conditions

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ABBREVIATIONS

ACC	1-Aminocyclopropane-1-Carboxylate
AMF	Arbuscular Mycorrhizal Fungi
ASC	Ascorbate
BA	6-Benzyladenine
BCAs	Biological Control Agents
CFU	Colony Forming Units
Chl *a*	Chlorophyll *a*
Chl *b*	Chlorophyll *b*
Chl t	Total Chlorophyll
CKs	Cytokinins
DHZ	Dihydrozeatine
EU	European Union
ET	Ethylene
FPP	Frozen Potato Peels and Pulp
FW	Fresh weight
GPX	Glutathione Peroxidases
GSH	Glutathione
GT	GreenTray®
IAA	Indole-3-Acetic Acid
IBA	Indole-3-Butyric Acid
iP	Isopentenyl Adenine
LLR	Length of Lateral Roots
LRD	Lateral Root Density
ME	Meat Extract
MOL	Sugar Cane Molasses

MP	Maize Protein
MS	Murashige & Skoog Medium
NAA	α-Naphthaleneacetic Acid
NLR	Number of Lateral Roots
NYDA	Nutrient Yeast Dextrose Agar
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PEP	Peptone
PGP01	Plant Growth-Promoting strain 01
PGP02	Plant Growth-Promoting strain 02
PGP03	Plant Growth-Promoting strain 03
PGPF	Plant Growth-Promoting Fungi
PGPM	Plant Growth-Promoting Microorganisms
PGPR	Plant Growth-Promoting Rhizobacteria
PP	Pea Protein
PRL	Primary Root Length
PS	PROSTAR 510A
REM	Rooting Elongation Medium
RIM	Rooting Induction Medium
SLs	Strigolactones
TDZ	Thidiazuron
TIS	Temporary Immersion System
TS	Tomato Seeds After Flotation
TSB	Triptone Soy Broth Medium
TRP	Tryptone
UHPLC-MS/MS	Ultra-High Performance Liquid Chromatography Tandem Mass Spectrometry

WB	Wheat Bran
WPM	Woody Plant Medium
WT	Wild-Type Genotype
YE	Yeast Extract

Summary/Resumen/Resum

Summary

In vitro tissue culture constitutes a very versatile technique to obtain large amounts of true-to-type and disease-free-plant materials. However, in some fruit tree crops, the poor *in vitro* rooting or acclimatization of some genotypes, or the high losses of plant material associated to endophytic contaminations may limit the effectiveness of the process. The use of microorganisms with plant-growth promoting ability might represent a sustainable alternative to overcome those limitations, knowing their ability to produce plant hormones or control pathogens growth. On the other hand, for the scale-up of the application to field conditions, it is of crucial importance to develop a product based on microorganism showing potential agronomical interest.

The three microorganisms *Pseudomonas oryzihabitans* PGP01, *Cladosporium ramotenellum* PGP02 and *Phoma* spp. PGP03 were isolated from *Prunus* and *Pyrus* contaminated *in vitro* cultures showing a greater growth than those non-contaminated. In seedlings obtained from *in vitro* nectarine rescued embryos (**Chapter 1**), *P. oryzihabitans* PGP01 promoted root development, favouring the acclimatization to greenhouse conditions. In *in vitro* micropropagated commercial rootstocks (**Chapter 2**), *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 increased the *in vitro* rooting percentage, from 56.3 to 100%, of the hard-to-root *Pyrus* rootstock Py12 explants treated with 3-indolebutyric acid. An effect of *P. oryzihabitans* PGP01 on root development of the *Prunus* rootstock RP-20 was observed. The *in vitro* ability of the three microorganisms to produce IAA supported these results. Using *Arabidopsis thaliana* defective mutants, the role of strigolactones (SLs) and glutathione (GSH) in the root events induced by *P. oryzihabitans* PGP01 was suggested (**Chapter 3**). In a liquid culture (**Chapter 4**), it was established a link between auxin levels in the medium and root development in the presence of *P. oryzihabitans* PGP01. Furthermore and regarding endophytes growth in the culture medium, the role of acidic pH to control their growth in RP-20 cultures was suggested in the presence of *C. ramotenellum* PGP02, being this assumption finally confirmed in **Chapter 5** in the absence of microorganisms. In this chapter, the micropropagation at low pH reduced endophytes population without affecting *in vitro* micropropagation. Finally, wastes based on potato, tomato and cereals industries were tested for the development of a cheap culture medium for *P. oryzihabitans* PGP01. The growth of this bacterium in a potato wastes-based medium provided a maximum of 4.4×10^9 CFU mL^{-1} without losing the plant growth-promoting activity (**Chapter 6**). The results obtained in the present thesis provide novel insights regarding the use of beneficial microorganisms as more sustainable alternatives to promote *in vitro* plant growth.

Resumen

El cultivo *in vitro* de tejidos vegetales es una técnica muy útil para obtener grandes cantidades de material genéticamente idéntico y libre de enfermedades. Sin embargo, esta técnica se ve limitada en algunas plantas frutales por la poca capacidad de enraizamiento y aclimatación de algunos genotipos, o por las pérdidas de material vegetal causadas por la presencia de contaminaciones endófitas. El uso de microorganismos que mejoran el crecimiento de plantas puede ser una alternativa muy interesante debido a su capacidad de producir hormonas vegetales o de controlar el crecimiento de patógenos. Es importante desarrollar productos basados en estos microorganismos para escalar su posible aplicación en campo.

Los tres microorganismos *Pseudomonas oryzihabitans* PGP01, *Cladosporium ramotenellum* PGP02 y *Phoma* spp. PGP03 se aislaron de cultivos *in vitro* contaminados de *Prunus* y *Pyrus* que mostraban un mayor crecimiento que los no contaminados. En plántulas obtenidas a partir de embriones de nectarina rescatados *in vitro* (**Capítulo 1**), *P. oryzihabitans* PGP01 indujo mayor desarrollo radicular que favoreció la aclimatación de las plantas en invernadero. En patrones comerciales micropropagados *in vitro* (**Capítulo 2**), *C. ramotenellum* PGP02 y *Phoma* spp. PGP03 mejoraron el porcentaje de enraizamiento *in vitro,* de un 56.3 a un 100%, de explantos tratados con IBA del portainjerto *Pyrus* Py12 difícil de enraizar. En este mismo capítulo, *P. oryzihabitans* PGP01 también promovió el desarrollo de las raíces del patrón de *Prunus* RP-20. Todos estos resultados podrían estar relacionados con la producción de IAA por parte de los tres microorganismos. Utilizando mutantes de *Arabidopsis thaliana,* se sugirió que los efectos en las raíces producidos por *P. oryzihabitans* PGP01 podrían estar mediados por estrigolactonas (SLs) y glutatión (GSH) (**Capítulo 3**). En cultivo con medio líquido (**Capítulo 4**), se observó que los efectos en la raíz producidos por *P. oryzihabitans* PGP01 podrían estar mediados por el contenido de auxinas en el medio de cultivo. En este mismo sistema de crecimiento en líquido, en presencia de *C. ramotenellum* PGP02, se sugirió el efecto de un pH bajo en el medio de cultivo sobre el crecimiento de microorganismos endófitos en plantas de RP-20. Esta hipótesis fue finalmente confirmada en el **Capítulo 5,** demostrando que un pH ácido en ausencia de microorganismos reducía la concentración de endófitos sin afectar la micropropagación *in vitro*. Finalmente, se probaron tres subproductos de la industria de la patata, tomate y cereales para elaborar un medio barato para la producción de *P. oryzihabitans* PGP01. El crecimiento de esta bacteria en un medio basado en subproductos de patata proporcionó un crecimiento máximo de $4{,}4 \times 10^9$ UFC mL^{-1} sin afectar la actividad biológica del mismo (**Capítulo 6**). Los resultados presentados en esta tesis proporcionan hallazgos muy novedosos acerca del uso de microoganismos beneficiosos como alternativas más sostenibles para promover el crecimiento de plantas *in vitro*.

Resum

El cultiu *in vitro* de teixits vegetals és una tècnica molt utilitzada per a l'obtenció d'importants quantitats de material genèticament idèntic i lliure de malalties. No obstant això, en alguns cultius de fruiters, aquesta tècnica es pot veure limitada per la baixa capacitat d'arrelament i aclimatació d'alguns genotips, així com per las grans pèrdues ocasionades per la presència de contaminacions endòfites. L'ús de microorganismes que milloren el creixement de plantes pot constituir una alternativa interessant gràcies a la seva capacitat de produir hormones vegetals i controlar el creixement de patògens. A més a més, també és important desenvolupar productes basats en aquests microorganismes per a l'escalat de la seva aplicació a camp.

En aquesta tesi, els tres microorganismes *Pseudomonas oryzihabitans* PGP01, *Cladosporium ramotenellum* PGP02 i *Phoma* spp. PGP03 es van aïllar de cultius *in vitro* contaminats de *Prunus* i *Pyrus* que mostraven un millor creixement que els no contaminats. En plantes produïdes a partir d'embrions de nectarina rescatats *in vitro* (**Capítol 1**), es va demostrar que *P. oryzihabitans* PGP01 augmentava el desenvolupament radicular que afavoria la seva aclimatació en hivernacle. En portaempelts comercials micropropagats *in vitro* (**Capítol 2**), *C. ramotenellum* PGP02 i *Phoma* spp. PGP03 milloraven el percentatge d'arrelament *in vitro*, d'un 56,3% a un 100%, en explants tractats amb IBA del portaempelt difícil d'arrelar Py12. En el mateix capítol, *P. oryzihabitans* PGP01 també promogué el desenvolupament de les arrels del porta-empelt de *Prunus* RP-20. Tots aquests resultats podrien estar relacionats amb la producció de IAA per part dels tres microorganismes. Mitjançant l'ús de mutants d'*Arabidopsis thaliana*, es va suggerir que les modificacions en les arrels induïdes per *P. oryzihabitans* PGP01 podrien estar mediades per les estrigolactones (SLs) i el glutatió (GSH) (**Capítol 3**). En un cultiu en medi líquid (**Capítol 4**), es va observar que els efectes en les arrels produïts per *P. oryzihabitans* PGP01 podrien estar mediats pel contingut d'auxines en el medi de cultiu. A més a més, utilitzant aquest mateix sistema, en presencia de *C. ramotenellum* PGP02, es va suggerir un efecte del baix pH en el medi de cultiu sobre el creixement d'endòfits en plantes de RP-20. Aquesta hipòtesi va quedar demostrada al **Capítol 5**, verificant que un pH àcid, en absència de microorganismes, reduïa la concentració d'endòfits sense afectar la multiplicació *in vitro.* Finalment, es van provar tres subproductes de la industria de la patata, tomàquet i cereals per a l'elaboració d'ún medi de cultiu barato per la producció de *P. oryzihabitans* PGP01. El creixement d'aquest bacteri en un medi basat en subproductes de patata es va obtenir un creixement màxim de 4.4×10^9 UFC mL^{-1} sense afectar la seva activitat biològica (**Capítol 6**). Els resultats obtinguts aporten noves aproximacions sobre l'ús de microorganismes beneficiosos com alternatives més sostenibles per a promoure el creixement de plantes *in vitro.*

Introduction

1. Plant growth-promoting microorganisms

As sessile organisms, plants living in soil are naturally exposed to the highly fluctuating environmental conditions. These conditions also include the changes in the nature of soil microorganisms. It is widely known that plants interacts with soil-borne microorganisms, establishing relations that can be classified as **beneficial or pathogenic** (Millet et al., 2010). In beneficial interactions, none of the two interacting organisms are damaged, whereas pathogenic relationships often end up with the death of the host (Kusari et al., 2012). In this thesis, particular attention to the beneficial interactions between plant and soil-borne microbes will be paid, leaving pathogenic relationships to one side. In the natural environment, most of beneficial interactions between plants and microbes take place in a narrow region of the soil called **rhizosphere**. This zone is highly influenced by the root system, and it has been discovered that it is much richer in microorganisms than the surrounding soil regions since plant roots secrete metabolites that can be used by microorganisms as nutrients (Chauhan et al., 2015; Lugtenberg & Kamilova, 2009; Vishwakarma et al., 2020). In fact, it is estimated that approximately 10^6-10^9 bacteria, and 10^5–10^6 fungi per gram of soil compete for the carbon metabolites derived from the roots in the rhizosphere (Chuberre et al., 2018). These interactions where the microorganisms are directly interrelating with the roots or even habit attached to them are called **rhizospheric interactions** (Vessey, 2003). On the other hand, other microbes display the ability to colonise plant tissues and live inside the host plants leading to **endophytic relationships** (Guerrero-Molina et al., 2012; Kusari et al., 2012). In both cases, plants could benefit these interactions by increasing their growth due to the improvement of the nutrient availability, the activation of plant defences or the suppression of the growth of pathogenic microorganisms (Calvo et al., 2014; Morales-Cedeño et al., 2021; Vishwakarma et al., 2020). These microorganisms with the ability to improve plant traits are generally classified within the cluster of **Plant Growth-Promoting Microorganisms** (PGPMs). In this group are included **Plant Growth-Promoting Rhizobacteria** (PGPR) and **Plant Growth-Promoting Fungi** (PGPF) (Figure 1). In this classification, **Arbuscular Mycorrhizal Fungi** (AMF) are also included due to their important role in alleviation of biotic and abiotic stresses (Evelin et al., 2009; Ważny et al., 2018).

Since the beginning of the 21st century, the increased use of pesticides in crop production due to the growth of population and the subsequent world food demand have raised a growing concern about their potential impact on human health (FAOSTAT, 2019). Linked to this challenge, the use of PGPMs could be presented as

a potential solution to modulate the harmful consequences derived from these chemical compounds as they can act as **biofertilizers** (Lugtenberg & Kamilova, 2009), **biological control agents** (Morales-Cedeño et al., 2021) or natural **phytostimulators** (Calvo et al., 2014). In addition, their role as alleviating agents of **abiotic stress** has also been reported (Alberton et al., 2020).

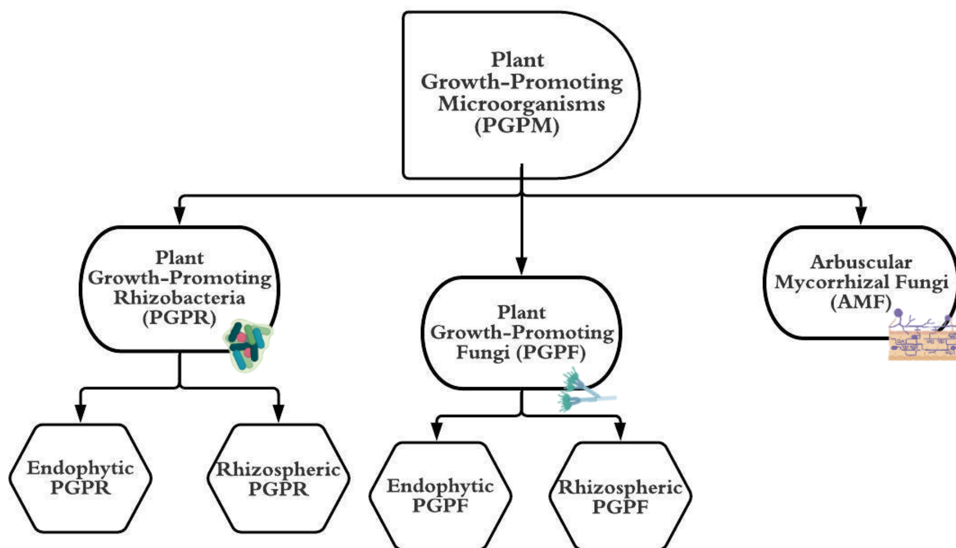

Figure 1. Scheme showing the classification of the different PGPMs. Created with LucidChart (https://www.lucidchart.com/pages/es) and BioRender (https://biorender.com/).

1.1. Plant growth-promoting rhizobacteria (PGPR)

Within the broad group of PGPMs, PGPR represents the one that has been studied in more depth by far. Generally, PGPR are considered as living-free bacteria that constitutes an indispensable part of the **rhizosphere** (2-5% of the rhizobacterial community), colonizing either the surface of the roots or even the intercellular spaces (Calvo et al., 2014; Kloepper & Schroth, 1978; Vessey, 2003). A huge amount of bacteria lives in the rhizosphere in close contact with root plants, most of them belonging to *Bacillus*, *Azospirillum* or *Pseudomonas* genus (López-Bucio et al., 2007a; Lugtenberg & Kamilova, 2009; Ortiz-Castro et al., 2020).

1.1.1. *Pseudomonas oryzihabitans*

In the present thesis, the bacterial strain used to conduct the experiments was *Pseudomonas oryzihabitans*, included in the *Pseudomonas* genus. Currently, around 144 species of Gram-negative bacteria belonging to this genera are known, making it the genus with the highest number of species (Gomila et al., 2015). *Pseudomonas* isolates have been frequently extracted from the roots, and different species belonging to this genus have been reported as the core of plant rhizobiome in many crops (Lucas García et al., 2003; Mirza et al., 2006; Qessaoui et al., 2019). In culture, *P. oryzihabitans* displays circular colonies ranging 0.5-1 mm that become shrivelled after 24-48 h (Gutiérrez-Bustos et al., 2009) (Figure 2A, B). As a curious fact, in medium supplemented with tyrosine, this bacterium is able to produce a melanin-like pigment that confers a dark shade to the colonies (Figure 2C).

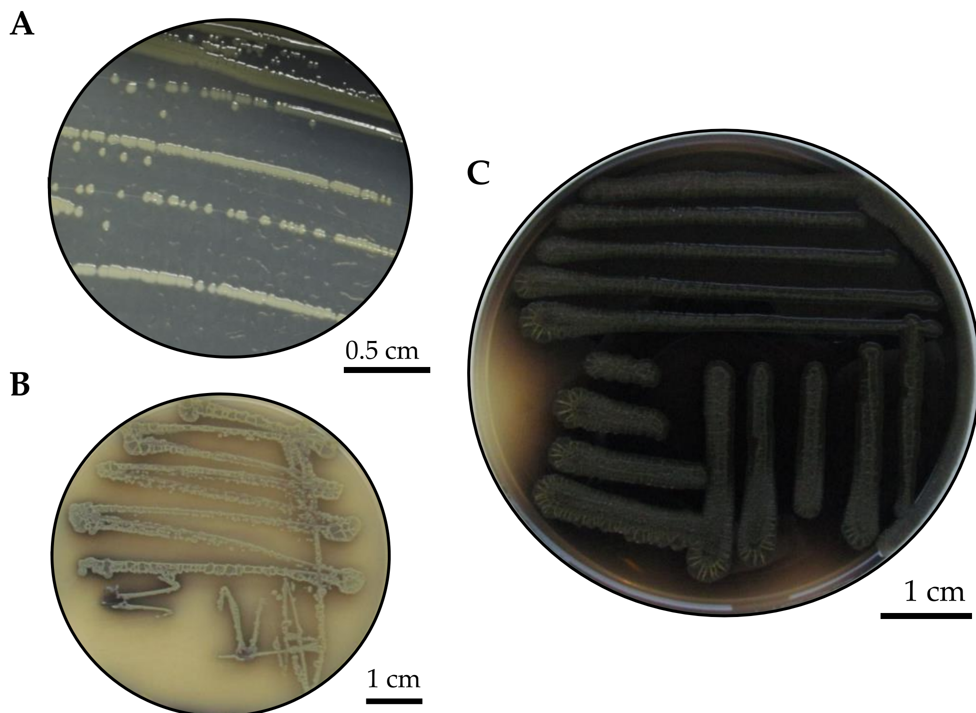

Figure 2. Shape and shade of *P. oryzihabitans* colonies during 24-48 h (A, B) and after 48 h (C) of *in vitro* growth on NYDA medium.

Concerning its role as PGPM, the very little amount of information available in literature about this bacterium makes difficult to get an overall idea of its role in plant-microbe interactions. In spite of that, several reports have demonstrated the

plant growth-promoting effect of this genus in different crops such as soybean or potato (Belimov et al., 2001, 2015; Kuzmicheva et al., 2017). Moreover, other studies reviewed by Gutiérrez-Bustos et al. (2009), have reported the implication of this microbe in the suppression of the growth of plant pathogens as well as different biotechnological processes such as vitamin C production or bioremediation. However, studies explaining the interaction of this bacterium with plants micropropagated *in vitro* have not been conducted thus far.

1.2. Plant growth-promoting fungi (PGPF)

Unlike the PGPR group, **plant growth-promoting fungi** (PGPF) constitutes a group of PGPM not so well explored. Nonetheless, in the recent years, the importance of PGPF in agriculture is considerably increasing as it has been demonstrated that they display similar mechanisms of plant growth-promotion than those described in PGPR (Hossain et al., 2017). Fungi included to this group are soil-borne filamentous fungi mainly belonging to *Trichoderma*, *Aspergillus* and *Penicillium* (Jahagirdar et al., 2019). However, this thesis project aims to increase the knowledge in this regard providing evidences about the potential role of two species belonging to ***Cladosporium*** (*Cladosporium ramotenellum*) and ***Phoma*** genus as PGPF.

1.2.1. *Cladosporium ramotenellum* and *Phoma* genus

The fungi used in this thesis project were *C. ramotenellum* and one species included in the *Phoma* genus (Figure 3A, B). *C. ramotenellum* belongs to *Cladosporium* genus, which in turn constitute one of the largest (more than 772 names) and heterogeneous genera of hyphomycetes widely dispersed in the environment (Bensch et al., 2012). Within this complex genus, numerous species are frequently isolated from the soil, so it is coherent to think that they can establish relations with plants. Especially for *C. ramotenellum*, it is known that it is included in the *Cladosporium herbarium* complex together with other species belonging to the same genus (Schubert et al., 2007). These fungal species have been reported as fruit pathogen in grapes (Manawasinghe et al., 2020; Swett et al., 2016) and pears (Muscat et al., 2020). As opposite, *C. ramotenellum* has been also found being part of the common microbiota present in leaf tissues of European Ash (Agan et al., 2020).

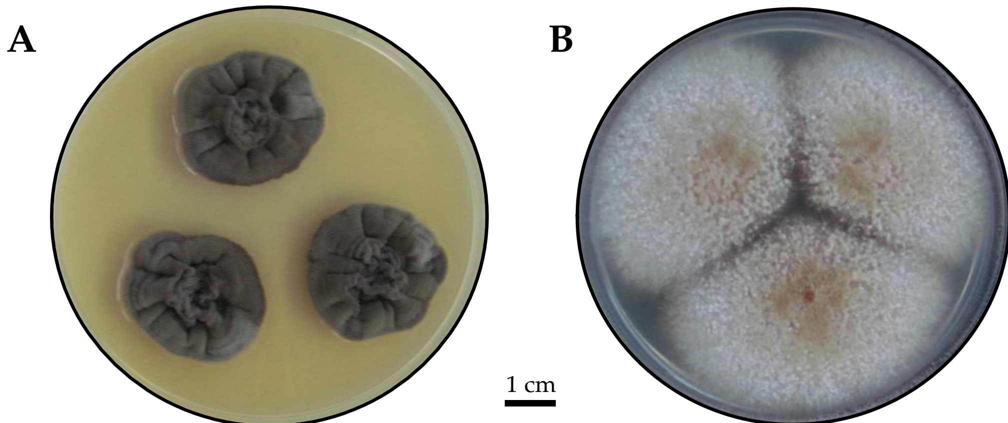

Figure 3. *C. ramotenellum* (A) and *Phoma* spp. (B) growing in PDA medium.

In general terms, species included in *Phoma* genus are classified as coelomycetous fungi, and as well as *Cladosporium* genus, they also present a great ecological diversity (Aveskamp et al., 2010). The majority of fungal species in this genus are included in the *Didymellaceae* family. However, it is considered a problematic genus, as taxonomists frequently have to deal with problems on the identification process due to unclear species boundaries (Aveskamp et al., 2010). Most of the species belonging to this genus have been found associated with land plants, establishing both pathogenic and beneficial relations depending on the *Phoma* species.

In both cases, several species from *Cladosporium* and *Phoma* genus with a plant growth-promoting ability have been described by several authors in rice (Hamayun et al., 2009a; Khan et al., 2014). Nevertheless, no studies using these two fungal species to promote plant growth of fruit tree plants have been previously published, and much less using *in vitro* tissue culture tools.

2. *Prunus* and *Pyrus* genus: Origin and agricultural importance

The *Rosaceae* represents large angiosperm family included in the Rosales order, containing about 3000 species, 3 subfamilies, and between 88-100 genera (Hummer & Janick, 2009). In this family, several plant genera producing fruits with important commercial and nutritional traits such as strawberries, apple, **pears, peaches** or almonds, are included (Verde et al., 2013; Xiang et al., 2017). Specifically, pears and peaches which belong to the *Pyrus* and *Prunus* genus, are two of the most important fruit producing plant genera in temperate regions (Wu et al., 2013; Xiang et al., 2017). Within *Prunus* genus, peach (*Prunus persica* (L.) Batsch) is one of the most

representative species, being widely cultivated for more than 4000 years (Faust & Timon, 1995). However, other plant varieties and species producing drupe fruits such as nectarine (*P. persica* cv nectarine), cherry (*Prunus avium*), plum (*Prunus domestica*) or almond (*Prunus dulcis*) are also included (Potter, 2012). *Pyrus* is also a very diverse genus including at least 22 pear species which can be divided into two groups: European and Asiatic pears (Wu et al., 2013). However, only *Pyrus communis*, *Pyrus bretschneideri*, *Pyrus pyrifolia, Pyrus ussuriensis* and *Pyrus sinkiangensis* are used as crops (Wu et al., 2013).

In fruit production terms, peaches and pears top the list of the most produced fruits all over the world (Byrne, 2012), being **Spain** one of the main producer countries with more than 1,000,000 and 300,000 tonnes of peach and pears, respectively (FAOSTAT, 2019). Within Spain, Catalonia ranked the first position in pear and nectarine production (129,062 and 173,870 tonnes, respectively), and the second in terms of the production of peaches (234,780 tonnes) (MAPA, 2019). In all cases, **Lleida** covers the largest amount of production in Catalonia (MAPA, 2019).

Due to the significant importance of these fruit crops, different agronomical and breeding strategies to ensure cultivar performance are increasingly being applied. These include the grafting of sensitive commercial varieties with **resistant rootstocks** to biotic and abiotic stresses, as well as the ***in vitro* embryo rescue** of early ripening varieties.

2.1. *Prunus* and *Pyrus* rootstocks: importance in agriculture

Grafting is an ancient technique used in agriculture for many woody perennial plant species for the production of high-value horticultural crops (Haroldsen et al., 2012). In this procedure, the **scion** (upper part of the plant) is combined with a **rootstock** (lower part of the plant) to improve their agronomic performance. However, the selection of an adequate rootstock through breeding programs is a complex process, and different criteria must be taken into consideration such as vigour (Zarrouk et al., 2005), compatibility with the selected cultivar (Felipe, 2009) or resistance to biotic and abiotic stresses (Colla et al., 2010; Nezami et al., 2014; Reig et al., 2020). Moreover, it is known that rootstocks also have an impact in fruit physical and chemical attributes (Radović et al., 2020; Reig et al., 2016; Shahkoomahally et al., 2020; Singh et al., 2020). For all these reasons, breeding programmes invest their efforts to the release of new resistant rootstocks to improve plant adaptation to soil as well as fruit quality.

Numerous rootstocks belonging to *Prunus* genus with outstanding agronomic properties have been released for commercial purpose. For instance, **GF-677** represents the most hybrid almond x peach (*Prunus amygdalus* x *P. persica*) hybrid rootstock used in the Mediterranean area, and its application relies on its tolerance to poor soil fertility, drought and lime-induced Fe chlorosis (Giorgi et al., 2005; Moreno, 2004; Nadal et al., 2013). Moreover, 'Adafuel' and 'Adarcias' are also almond x peach hybrid rootstocks released at the late 20th century, and they were selected because of their resistance to several pathogens including powdery mildew (*Sphaeroteca pannosa*) or shot hole disease (*Coryneum beijerinckii*) (Cambra, 1990; Moreno & Cambra, 1994). On the other hand, the hybrid rootstock plum x almond (*Prunus domestica* x *P. amygdalus*) 'Replantac', also known as **'Rootpac ®'** has been also shown as tolerant to Fe-lime induced chlorosis. This fact, along with its high productivity yield makes this cultivar ideal for replanting situations (Pinochet, 2010). In the present thesis, *in vitro* micropropagated shoots of the Rootpac® RP-20 has been used as plant material used in several chapters to test the effect of three rhizosphere microorganisms.

Regarding *Pyrus*, huge amounts of commercial rootstocks with agronomic interest are also available. In this sense, Lordan et al., (2017) performed a field trial using the three *Pyrus communis* rootstocks Old Home × Farmingdale **(OHF) 97**, **OHF87** and **Pyrodwarf** to increase the performance of 'Taylor's Gold Comice' and 'Concorde' pear cultivars. In both experiments, no differences among the three rootstocks were obtained in terms of production yield. Moreover, some other *Pyrus* rootstocks that have not been released yet also exist. This is the case of the two rootstocks used in the second chapter of this PhD project, **Py170** and **Py12**. While Py170 represents an hybrid between "Pyriam" OH11 and *Pyrus amygdaliformis* (Simard & Michelesi, 2002), Py12 derived from the open pollination of *P. communis* cv. Williams. Both of them are in their last phase of selection of an IRTA pear rootstock-breeding programme to gain tolerance to lime-induced chlorosis and reduced vigour (Dolcet-Sanjuan et al., 2008).

2.2. *In vitro* embryo rescue

In fruit tree crops, the early ripening trait is one of the main goals of many breeding programmes (Mancuso et al., 2002; Sundouri et al., 2014). For that purpose, interspecific hybridization between early ripening parents represent one of the main tools to ensure the transference of this valuable trait of species of commercial interest (Liu et al., 2007; Wu et al., 2021). However, seeds from many crosses suffer embryo

abortion and therefore, loss of germination (Uma et al., 2011). As a solution, the culture of these immature embryos in an aseptic environment could represent a valuable approach to promote a sustainable plant development. This methodology has been successfully implemented in kiwifruit (Wu, 2012; Wu et al., 2012), banana (Uma et al., 2011), citrus (Viloria et al., 2005; Xie et al., 2019), grape (Zhu et al., 2020), cherry (Wu et al., 2021) and peach (Liu et al., 2007; Sinclair & Byrne, 2003) breeding programmes, being this procedure different depending on the plant species. For instance, Uma et al. (2011) performed the *in vitro* culture of banana embryos through callus formation by adding 6-benzyl-adenine (BA) and indole-3-acetic acid (IAA) to the culture medium. In grape *in vitro* embryo cultures, the same hormonal combination was shown to be the best option to ensure embryo germination and plantlet development (Zhu et al., 2020). Moreover, Wu et al., (2021) studied the effect of a pre-treatment by low temperature on *in vitro* germination of cherry embryos.

In peach (*P. persica* L.) cultivars, the process seems to be slightly more complex as several factors such as embryo size or the culture recipient represent key factors to seriously consider when carrying out *in vitro* embryo rescue. While small embryos have a size ranging 5-10 mm, large embryos are >10 mm long (Figure 4A, B).

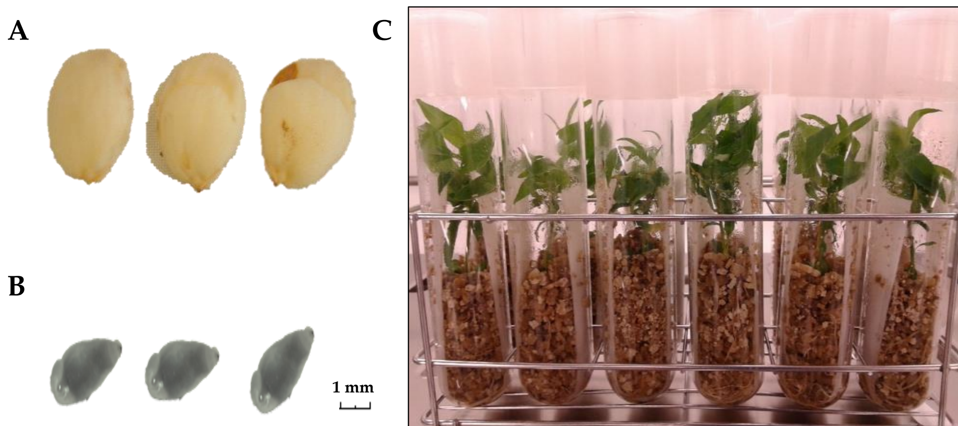

Figure 4. Large (A) and small (B) peach embryos, as well as subsequent plantlets derived from peach *in vitro* rescued embryos using vermiculite (C).

It is important to highlight that a cold pre-treatment under dark conditions called **stratification phase** is needed to break the dormancy of both small (5-10 mm long) and large (>10mm) embryos (Anderson & Byrne, 2002). The most extended procedure to culture *in vitro* peach is the introduction of embryos in glass tubes that contains the nutrients favouring germination after stratification (Anderson et al., 2006). The medium most commonly used for peach embryo rescue is the Woody Plant

Medium (WPM) (McCown & Lloyd, 1981). This medium is supplemented with gibberellins (GAs) and BA to promote the break of embryo dormancy of small embryos; however, plant regulators are not added in the WPM for the *in vitro* rescue of large embryos. Also, the addition of elements such as vermiculite (Figure 4C) to the culture medium it has been shown as a useful approach to favour peach embryo germination and acclimation to greenhouse and field conditions (Dolcet-Sanjuan et al., 2017).

3. *In vitro* tissue culture technique

In vitro tissue culture represents a worldwide implemented technique by which cells, tissues, organs or the whole plant are grown under aseptic conditions in a **controlled and enriched-nutrient environment** to produce clones of plants or new genetic variation (Hussain et al., 2012). This procedure is mainly based on the concept of the **totipotency** of plant cells whereby a single cell is able to express the whole genome by cell division (Thorpe, 2007). The *in vitro* culture of plant tissues has gone through different phases of evolution, being initiated as an instrument merely applied for research purposes to finally become an exploited technique in plant production industry and breeding programs (Akin-Idowu et al., 2009). Nowadays, it has become an indispensable approach in agriculture due to its great variety of applications including the introduction of homogenous free-disease plant material (Hu et al., 2015; Wang et al., 2018), the establishment of new stable genotypes by somaclonal variation (Wang & Wang, 2012), or the regeneration of plants using *in vitro* embryo cultures, or the generation of doubled haploid lines as source of completely homozygous parental lines, indispensable for the hybrid seed production industry.

3.1. *In vitro* culture steps for plant micropropagation

The first step of *in vitro* tissue culture involves the selection of the plant tissue from an *ex vitro* cultured mother plant (Hussain et al., 2012). The selected *in vitro* tissue receives the name of **explant**. Once selected, the explants are submitted to a surface sterilization process with chemical disinfectants before its introduction in the culture medium (Husain & Anis, 2009; Tilkat et al., 2009). After sterilization, the following steps of micropropagation are (1) **establishment** of shoot tip cultures, (2) **multiplication** of explants, (3) *in vitro* **rooting** and (4) **acclimatization** of the rooted plantlets as a final step of the process (Figure 5).

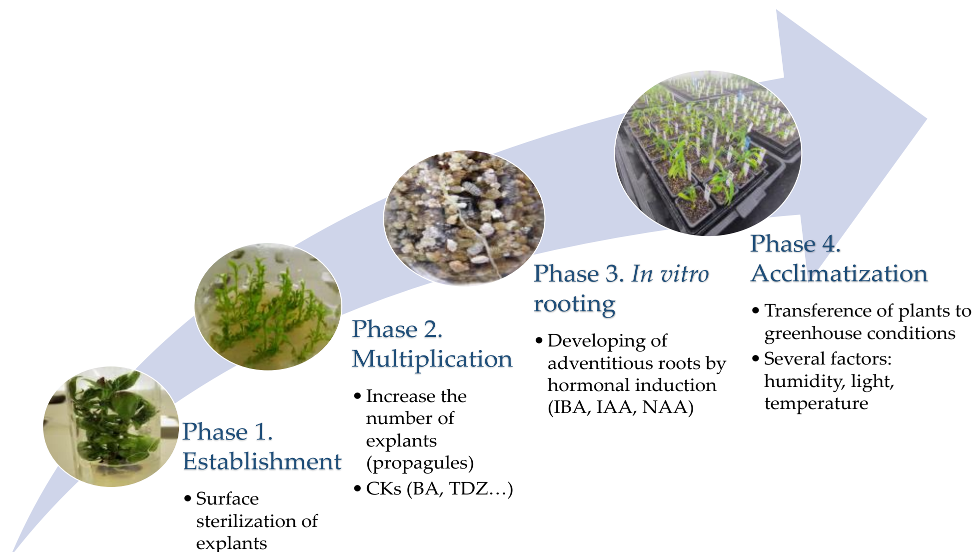

Figure 5. Plant *in vitro* tissue micropropagation stages.

3.1.1. Establishment of the cultures

The first step of the *in vitro* micropropagation involves the surface sterilization of explants (Hussain et al., 2012). Different procedures in order to obtain sterile plant material have been followed using chemicals disinfectants such as sodium hypochlorite (NaOCl), mercuric chloride ($HgCl_2$) or isothiocyanate compounds (Lazo-Javalera et al., 2016; Shekhawat et al., 2015; Singh & Gurung, 2009). After sterilization, sterilised explants are planted in the culture medium to induce multiplication, incubating the cultures to the required conditions depending on the plant species.

3.1.2. Multiplication of explants

The multiplication phase represents the second step of the *in vitro* manipulation of plant tissue cultures, and it is destined to increase the explant production. In this step, the number of propagules is progressively increased with the different subcultures in the medium supplemented with plant growth regulators, mainly cytokinins (CKs) (Saini & Jaiwal, 2002). From the different existing CKs, BA has been shown as the most commonly used CKs for a successful multiplication of most plants, including some as diverse as pistachio (Tilkat et al., 2009), bakain (Husain & Anis, 2009), *Stevia rebaudiana* (Sivaram & Mukundan, 2003) or *Clitoria ternatea* (Singh & Tiwari, 2010). However, others CKs including thidiazuron (TDZ), kinetin,

adenine or zeatin have been also employed for the multiplication of other plant species such as *Capsicum annum* (Peddaboina et al., 2006) or *Cassia angustifolia* (Siddique & Anis, 2007). In some cases, the multiplication and rooting in the same culture medium have been also studied by the combined addition of CKs and auxins (Siddique and Anis, 2007; Wiszniewska et al., 2016).

3.1.3. *In vitro* rooting

Of the different steps of *in vitro* micropropagation, the formation and development of **adventitious roots** represents the most challenging step by far in woody species (Arab et al., 2018; Quambusch et al., 2016). In order to develop an efficient rooting protocol, it is important to distinguish between two processes clearly differentiated: root induction and root elongation. Normally, root induction and elongation take place in different culture media, named as root induction medium (RIM) and root elongation medium (REM), respectively. For root induction, the supplementation of the Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) with **auxins** constitutes the most ancient procedure followed to initiate the development of roots in *in vitro* explants (Dolcet-Sanjuan & Claveria, 1995; James & Thurbon, 1979; Lane & McDougald, 1982). However, the effectiveness of this process is highly dependent on the plant genotype as well as the type and dose of auxin. Iglesias et al. (2004) proved that the use of the auxin **indole-3-butyric acid** (IBA) led to a higher number of shoots forming roots than with **α-naphthaleneacetic acid** (NAA). In the same study, different concentrations of IBA were tested, showing that 10 µM was the most successful dose of auxin to induce rooting. Furthermore, Dolcet-Sanjuan et al. (2004) reported a differential response of auxin type and concentration in terms of *in vitro* rooting in different walnut clones. Another factor that it is also important to consider is the composition of the culture medium. In this sense, the use of ½MS medium has resulted as a useful approach to induce an efficient *in vitro* rooting (Amiri & Elahinia, 2011). Generally, root elongation is performed in a MS-free auxin medium, and in some works is promoted with the addition of vermiculite as substrate to favour root development (Jay-Allemand et al., 1992; Kalinina & Brown, 2007).

In the last years, the European Commission has implemented some restrictions aimed at reducing the use of chemicals, including auxins, in plant production. For that reason, the pursuit of new alternatives to the use of synthetic growth regulators are urgently needed (Elmongy et al., 2018; Pacholczak et al., 2012). In this regard, the use of microorganisms with plant growth-promoting ability may

represent an interesting approach due to their potential role in producing plant hormones (Calvo et al., 2014).

3.1.4. Acclimatization

The use of *in vitro* plants with commercial purposes includes their successful adaptation to the field conditions. The transference of plants from *in vitro* to *ex vitro* conditions (**acclimatization or hardening**) is a crucial step that represents the beginning of the autotrophic life of plants (Dobránszki & Teixeira da Silva, 2010). In this process, i*n vitro* plantlets are transferred to a new substrate, and they have to cope with the stressful conditions of the environment (Hussain et al., 2012). It is well-known that different parameters including the **relative humidity, temperature and light** on the acclimatization environment could negatively affect the yield of the acclimatization process (Chandra et al., 2010; Hazarika, 2006; Maleki Asayesh et al., 2017). For that reason, plant hardening must be carried out in a gradual manner to ensure normal plant growth and development (Hussain et al., 2012).

3.2. Systems of *in vitro* culture

The conventional protocols for plant micropropagation normally include the use of small flasks with semi-solid medium that allows culturing a small amount of explants (Mehrotra et al., 2007). The high amount of culture material needed (gelling agents), as well as the intense manipulation of the cultures required, makes this procedure considerably **time-consuming** in a longer term. In order to solve this issue, the implementation of **bioreactors** for plant *in vitro* culture is presented as a valuable alternative to reduce the intensive labour of *in vitro* micropropagation process (Pereira-Lima et al., 2012). Moreover, using bioreactors, it has been also demonstrated that the micropropagation rate is highly improved (Alvard et al., 1993; Escalona et al., 1999). Since the early 90s', several types of bioreactors for plant micropropagation have been developed, including the temporary immersion system (TIS) bioreactors.

In TIS bioreactors, *in vitro* explants are temporarily immersed in the culture medium (Figure 6). For that, plants and medium are placed using two glass or plastic vessels, and their interaction is ensured by the transference of the culture medium to the flask containing the plant material driven by the positive pressure of an air pump (Pereira-Lima et al., 2012). In this system, the close interaction of the liquid medium with plant material, and the forced aeration of the recipient containing *in vitro*

explants promote nutrient assimilation and provides an adequate source of oxygen that results in an increase of the culture growth (Pereira-Lima et al., 2012). In addition, this system it has been implemented in some recalcitrant genotypes to avoid the problem of **hyperhydricity** of explants (Godoy et al., 2017; Lotfi et al., 2020). This fact together with the high rates of multiplication and the greater growth of cultures make the TIS bioreactor a useful procedure for plant micropropagation.

As a result of a work developed in the Plant *in Vitro* Culture Laboratory at IRTA Fruitcentre, a new TIS bioreactor, named GreenTray (GT) and patented by IRTA (ES 2 763 637 B1; WO 2020/109637 A1) (Dolcet-Sanjuan & Mendoza, 2018), was available for the present Thesis work (Figure 7).

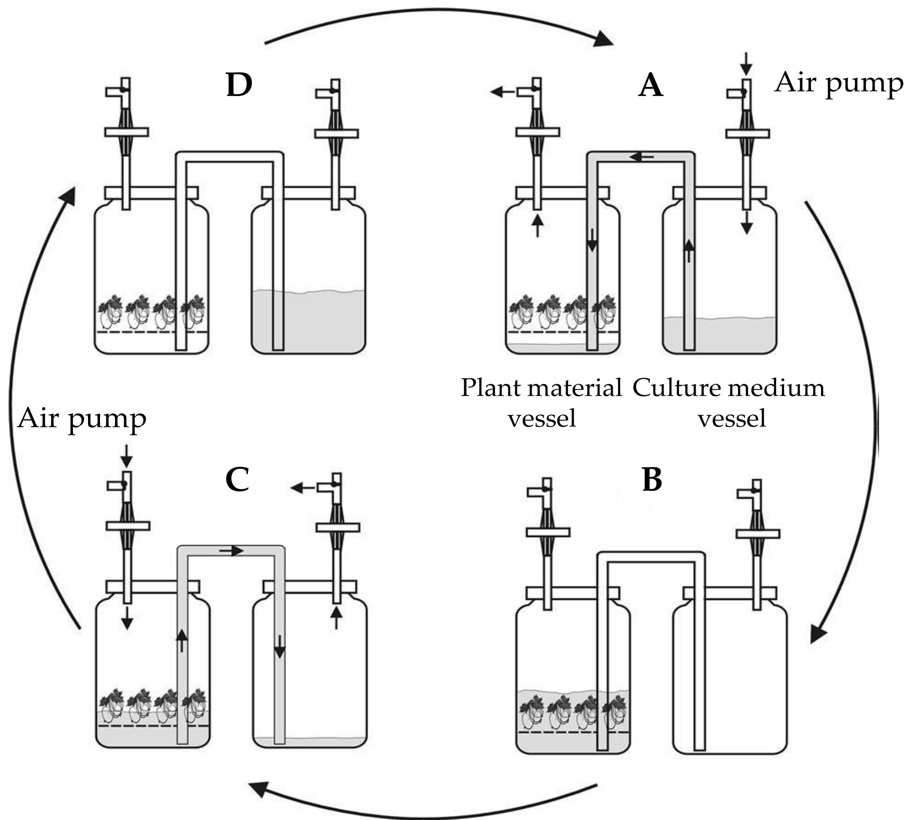

Figure 6. Functioning of a bioreactor with TIS system. Firstly, the culture medium is moved to the plant material vessel driven by the airflow imposed by the air pump **(A)**; Plant tissues and culture medium interact in the plant material vessel during a short period of time **(B)**; Culture medium is pushed back to its vessel by an air pump in the plant material vessel **(C)**; The air flux stops when the culture medium is again in its vessel **(D)** (adapted from Georgiev et al., 2014).

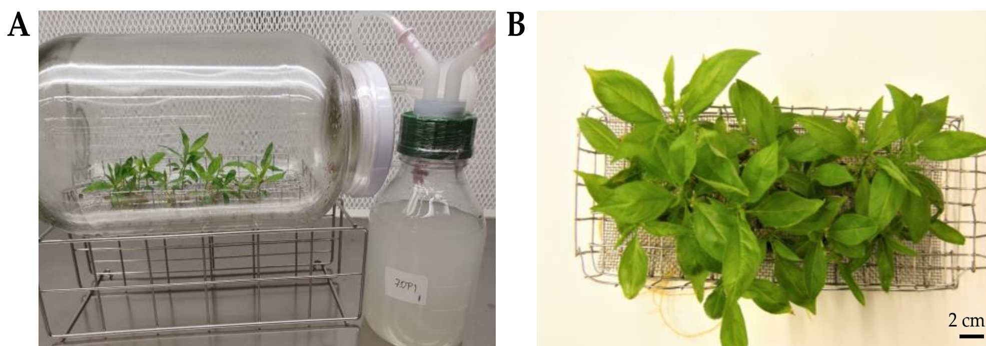

Figure 7. GreenTray® bioreactor containing *in vitro* micropropagated plants.

3.3. Endophytic contaminations in *in vitro* cultures

As previously mentioned, explants micropropagated *in vitro* develop in a culture medium under aseptic conditions, and the presence of microorganisms was attributed to contaminations due to an inappropriate explant manipulation. Nonetheless, the advances made through the last years in this regard have led to abandon this assumption as it has been proved that in spite of the surface sterilization treatment, *in vitro* cultures are not free of microorganisms (Orlikowska et al., 2017b). The internal part of plants are colonized by an important quantity of microbes, commonly known as **endophytes**. In *in vitro* cultures, the presence of this type of contaminations could be detected at the multiplication stage as they can be released to the culture medium or even grow at the basis of the explants (Figure 8). In all cases, identification of endophytes by colony shape is not an easy task due to the possible coexistence of difference species with similar morphological attributes. For that reason, sensitive procedures such as isolation by single pure culture, and subsequent sequencing of a region of the 16S rDNA are required for an adequate identification (Quambusch et al., 2014).

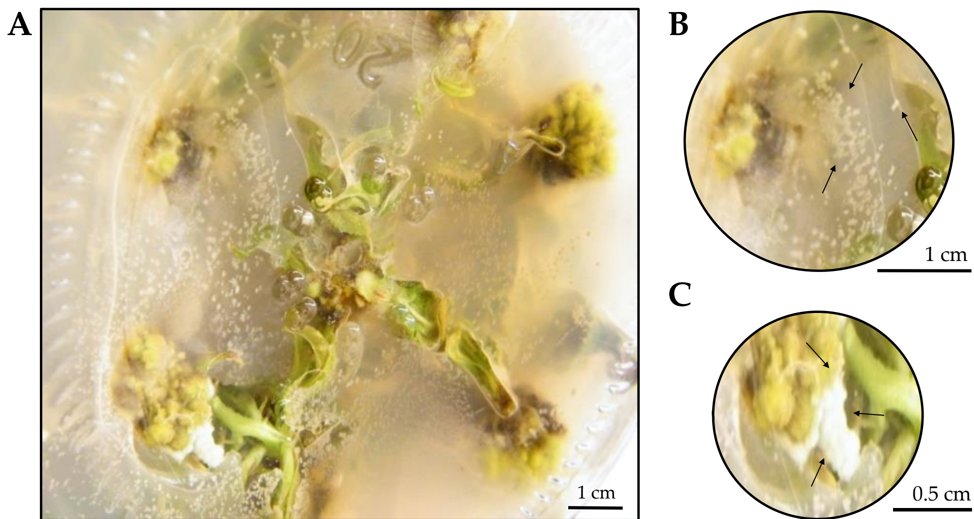

Figure 8. Endophytes growing on the surface of the culture medium (A, B) or in the shoot basis (C).

According to Petrini (1991) and Wilson (1995), endophytes are described as microorganisms with the ability of living within plants throughout the whole, or only a part of their life cycle without triggering disease symptoms. Most of them are considered bacteria belonging to *Methylobacterium* and *Curtobacterium* genus

(Panicker et al., 2007; Pohjanen et al., 2014). Following this definition, it seems logic to believe that these contaminations would not interfere on the *in vitro* explant performance. Nevertheless, the reality is that these microorganisms may affect *in vitro* cultures development. Whether this alteration resulted in a positive or negative effect remains being a subject of controversy. Several studies have reported the negative impact of endophytic contaminations on *in vitro* cultures during the last years (Dunaeva & Osledkin, 2015; Lotfi et al., 2020; Thomas, 2004, 2011). In addition, some endophytic contaminants lead to the loss of valuable research material due to they can overrun the cultures (Cassells, 2012). In those cases, endophytes elimination represents the highest priority to preserve plant material. In this sense, different strategies including the addition of antibiotics or other chemical compounds to the culture medium cover the greatest proportion of studies approached (Khan et al., 2018; Lotfi et al., 2020; Shehata et al., 2010). On the other hand, it has been demonstrated that some bacterial endophytes show beneficial effects in *in vitro* cultures improving multiplication and rooting of recalcitrant genotypes, as well as controlling the growth of other harmful microorganisms (Kavino & Manoranjitham, 2018; Quambusch et al., 2014; Zawadzka et al., 2014). Considering the above, the biological control of *in vitro* contaminants that negatively affect the *in vitro* explants performance using microbial isolates that improve plant growth process may represent an interesting approach to be targeted.

4. Use of PGPMs to improve *in vitro* micropropagation technique

As the years go by, the *in vitro* tissue culture techniques, especially plant micropropagation, has been consolidated as the most successful example of methodology that has been highly exploited with commercial purposes (Hussain et al., 2012). Nonetheless, this technique is somewhat constrained by the high economical losses associated with the presence of *in vitro* pathogens or by the inability to cope with the subsequent environmental conditions (Cassells, 2012; Williamson et al., 1998). For these reasons, the development of strategies that improve the *in vitro* plant establishment has become imperative in order to minimise the high costs linked to the large losses of plant material. In this context, the *in vitro* application of microorganisms with the ability to form beneficial relationships with plants may be presented as a viable opportunity to protect *in vitro* cultures while promoting their growth and development. The inoculation of *in vitro* cultures with beneficial microorganisms, including PGPR and AMF, have been the focus of several reviews (Orlikowska et al., 2017b; Rai, 2001) and book sections (Vestberg et al., 2002; Vestberg

& Cassells, 2009). However, the introduction of bacteria to increase plant *in vitro* tissue cultures efficiency has been implemented not only with commercial purposes to increase the yield of plant production, but also in research as a useful tool for plant breeding. In this case, the clearest example of the application of microorganisms to improve plant *in vitro* tissue cultures efficiency is the *Agrobacterium tumefaciens*-mediated transformation (Orlikowska et al., 2017b). This soil-borne bacterium has been considered as a universal vector for the introduction of foreign genetic information in plants with the aim to obtain transformed plants (Ceasar & Ignacimuthu, 2011; Jaiwal et al., 2001). Thanks to this procedure, many protocols for the *in vitro* regeneration of different plant crops including canola (Cardoza & Stewart, 2003), finger millet (Ceasar & Ignacimuthu, 2011), cowpea (Chaudhury et al., 2007), barley (Trifonova et al., 2001) or apricot (Petri et al., 2008) have been reported.

In relation to *in vitro* micropropagation, one of the first studies that reported the use of microorganisms to improve the effectiveness of this technique was conducted by Digat et al. (1987). In this study, it was demonstrated that *Pseudomonas fluorescens* and *Pseudomonas putida* existing in artificial substrates were able to attach *in vitro* plant roots, improving plant acclimatization. Since then, significant advances have been made in the development of *in vitro* tissue culture techniques which have considerably increased the knowledge about effects of microorganisms in *in vitro* tissue cultures. For instance, many authors have reported the use of beneficial microorganisms to improve the effectiveness of the **multiplication stage**. Some of these microorganisms are obtained from other cultures that act as source of microbe inoculants. For instance, Zawadzka et al. (2014) isolated three bacterial species (*Paenibacillus glucanolyticus*, *Curtobacterium pusillum* and *Methylobacterium extorquens*) from hosta or raspberry *in vitro* tissues. After isolation, these three microbial strains were proved for their ability to improve the multiplication rate of these two cultures as well as rose and gerbera. The authors concluded that while the inoculation with *C. pusillum* promoted an increase in axillary shoots in all four genotypes, the increase on the number of axillary shoots was genotype-dependent for the other two bacterial strains *M. extorquens* and *P. glucanolyticus*. In other cases, these microorganisms with a positive effect in multiplication are found as natural colonisers of microplants. In different *P. avium* genotypes, it was established a relation between the endophytic microbial strains colonising the explants and the micropropagation success (Quambusch et al., 2014). Furthermore, other *in vitro* processes such as seed germination and plant regeneration have also benefited by using microorganisms. In this sense, Costa Júnior et al. (2020) demonstrated that the incubation of *Allium*

sativum cv *Gigante roxo* meristems with *Enterobacter cloacae* and *Burkholderia cepacia* improved the growth and development of the subsequent plantlets in comparison to those plants which were not inoculated. Moreover, the presence of *Epichloë* spp. endophytes in *Bromus auleticus* seeds improved *in vitro* germination, callus induction as well as *in vitro* regeneration of plantlets (Regalado et al., 2018).

Nevertheless, the ***in vitro* adventitious root development** is undoubtedly the process on which many researchers have focused most of their efforts. *In vitro* rooting of explants is the most challenging process of *in vitro* micropropagation, and it is crucial to ensure plant survival (Wiszniewska et al., 2016). In this case, the application of beneficial microorganisms has been widely studied, and the large number of studies available in the scientific literature provide sufficient evidence to support the use of PGPM to improve the efficiency of this process. The introduction of microorganisms in *in vitro* culture to stimulate rooting have been successfully reported in many plant species including *Helleborus* (Orlikowska et al., 2017a), photinia (Larraburu et al., 2007) or cherry (Quambusch et al., 2016), among others. In these studies, the authors combined the use of hormones with the inoculation with microorganisms. However, in some studies, the use of PGPMs allowed to minimise the costs of the propagation process through the removal of some compounds from the culture medium. For example, in banana (*Musa* spp.) micropropagated plantlets, the incorporation of a combination of bacterial strains allowed the omission of minerals and salts from the growing media (Kavino & Manoranjitham, 2018). In this study, plants with higher number of roots per shoot were obtained. In another study carried out by Burygin et al. (2019) using a hormone-free medium, potato plants cultured in combination with a strain of *Ochrobactrum cytisi* displayed a higher number of roots than non-inoculated plantlets. In addition, Luziatelli et al. (2020) further explored this issue proving that the auxins produced by *Pantoea agglomerans* were able to induce an earlier *in vitro* root response than those growing on the medium with synthetic auxins.

For many microbes, the inoculation of explants with beneficial microorganisms is not always viable during the micropropagation process since different parameters including the inoculation density or temperature should be monitored (Orlikowska et al., 2017b). In those cases, it might be reasonable to apply them at the **acclimatization or hardening stage** to ensure the adaptation of *in vitro* plantlets to environmental conditions. It is known that biohardening of plants in the presence of beneficial microorganisms could activate mechanisms of systemic

resistance to help plants to cope with stressful conditions (Harish et al., 2008; Rajamanickam et al., 2018). In this sense, bacteria belonging to *Bacillus* and *Pseudomonas* genus have been studied for its ability to promote *ex vitro* hardening to a greater extent. In banana (*Musa* spp.) plantlets, the inoculation of micropropagated plantlets under field conditions with bacteria belonging to *Bacillus* genus led to a greater performance of plants in terms of plant growth and resistance to pathogens (Jaizme-Vega et al., 2004; Rajamanickam et al., 2018; Suada et al., 2015). Furthermore, other authors have reported the positive impact on *ex vitro* hardening of tea micropropagated plants in the presence of *Bacillus* as well as *Pseudomonas* spp. bacteria (Pandey et al., 2000; Thomas et al., 2010). These bacteria have also promoted *ex vitro* acclimatization in plant species from lesser-known genera as is the case of the medicinal plant *Picrorhiza kurrooa.* Trivedi & Pandey (2007) concluded that bacterial isolates from these two genera improved plant survival and growth while supressing the growth of pathogenic fungi. Moreover, although little mentioned, the role of AMF on the enhancement of the adaptation of *in vitro* plantlets to soil conditions it has also extensively reported (Vestberg et al., 2002). In this regard, the symbiotic relationship established in soil between *in vitro* plants and AMF improves acclimatization, observing an increase in plant growth and biochemical attributes in many plant species (Mathur et al., 2018; Yadav et al., 2013).

In the light of the above, many evidences have been provided in favour of the introduction of microorganisms in aseptic *in vitro* tissue cultures to improve the efficiency of plant propagation techniques. Specially, the use of numerous microorganisms including fungi and bacteria with a plant growth-promoting activity have represented an outstanding breakthrough in the field of *in vitro* micropropagation, leading to efficient plant production protocols that are used for commercial purposes. Although the different ways by which PGPM improve *in vitro* and *in vivo* plant performance have been briefly commented in this section, a more detailed review of the described mechanisms underlying these effects will be presented in the next section of this thesis report.

4.1. *In vitro* mechanisms of action of PGPM

In the previous section, the impact of the use of PGPMs in plant *in vitro* micropropagation was reviewed. However, due to the high opportunities that plant *in vitro* tissue culture offers, this technique has been widely used as a model to study the different pathways used by PGPMs to enhance plant growth and development.

In this sense, some of the most described **plant growth-promoting mechanisms** (Figure 9) are described as follows:

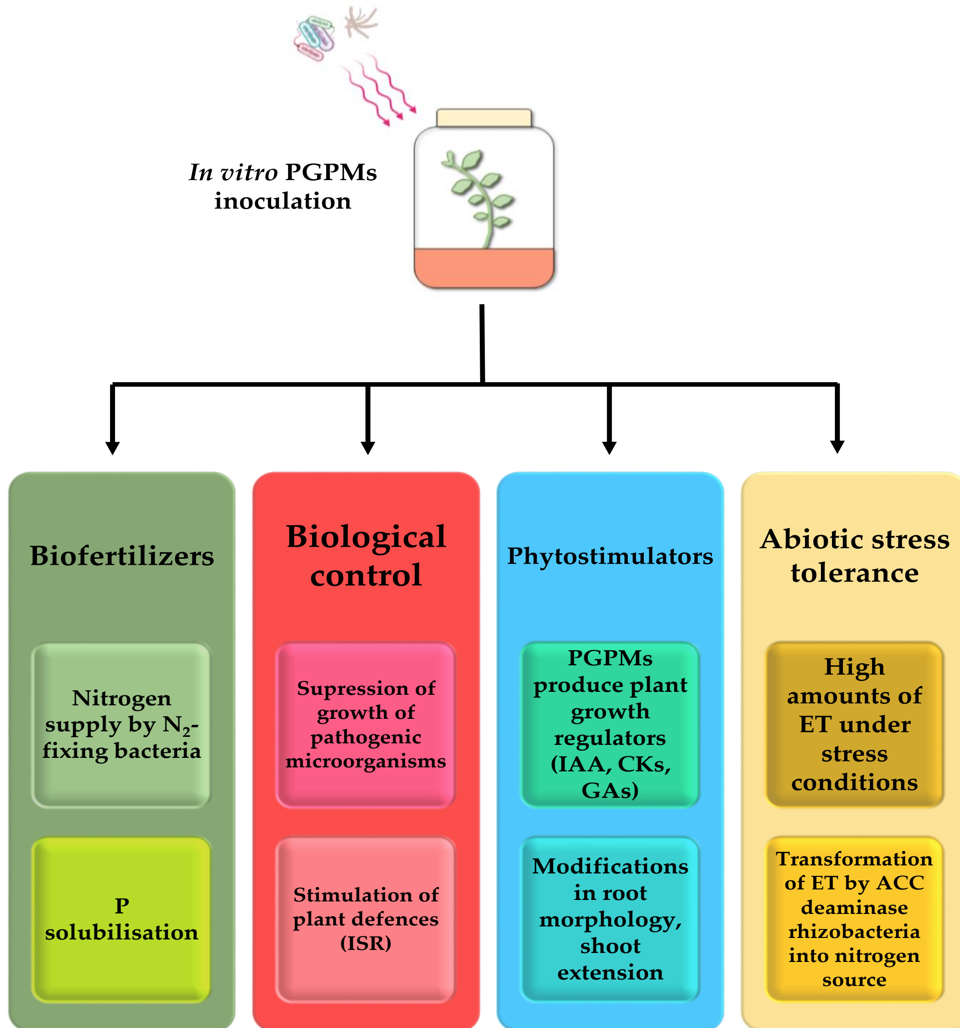

Figure 9. Described mechanisms used by PGPMs to stimulate plant growth in *in vitro* micropropagated plants.

a) At first, they can promote plant growth by supplying the plant with nutrients acting as **biofertilizers**. This is a very common mechanism observed in leguminous plants such as soybean, pea or peanut in response to the interaction with bacteria belonging to *Rhizobium* or *Bradyrhizobium* genus (Lugtenberg & Kamilova, 2009). Nevertheless, this ability has been also attributed to other bacterial genera (Scherling et al., 2009). For example, Sajjad Mirza et al. (2001) reported that the improvement in

plant growth observed in sugar cane *in vitro* plantlets after the inoculation with one strain of *Enterobacter* spp. was related to the ability of this bacterial strain to fix nitrogen. In a subsequent work, Oliveira et al. (2002) also isolated five endophytic bacterial species that contributed to nitrogen fixation in sugar cane micropropagated plantlets. In *Oryza sativa* L., it was observed that out of forty-two *Azospirillum* species studied, *Azospirillum amazonense* was able to promote plant growth by fixing nitrogen rather than using hormonal mechanisms as in the other *Azospirillum* species studied (Rodrigues et al., 2008). Moreover, many authors have reviewed that AMF also play a role in plant nutrition (Vejan et al., 2016; Vestberg & Cassells, 2009). Likewise, it has also been reported the ability of different microbial species to solubilise other nutrients such as phosphate, facilitating its assimilation for plant roots and promoting plant growth (Peix et al., 2003; Whitelaw et al., 1999).

b) On the other hand, PGPMs can also favour plant growth due to their potential role as **biocontrol agents** (BCAs) (Figure 9). It is well established that PGPMs, mainly belonging to *Bacillus* and *Pseudomonas* genus, are able to compete with other pathogenic microorganisms, supressing their growth (Morales-Cedeño et al., 2021). In *in vitro* tissue cultures, this role of PGPMs has been widely studied in the pathosystem composed by *in vitro* plants of banana and *Fusarium oxysporum*. Ayyadurai et al. (2006) concluded that the strain FP10 of *P. aeruginosa* increased plant growth while reducing the vascular discoloration caused by this fungus. In a more recent study, Kavino & Manoranjitham (2018) reported that the bacterization with strains from *Pseudomonas* and *Bacillus* genus resulted in a 78% disease reduction of the *Fusarium* wilt. In other *in vitro* plant-pathogen systems, some strains of *P. fluorescens* considerably reduced the *Verticillium dahliae* wilt incidence in *in vitro* rooted olive plantlets (Mercado-Blanco et al., 2004). Different mechanisms of biological control have been proposed, most of them related with the production of antimicrobial molecules or the stimulation of plant innate defences in the response called **induced systemic resistance** (ISR) (Morales-Cedeño et al., 2021). This response involves the activation of defence enzymes that confers plants resistance to pathogen attacks (Rajamanickam et al., 2018).

c) It is quite interesting to remark the role of some of these microorganisms as natural **phytostimulators**. In this sense, microbial inoculants are able to alter plant growth and development by the production of plant growth regulators such as **auxins, GAs or CKs** (Drogue et al., 2012; Vessey, 2003). The role of **IAA** in plant-microbe interactions has been studied in a greater extent than the other plant growth regulators (Calvo et al., 2014; Vessey, 2003). In *in vitro* plant-microbe interactions, it

has been described the ability of several bacterial species to produce auxins (Arkhipova et al., 2020; Burygin et al., 2019; Dias et al., 2009). This hormone is involved in many plant functions such as apical dominance or differentiation of vascular tissue; however, in plant-microbe interactions, special attention has been paid in its implication in root development events, and more specifically in modifications in root morphology (Calvo et al., 2014; Vessey, 2003). For instance, in *Arabidopsis thaliana* seedlings, studies have demonstrated that IAA-producing bacteria are able to induce root plasticity stimulating **lateral root development** (Contesto et al., 2010; Iqbal & Hasnain, 2013; Zamioudis et al., 2013). As a result, root morphological changes induced by the PGPM-produced IAA lead to an enhancement of nutrient uptake from the soil or root exudation (Masciarelli et al., 2013; Spaepen & Vanderleyden, 2011). Different mechanisms by which PGPMs produce auxins have been proposed. Some bacteria including *Azotobacter paspali* promote plant growth by the direct production of IAA (Lugtenberg & Kamilova, 2009), but, in other microbes, this auxin production is strictly dependent on the tryptophan present in root exudates (Lugtenberg & Kamilova, 2009; Spaepen & Vanderleyden, 2011).

On the other hand, several studies have demonstrated that other plant hormones including CKs or GAs are also produced by PGPMs; however, the lack of studies concerning the role of this hormones using *in vitro* tools make difficult to obtain an overall idea about the role of these hormones in *in vitro* plant-microbe interactions. CKs include a huge group of plant hormones with the ability to promote plant cell division and leaf expansion (Calvo et al., 2014). The ability of PGPMs to produce CKs to promote plant growth was confirmed by García de Salamone et al. (2001). Together with auxins, these hormones regulate root development promoting lateral root initiation (Aloni et al., 2006). Arkhipova et al., (2005) concluded that inoculation of lettuce plants with the CKs-producing *Bacillus subtilis* induced a 30% increase of root weight related to those non-inoculated. In addition, GAs are hormones mainly involved in the elongation of the stem (Vessey, 2003), and huge information about the production of these plant growth regulators by PGPMs is available in scientific literature (Hamayun et al., 2009b, 2010; Khan et al., 2009). Khan et al. (2014) reported that the inoculation of two GAs-deficient rice mutants with two fungal strains increased shoot length regarding non-inoculated plants.

d) Related to the above, it is well documented that these beneficial microorganisms have shown to be especially effective in the mitigation of the negative effects caused by **abiotic stresses** such as drought or salt stress (Arkhipova et al., 2020; Saravanakumar & Samiyappan, 2007). Under these conditions, PGPMs that contains

the 1-aminocyclopropane-1-carboxylate (ACC) deaminase can metabolise the high ethylene (ET) levels produced transforming it into ammonia, among others, facilitating the survival of plants (Belimov et al., 2015; Orozco-Mosqueda et al., 2020). Evidence has also been provided that PGPMs which possess ACC activity are able to make *in vitro* plants more tolerant to the presence of high concentrations of heavy metals (Ali et al., 2021).

5. Use of the model plant *Arabidopsis thaliana* in aseptic conditions for the study of plant-PGPM interactions

As plants and PGPMs living in their natural environment mainly interact in the rhizosphere, the studies on the impact of these microorganisms in roots are gaining considerable importance in research. Following this reasoning, the use of the model plant *A. thaliana* may be presented as a facilitating tool to increase the knowledge in the field of PGPMs because of the simplicity of its root system as well as the broad range of molecular tools developed for this plant species (Kellermeier et al., 2014; Shekhar et al., 2019). As in most plant species, the first structure that appears after germination of *A. thaliana* is the radicle, from which primary root starts to develop (Figure 10). This root system is commonly named as **allorhizic** (Shekhar et al., 2019). On the contrary, in homorhizic systems, post-embryonic secondary roots that develop adventitious roots, dominate root system architecture after germination (Shekhar et al., 2019) (Figure 10).

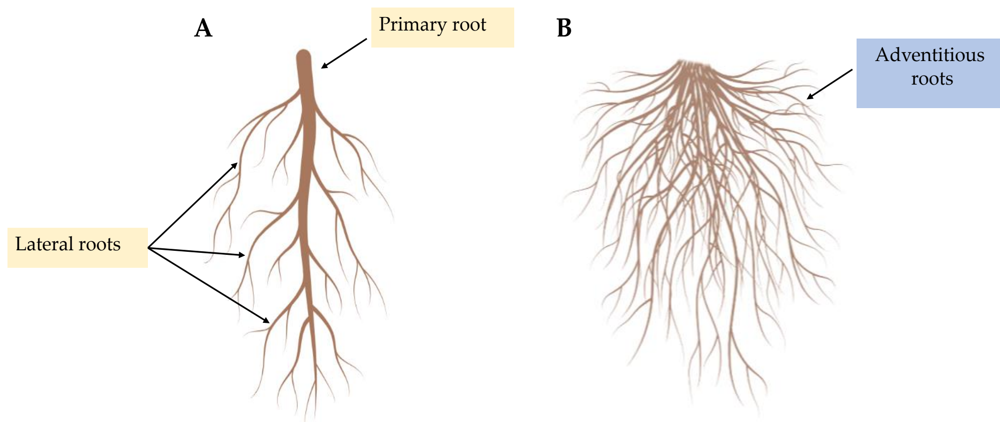

Figure 10. Structure of an allorhizic (A) and homorhizic (B) root systems. Created with BioRender (https://biorender.com/).

Thus, growing *A. thaliana* plants under aseptic conditions using MS medium in plates might be helpful to follow the evolution of the root architecture system in

the presence of PGPMs. By this system, Zamioudis et al. (2013) demonstrated that different strains of *Pseudomonas* spp. were able to promote plant growth as well as root plasticity. These authors stated that one of the bacterial strains belonging to the species *P. fluorescens* inhibited primary root development but stimulated **lateral roots and hairy root formation**. Contradictorily, Iqbal & Hasnain (2013) studied the effect of one strain *Aeromonas punctata* and they concluded that this bacterium increases primary root length as well as lateral root density.

In addition, by this methodology, valuable information regarding the mechanisms underlying the root modifications induced by PGPMs has been obtained. In this case, ***A. thaliana* mutants** lacking molecules or transcription factors involved in signalling processes are needed. Most of them place plant hormones such **auxins, ET and jasmonic acid** at the centre of the root development pathways. In a study performed by Contesto et al. (2010), the response of two mutants deficient in IAA transport and signalling with one strain of *Phyllobacterium brassicacearum* revealed that these two pathways are required for the response to this bacterium. Similar conclusions can be extracted from a very comprehensive study conducted by Zamioudis et al. (2013) in which it was demonstrated that some bacteria belonging to *Pseudomonas* spp. are able to trigger root morphology changes in *Arabidopsis* roots mediated by signalling pathways controlled by auxins, ET and JA. Recently, using auxins signalling deficient mutants, Ortiz-Castro et al. (2020) shed more light to the PGPMs-induced root morphological changes demonstrating that *P. fluorescens* and *P. putida* are able to promote Arabidopsis root development by the release of bioactive cyclodipeptides with auxin-like activity. However, other authors such as López-Bucio et al. (2007) reported that three auxins (*aux1-7*, *eir1* and *axr4*) and two ET (*etr1* and *ein2*) mutants showed normal growth and development in response to the inoculation with *Bacillus megaterium*, suggesting that this bacterium could use both auxin and ethylene-independent systems to enhance plant growth. On the other hand, *A. thaliana* seedlings cultured in germ-free MS medium also served as a model for the study of tolerance to abiotic stresses in the presence of microorganisms. In this regard, Chu et al. (2019) reported that *P. putida* was able to favour Arabidopsis plant survival under salt stress conditions.

On the other hand, many authors have also used this procedure to unravel the mechanisms involved in root architecture development in the absence of PGPMs. For instance, Passaia et al. (2014) revealed the importance of the redox control mediated by glutathione peroxidases (GPX) in the hormonal-mediated control of

lateral root development. In the same year, this fact was corroborated by Márquez-García et al. (2014). However, these authors went one step further, and they established a link between **strigolactones** (SLs) and the reduced glutathione (GSH) homeostasis. In the last years, SLs are becoming increasingly important hormones in the plant sciences field as it is known that they interact with auxins for the control of branching (Gomez-Roldan et al., 2008). Furthermore, these hormones are also involved in responses to environmental stimuli. However, very little is available concerning the role of SLs in root architecture pathways in the presence of PGPMs.

6. From *in vitro* to field conditions: Developing a microbe-based product

In the context of this Ph.D. project, once it has been proved that the biological activity of the tested microorganisms showed a significant impact under laboratory conditions, the development of a product based on these microorganisms of interest for commercial purposes should start to be considered. To ensure the success of a microbe-based product, the following steps must be followed: (1) the **production** of large amounts of microbial population in an economic medium; (2) the maintenance of the viability of the microbial product through **formulation**, and (3) their appropriate **packaging** to maximize their shelf life as much as possible (Teixidó et al., 2020) (Figure 11).

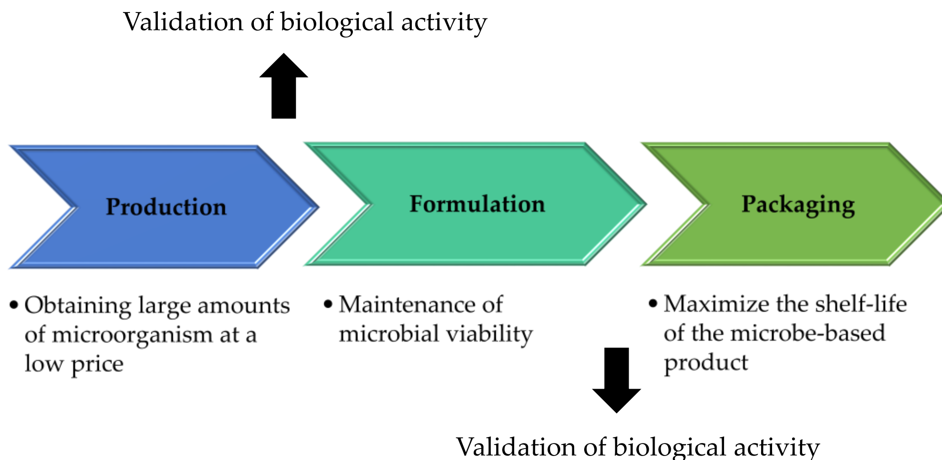

Figure 11. Flux diagram briefly explaining the development of a microbial-based product.

Focusing primarily on the first step, production of a microorganism with potential applications in industry is an important step in the development of the product. In this step, obtaining large amounts of microbial population developing an economic culture medium that ensures the maintenance of its biological activity are

the main requirements when developing a mass production protocol (Droby et al., 2016). In this sense, the re-utilization of agricultural food wastes or industry by-products may constitute a potential alternative to help to achieve this objective. Nowadays, it is known that an approximate amount of **88 millions of tonnes of food wastes** are generated within the European Union (EU) in a year, with associated economic cost estimated at 143 billion of euros (Stenmarck et al., 2016). To reduce these high economic losses, the EU has promoted different programmes to enhance the revalorization of agro-food wastes in other production processes (Mirabella et al., 2014). For example, by-products originated during **potato, tomato, cereals and olive** harvesting and processing have resulted in a great interest, as they are products highly consumed in the European market. It is known that the wastes generated in these crops industries are rich in different bioactive compounds such as starch and lignocellulose compounds that could be potentially used as valuable ingredients for other purposes (Fritsch et al., 2017). This fact, in turn, may open new horizons in the field of the development of microbe-based products, as these compounds might be included in the culture medium to satisfy the basic requirements for cell survival and maintenance. Previously, the use of by-products coming from other industries have resulted effective for the production of microorganisms (Costa et al., 2001; Yánez-Mendizábal et al., 2012). However, the number of studies reporting the use of agro-industrial wastes coming from potato, cereals or tomato are scarce in literature, giving an added value to the study in this thesis project.

On the other hand, the low-cost medium must satisfy the microbial nutritional requirements to ensure their **scale-up production**. Scaling-up of the production of a microbe production represents a difficult process, and it is known that its efficiency is highly influenced by different factors including temperature, pH, aeration and agitation (Churchill, 1982; Stanbury et al., 2017). In this step, the fermentation system is also important as many fungi could present problems of production when growing in tank bioreactors. For that reason, bacteria and yeasts are produced in liquid-phase cultures, while solid-phase cultures are usually reserved for fungi production (Teixidó et al., 2011). Liquid-phase cultures for bacterial production normally take place in stirred tank bioreactors. However, substrates such as peat or vermiculite are used for conidial production in solid streams (Larena et al., 2004; Teixidó et al., 2011).

As conclusion, the development of a microbe-based product starts with the optimization of the medium components in flasks conditions, and it follows with the

optimization of the growth conditions (temperature, pH, aeration and agitation) in solid or liquid bioreactor systems. As a final step, the validation of the biological efficacy of the final product must be measured to corroborate the feasibility of the low cost medium (Teixidó et al., 2011) (Figure 12). Following this procedure, optimized production protocols for different microorganisms with a biological control activity including *P. agglomerans* CPA-2 (Costa et al., 2001), *B. subtilis* CPA-8 (Yánez-Mendizábal et al., 2012) as bacteria, *Candida sake* CPA-1 (Abadias et al., 2003) or *Rhodotorula minuta* (Patiño-Vera et al., 2005) as yeasts and *Penicillium frequentans* 909 (De Cal et al., 2002), *Epicoccum nigrum* (Larena et al., 2004) and *Ampelomyces quisqualis* CPA-9 (Carbó et al., 2020) as fungi have been described. However, very little information regarding mass production protocols for PGPMs is available in literature, being this study one of the first reports in this field according to our knowledge.

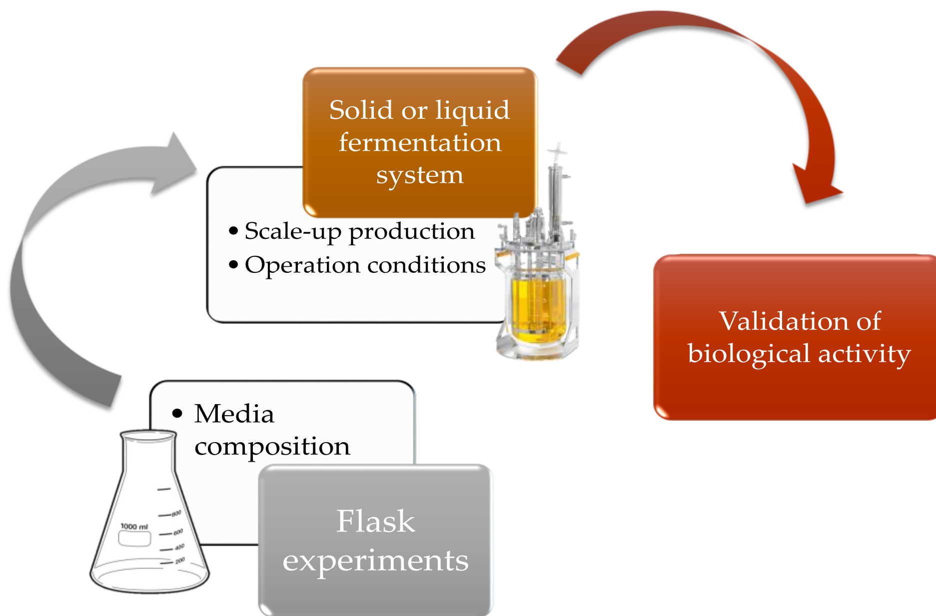

Figure 12. Steps for the mass production protocol of a microbe-based product.

7. References

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Objectives

Initial experiments conducted within the framework of the present thesis arose from the isolation of three microorganisms that appeared as contaminants of fruit tree *in vitro* cultures, and which presumably promoted *in vitro* plant growth when compared with non-contaminated cultures. The three isolates corresponded to the bacterium *Pseudomonas oryzihabitans* PGP01 and the two fungi *Cladosporium ramotenellum* PGP02 and *Phoma* spp. PGP03. Reports about their role as PGPMs for fruit trees cultured *in vitro* are very scarce in the literature. For these reasons, the general objective of this thesis project was first to **characterize the effects of these three microorganisms on *in vitro* plant growth and development of fruit trees belonging to *Prunus* and *Pyrus* genus, and second to unravel of the physiological pathways associated with such growth promotion**. To achieve these goals, the following specific objectives were proposed:

1. **To describe the effects of *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 on *in vitro* cultured plants**

1.1. To evaluate their impact on germination, subsequent *in vitro* plantlet development and adaptation to greenhouse conditions of *in vitro* rescued embryos of three independent crosses of early ripening nectarine (*P. persica* cv Nectarine).

1.2. To determine their effects on *in vitro* root induction and development, and *in vitro* plantlet growth of three rootstocks, the commercial Rootpac® (RP-20), and the selections Py170 and Py12, belonging to *Prunus* and *Pyrus* genus, respectively.

1.3. To study the effects of *P. oryzihabitans* PGP01 on the root architecture of the model plant *A. thaliana.*

1.4. To elucidate the effects of *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 in plant growth and development of *Prunus* RP-20 plantlets, cultured in liquid medium using a temporary immersion system (TIS) bioreactor.

2. **To identify the mechanisms underlying the growth promotion caused by these microorganisms**

2.1. To determine the ability of the three microorganisms to produce auxins in *in vitro* cultures.

2.2. To evaluate the role of plant hormones including strigolactones (SLs) and antioxidant molecules such as ascorbate (ASC) and glutathione (GSH) in the interaction of *P. oryzihabitans* PGP01 with *A. thaliana* defective mutants.

2.3. To determine the differential phytohormonal composition of the culture media after co-culture of RP-20 plantlets with *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02, in TIS bioreactors.

2.4. To control the growth of endophytic contaminants in RP-20 plantlets cultured in TIS bioreactors in the presence or absence of *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02.

3. **To develop an economic mass production protocol for the bacterium *P. oryzihabitans* PGP01 by the revalorisation of agro-food industries by-products**

3.1. To define the optimal culture composition and conditions in flasks experiments (temperature, by-product, nitrogen source).

3.2. To scale-up the bacterial production in 2 L bioreactors, optimising the medium composition and process conditions.

3.3. To validate the maintenance of the biological activity of *P. oryzihabitans* PGP01 on *in vitro* cultured plantlets, when growing in the optimized medium.

Thesis structure and methodology

To meet all the aforementioned objectives, a multidisciplinary approach was carried out in the present thesis, combining methodologies from the field of plant *in vitro* culture and microbiological techniques to enrich knowledge of *in vitro* plant-microbe interactions. All the results are collected in six chapters, grouped in 4 main points: microorganism's isolation, effects on *in vitro* plant growth and development, mechanisms of action and the production of *P. oryzihabitans* PGP01 (Figure 1). Each of these chapters have resulted in the release of a scientific article.

1. Microbes isolation and description of the effects of microorganisms in *in vitro* plant growth and development

This thesis project was initiated due to the presence of microbial contaminants detected in *Prunus* and *Pyrus in vitro* cultures. Those contaminations claimed our attention since they promoted *in vitro* plant growth compared to those non-contaminated. The identification of these microbial contaminants revealed that three microorganisms were involved in this plant growth promotion, classified as *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03. After identification, the effects of these microorganisms in *in vitro* plant growth promotion were tested using several plant materials:

- ***In vitro* rescued embryos (Chapter 1)**

First, a preliminary inoculation of the three microorganisms was carried out in pear *in vitro* fully developed plantlets obtained by *in vitro* embryo rescue (Figure 2). Then, the three microorganisms were applied to nectarine (*P. persica* L cv. Nectarine) *in vitro* rescued embryos from three independent crosses, harvested in different years. Nectarine embryos from the first cross were inoculated with *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp PGP03. However, the second and third crosses were only inoculated with *P. oryzihabitans* PGP01 (Figure 2). In all cases, the response in terms of germination, growth and adaption of subsequent plantlets to environmental conditions was evaluated. Then, culture conditions of developing embryos were submitted to different stages, modifying temperature and light conditions. During the *in vitro* embryo rescue process, the evolution of the three microorganisms, along with the percentage of germinated embryos, was evaluated at the different stages. The effects of the co-culture of embryos and microorganisms on *in vitro* plantlet development were determined before acclimation to greenhouse conditions by measuring parameters such as plant fresh weight (FW), number of leaves, root volume, root length, stem length and the quantum yield (QY). Finally, the evaluation of the impact of the three microorganisms in the acclimatization process was carried out by calculating the percentage of living plants (Survival rate), as well as the growth after 4 weeks on acclimatization tunnels.

- ***In vitro* micropropagated plantlets (Chapter 2)**

In this case, commercial rootstocks belonging to *Prunus* (RP-20) and *Pyrus* (advanced rootstock selections Py170 and Py12) genus were employed to study the effects of the three microorganisms on *in vitro* rooting and subsequent plant development. In *in vitro* woody plant cultures, this process represents the main obstacle to ensure plant survival (Wiszniewska et al., 2016). For that, two independent procedures were followed to study whether the three microorganisms were able to improve the *in vitro* rooting percentage of two easy-to-root rootstocks (RP-20 and Py170) and one hard-to-root rootstock (Py12) (Figure 3A). In the first procedure, the coexistence of the three microorganisms and rootstocks took place after root induction of explants with 10 μM indole-3-butyric acid (IBA) **(Method 1)**. In contrast, in the **Method 2,** induction with IBA was not performed to study if these microorganisms were able to improve the *in vitro* rooting of explants. In addition, two different inoculation procedures were also followed. In the Method 1, *in vitro* explants were directly inoculated with 1 mL of microbial suspension. However, in the Method 2, explants were dipped in 1 ml of microbial suspension during 5 minutes. In both cases, *in vitro* co-culture of plants and microbes was carried out adding vermiculite to the culture medium. The rooting percentage was calculated in the two methods after 8 weeks of co-culture, and the impact on growth and development was also evaluated by measuring biometrical parameters as in Chapter 1.

On the other hand, *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 were also evaluated for their ability to stimulate *in vitro* plant growth of RP-20 and Py170 fully developed plantlets (Figure 3B). In this case, plants were root induced with IBA and then transferred to a medium without IBA to induce root development. Then, as in *in vitro* rooting experiments, the co-culture with microorganisms took place in a culture medium with vermiculite, being analysed the effects of the inoculation after 8 weeks of culture.

- ***A. thaliana* Wild-Type Col-0 genotype (Chapter 3)**

To corroborate the observed effects of *P. oryzihabitans* PGP01, the root phenotype of the Wild-Type (WT) genotype Col-0 of *A. thaliana* was analysed in the absence/presence of this bacterium (Figure 4). For that purpose, seeds were germinated in MS medium under sterile conditions. After germination and growth, Col-0 seedlings were treated with *P. oryzihabitans* PGP01, and the evaluation of the effects in roots was carried out by measuring the primary root length (PRL), number of lateral roots (NLR), length of lateral roots (LLR) and the lateral root density (LRD) with ImageJ version 1.52p (National Institutes of Health).

2. Mechanisms underlying plant growth promotion

Within this objective, the main goals approached were the elucidation of plant hormones, antioxidant molecules and the ability to control the growth of *in vitro* endophytic contaminants

- **Hormones and redox processes (Chapters 2, 3 and 4)**

To unravel the role of plant hormones and the antioxidant system in root development in the presence of *P. oryzihabitans* PGP01, *A. thaliana* mutants which were defective on SLs signalling or synthesis (*max2-3*, *max3-9* and *max4-1*), ASC (*vtc2-1* and *vtc2-2*) and GSH (*cad2-1*, *pad2-1* and *rax1-1*) were employed (Figure 4). In addition, other mutants involved in redox responses such as *RRTF* and *Ov32* were also tested. All the mutants were germinated in MS medium under sterile conditions. The inoculation with *P. oryzihabitans* PGP01 and the evaluation of the root phenotype was carried out following the same procedure as with Col-0. To complement these experiments, a RNA sequencing analysis was performed in *A. thaliana* Col-0 roots inoculated and non-inoculated with *P. oryzihabitans* PGP01 **(Chapter 3)**.

In addition, the ability of *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 to produce IAA was tested in microbial cultures by spectrophotometry (**Chapter 2**). In this experiment, supernatants of microbial cultures supplemented with tryptophan were mixed with the Salkowski reagent, and the IAA concentration was determined by measuring the absorbance at 530 nm of the coloured product obtained in this reaction (Figure 5A). Also, changes in the CKs isopentenyl adenine (iP), dehydrozeatine (DHZ), IAA and abscisic acid (ABA) were analysed in the culture medium of GreenTray® bioreactors (**Chapter 4**) in the presence of *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 as well as in the presence of *P. oryzihabitans* PGP01 at pH 5.7 and 7 (Figure 5B). For these analysis, an ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) was used.

- **Use of GreenTray® bioreactors to study the ability to control the growth of endophytic contaminations and hormonal changes in the culture medium (Chapter 4)**

The interaction between *Prunus* RP-20 *in vitro* plantlets and the two microorganisms *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 was also evaluated in the GreenTray® bioreactor. First, this system was tested for its feasibility to improve RP-20 *in vitro* micropropagation in a preliminary experiment (Figure 6). After 6 weeks of culture in this system, the shoot multiplication evaluation was conducted measuring the **multiplication rate** (Number of shoots per initial explant), **shoot length and shoot FW**, and compared to that obtained when culturing RP-20

explants in MS medium in agar flasks with agar (Figure 6). The multiples advantages that GreenTray® bioreactor offers in *in vitro* culture systems were used to study the role of *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 as biological control agents of *in vitro* endophytic contaminations and the hormonal changes in the culture medium (Figure 7). In this case, RP-20 shoots treated with 10 µM of IBA to induce rooting were cultured in the GreenTray® bioreactor. The response to the inoculation with *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 was evaluated in **two independent trials**. In a first trial, both *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 were inoculated at $1x10^3$ CFU mL^{-1} and $1x10^5$ sp mL^{-1}, respectively. The co-culture process took place at pH 5.7, which is the common pH to grow *in vitro* plants. In the second trial, GT bioreactors were only inoculated with *P. oryzihabitans* PGP01 at $1x10^6$ CFU mL^{-1}. In this case, the pH of the MS medium was set at 5.7 and 7, being the latter more favourable for bacterial growth. In all trials, population dynamics of *P. oryzihabitans* PGP01 and/or *C. ramotenellum* PGP02, together with the dynamics of endophytic population were evaluated in GreenTray® bioreactor taking samples of the culture medium at 0, 1, 2, 5, 7, 9, 12 and 15 days post-inoculation to evaluate whether these microorganisms are able to control or supress endophytes growth.

- **Effect of the pH in the control of endophytic contaminations in RP-20 explants cultured in GreenTray® bioreactors in the absence of microorganisms (Chapter 5)**

In this experiment, the main objective was to study if changes in the pH of the culture medium control endophytes growth in *Prunus* RP-20 explants in GreenTray® bioreactors without affecting micropropagation (Figure 7). For that purpose, the pH of the Murashige and Skoog (MS) medium used for *in vitro* micropropagation was adjusted to 3 and 7 using different proportions of a buffer solution based on 0.1 M citric acid and 0.2 M Na_2HPO_4. The dynamics population of endophytes was monitored in the culture medium at 0, 1, 3, 6 and 8 days after culture. At the end of the process, the effects of the culture at different pHs in *in vitro* explants behaviour was studied by measuring the number of shoots, shoot FW, shoot length as well as the chlorophyll content in leaves of RP-20 explants.

3. Production of *P. oryzihabitans* PGP01 using industry by-products (Chapter 6)

The production protocol was only developed for the bacterium *P. oryzihabitans* PGP01 due to the consistent effect as root development promoter observed in the previous chapters. The procedure followed is shown in Figure 9. First, several aspects related to the production process such as the culture temperature or the composition of the culture medium (by-product and nitrogen source) were optimized in flasks experiments. Carbon and mineral sources as well as the optimal

fermentation conditions were adjusted in the scale-up process to 2 L bioreactors Finally, *P. oryzihabitans* PGP01 grown in the wastes-based medium was inoculated to fully developed RP-20 *in vitro* plantlets to test its roles as root development enhancer and ensure biological activity maintenance.

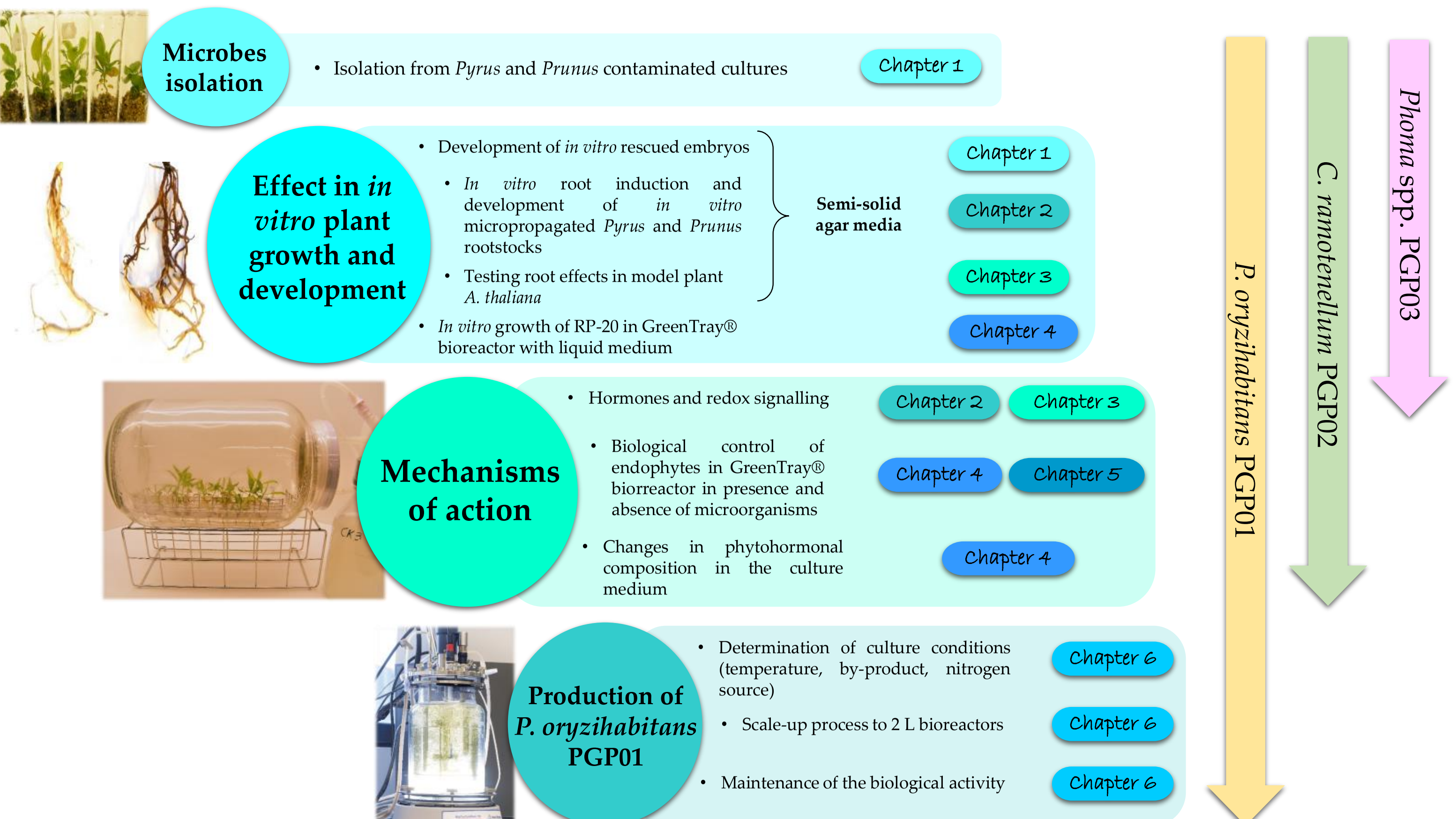


Figure 1. Structure of the thesis project.

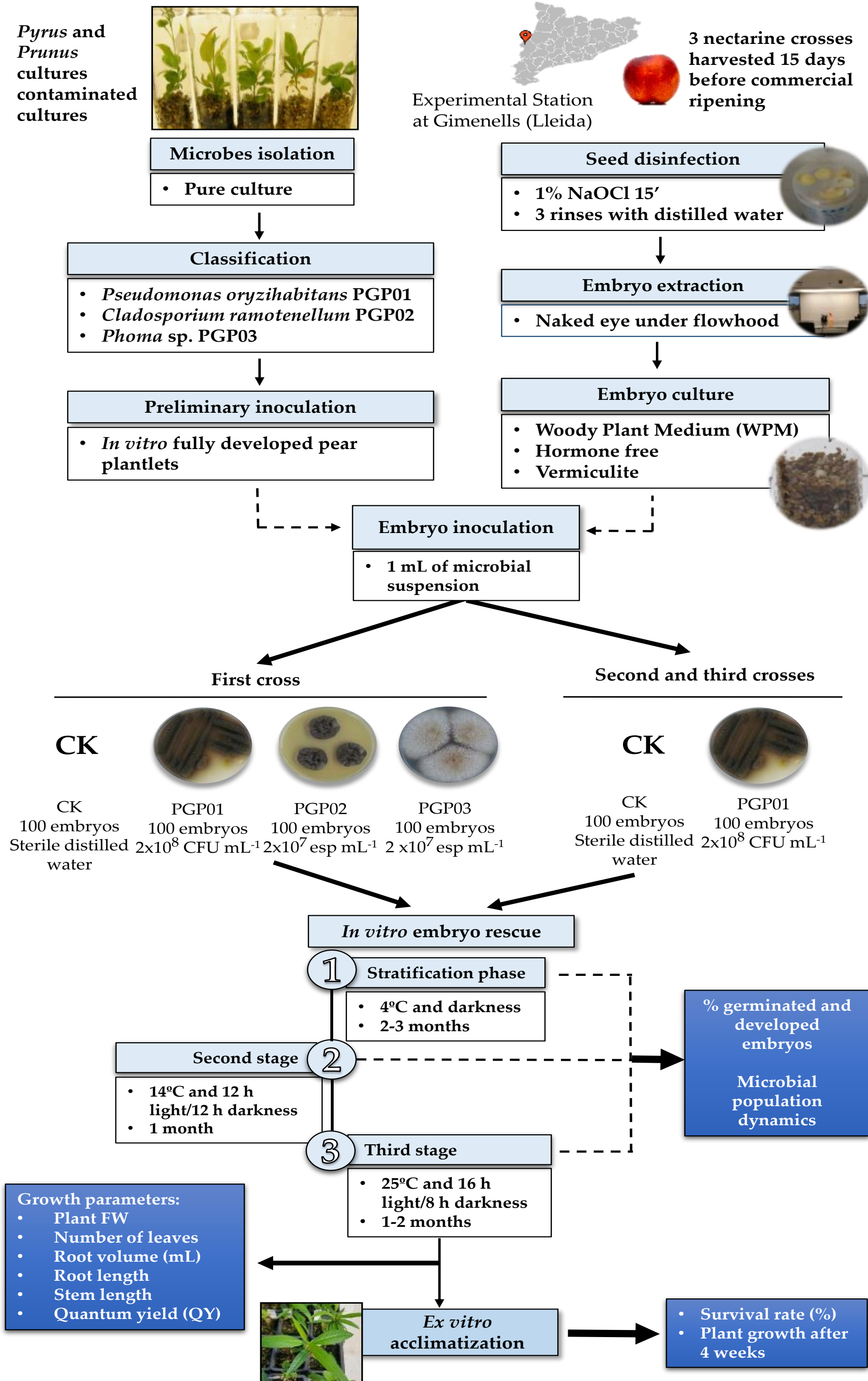


Figure 2. *In vitro* rescue of nectarine embryos in the presence of *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03.

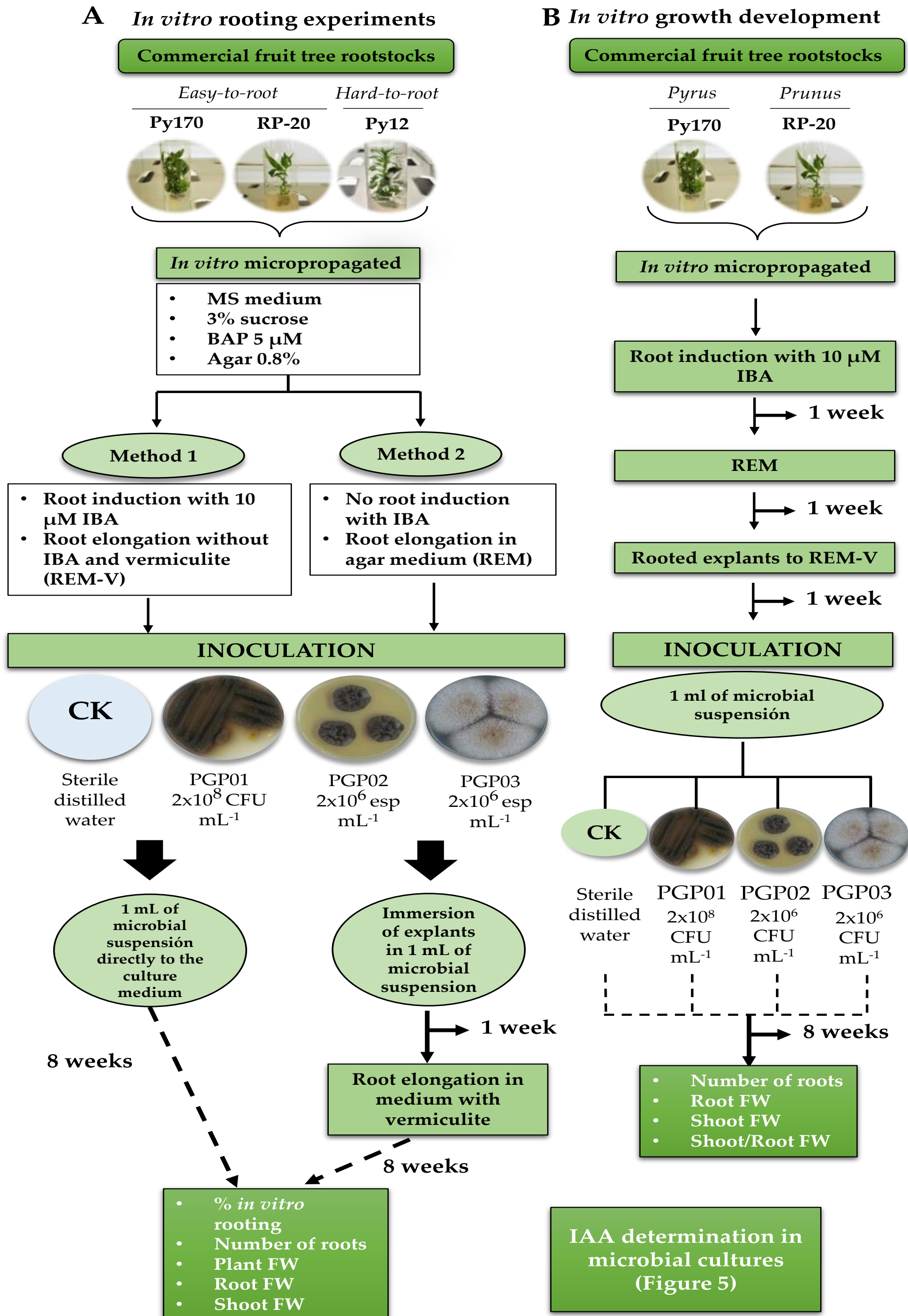


Figure 3. Effects on *in vitro* rooting (A) and *in vitro* plantlet growth and development (B) of commercial *Prunus* and advanced selections *Pyrus* rootstocks inoculated with *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03.

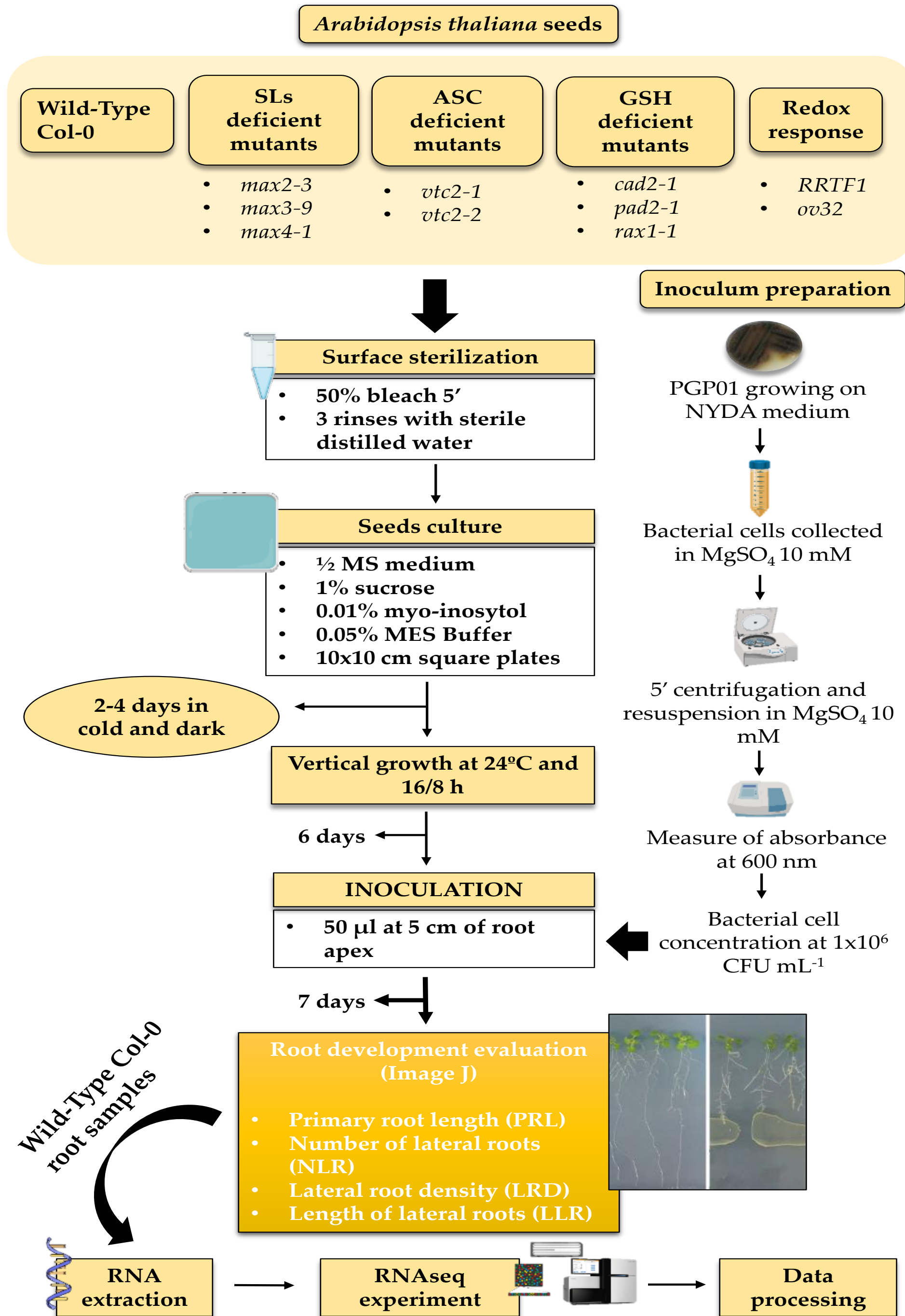
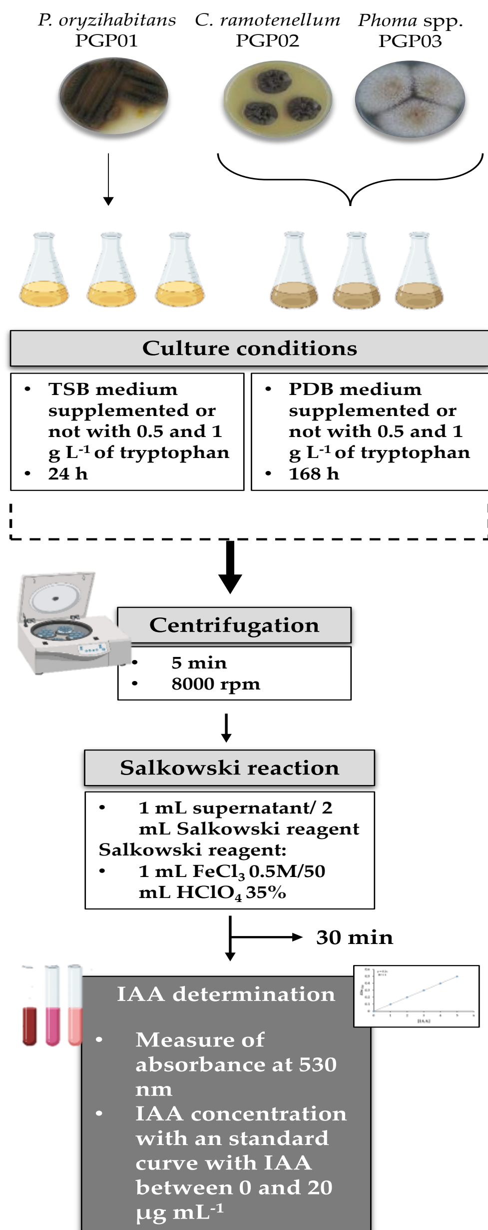


Figure 4. Protocol of inoculation with *P. oryzihabitans* PGP01 and root phenotype evaluation of *A. thaliana* Col-0 and mutants lacking hormones (SLs), antioxidant molecules (ASC and GSH) and redox effectors (*RRTF* and *ov32*) in the presence of *P. oryzihabitans* PGP01. Icons extracted from BioRender (https://biorender.com/).



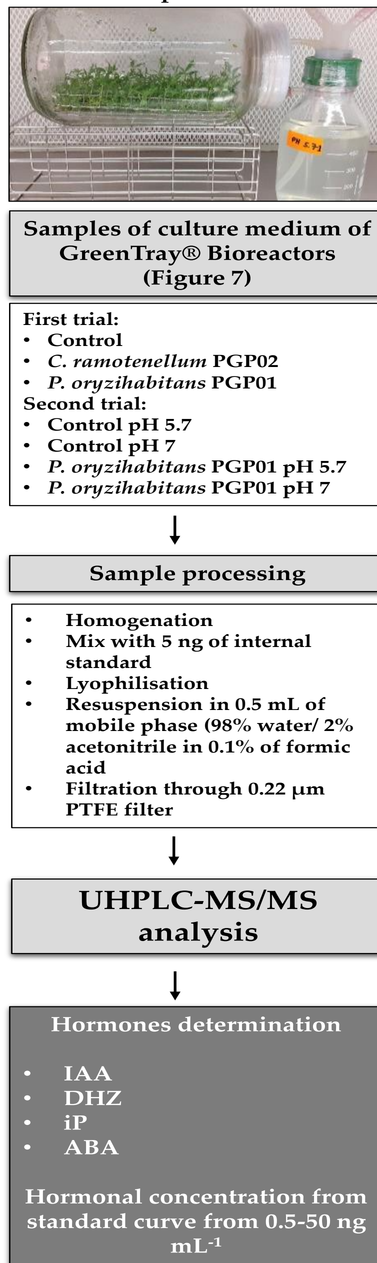


Figure 5. Determination of IAA in *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 cultures (A) and determination of IAA, DHZ, iP and ABA using UHPLC-MS/MS in samples of the culture medium of GreenTray® TIS bioreactors in the presence of *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02, and *P. oryzihabitans* PGP01 at pH 5.7 and 7 (B). Icons extracted from BioRender (https://biorender.com/).

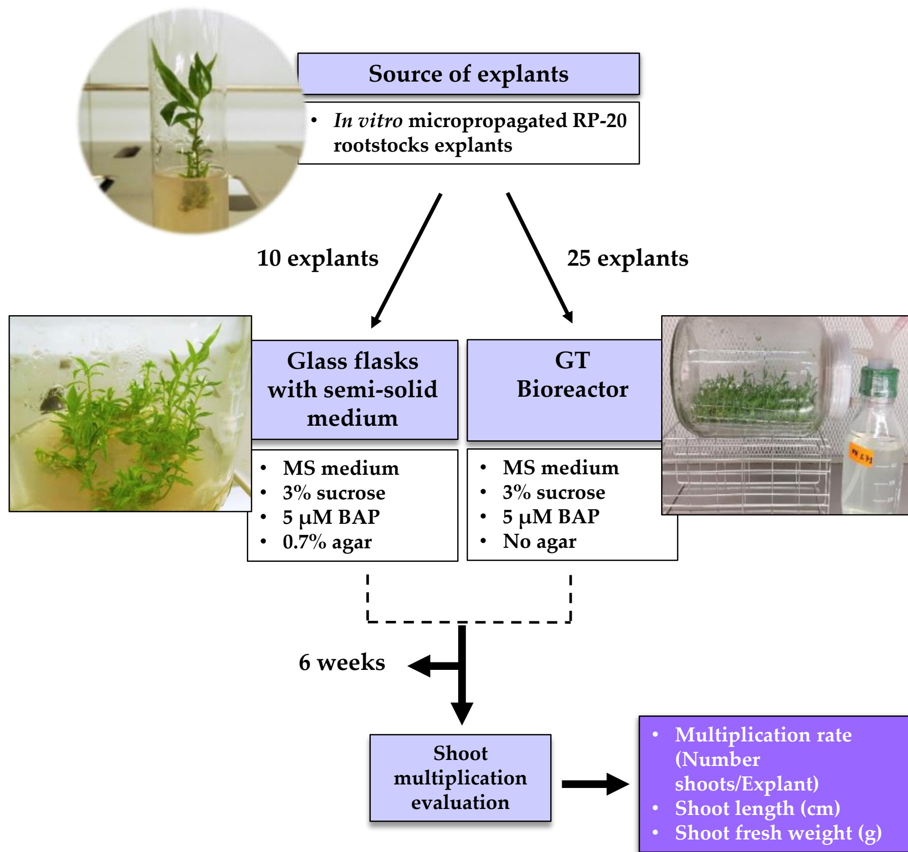


Figure 6. Use of GreenTray® bioreactor for *in vitro* RP-20 multiplication.

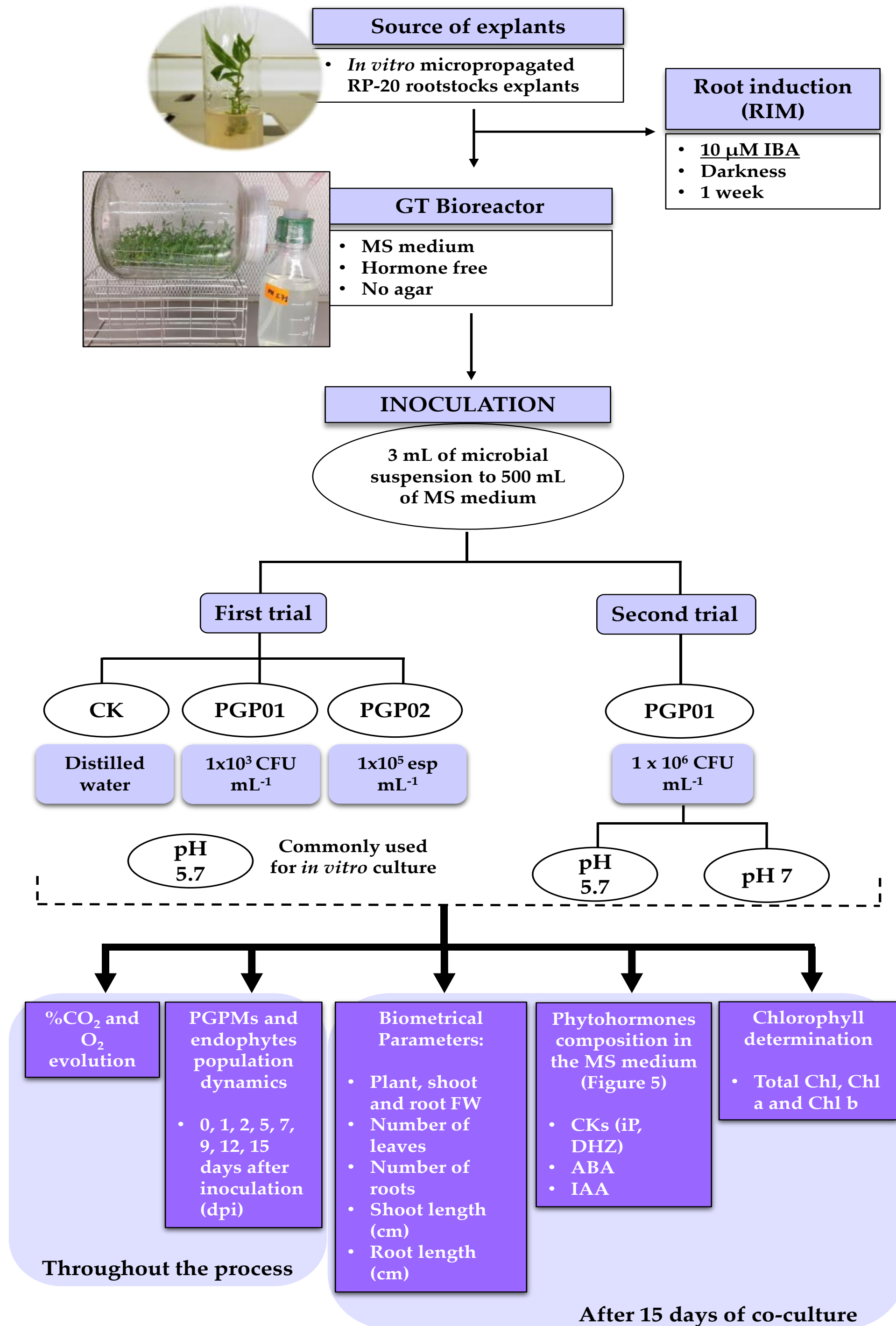


Figure 7. Use of GreenTray® bioreactor to analyse the biological control activity of *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 of endophytes growth as well as the changes in hormonal status in the culture medium.

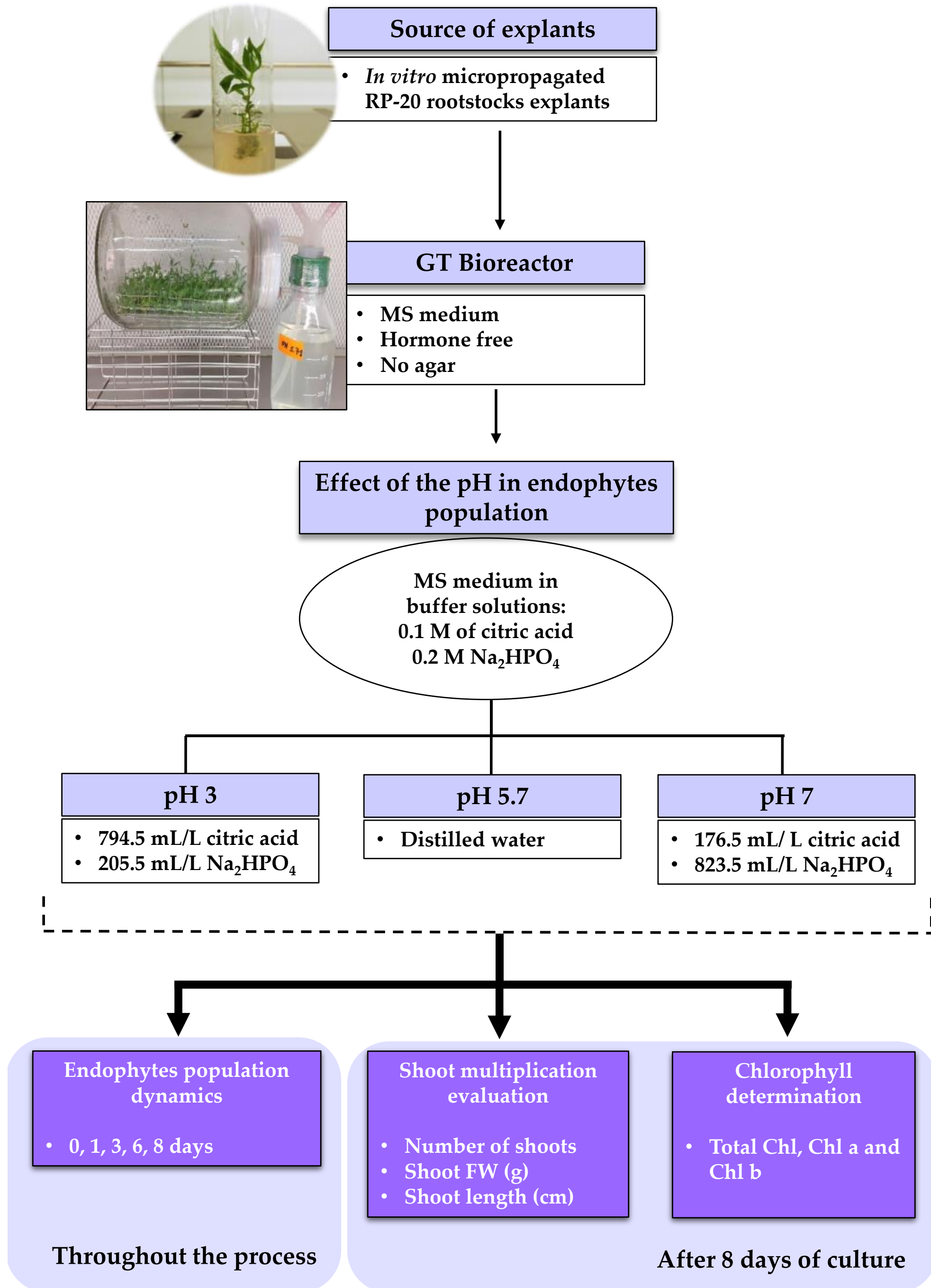


Figure 8. Effect of the pH on the dynamics population of endophytic contamination and RP-20 *in vitro* micropropagation in GreenTray® bioreactors.

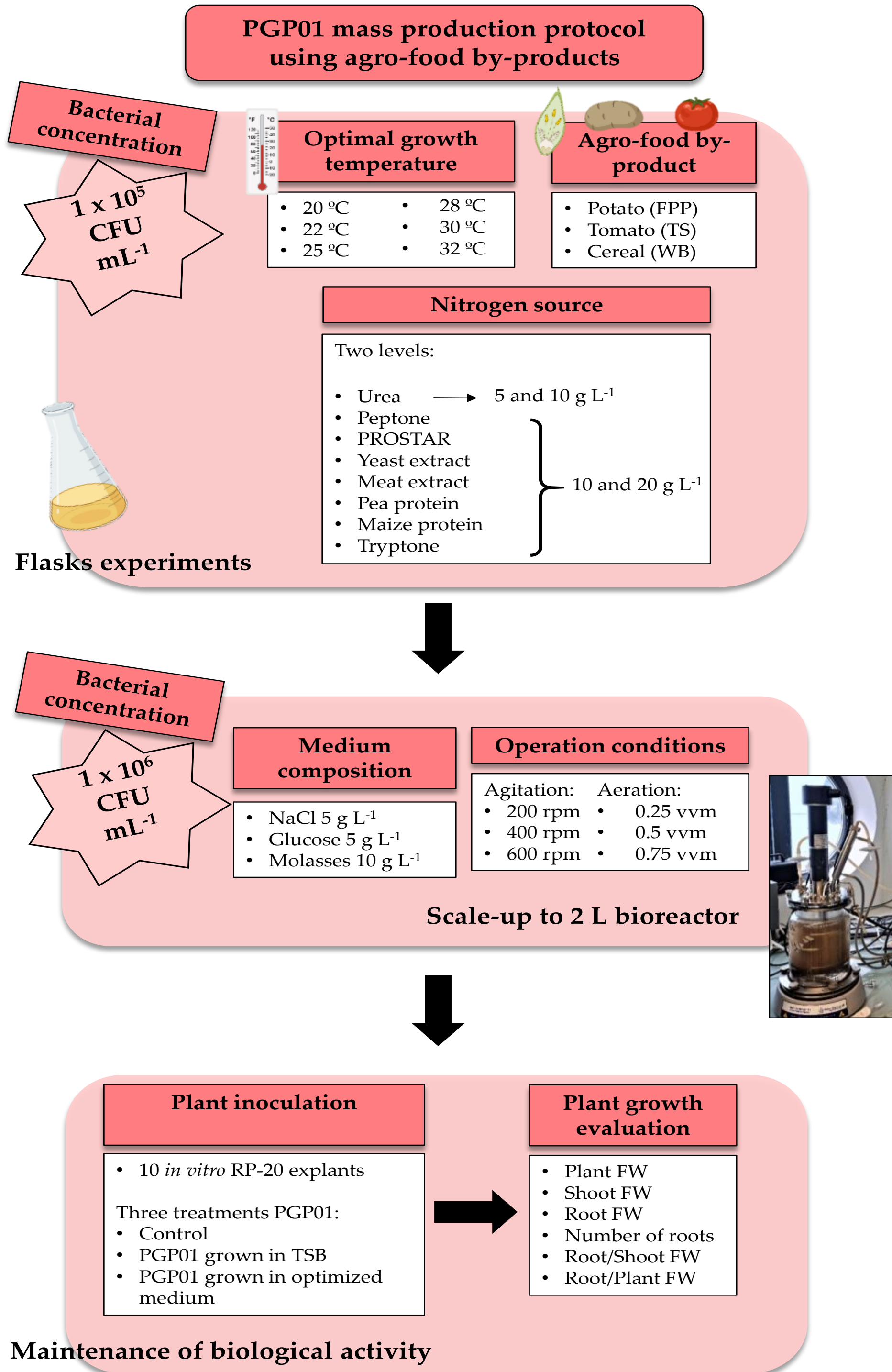


Figure 9. Economic and sustainable production of *P. oryzihabitans* PGP01 using agro-food by-products. Icons extracted from BioRender (https://biorender.com/).

Chapters

Chapter 1

Inoculation of *in vitro* cultures with rhizosphere microorganisms improve plant development and acclimatization during immature embryo rescue in nectarine and pear breeding programs

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Abstract

In the present study, two fungi *Cladosporium ramotenellum* strain PGP02 and *Phoma* spp. strain PGP03 and the bacterium *Pseudomonas oryzihabitans* PGP01 were isolated from *Pyrus* and *Prunus in vitro* rescued embryos, whose plantlets showed a better growth than non-contaminated cultures. Upon identification, concentrated solutions of the three microorganisms were applied to pear (*Pyrus communis* L.) *in vitro* rooted plantlets, increasing in different ways biometric parameters such as plant fresh weight (FW), stem length and root length. Then, these microorganisms were tested in embryos derived from three directed crosses between early ripening nectarine varieties (*Prunus persic*a cv. Nectarina). In a first cross, *in vitro* cultured embryos were inoculated with both fungi, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03, at $2x10^7$ sp mL^{-1}, and the bacterium *P. oryzihabitans* PGP01, at $2x10^8$ CFU mL^{-1}. In the following crosses, only the bacterium *P. oryzihabitans* PGP01, at $2x10^8$ CFU mL^{-1}, was employed. The effects on number of germinated embryos, development of the subsequent plants, after 24 weeks of *in vitro* culture, as well as their *ex vitro* acclimatization performance were analysed. These microorganisms had no effect on the germination efficiency of nectarine embryos. However, the presence of the bacterium *P. oryzihabitans* PGP01 modified root system architecture in the three crosses, increasing root volume and thickness, which in consequence enhanced the acclimatization efficiency to soil in those crosses with poor acclimation efficiencies. These results enforce a breakthrough in the use of microorganisms along the *in vitro* embryo rescue used in early ripening peaches and nectarines breeding programmes, and the production of plants more resistant to the stressful conditions imposed by the acclimatization to soil.

Keywords: Acclimatization process; Bacterium; Fungi; *In vitro* embryo rescue; Plant growth promotion; Root morphology.

1. Introduction

Peach (*Prunus persica* L.) and pear (*Pyrus communis* L.) are two of the most popular fruit trees cultured in temperate growing regions (Heidari et al., 2019; Topp et al., 2008). In 2017, the worldwide production of both peach and pear exceeded twenty millions of tones, being Spain one of the main producing countries (FAO 2017). Moreover, Catalonia ranks the first and second region of Spain in terms of surface of production of peaches and pears, respectively (MAPAMA 2017).

Given the above, the application of *in vitro* culture techniques are useful to provide large number of disease-free and true-to-type plant materials (Ko et al., 2018). In addition, *in vitro* embryo rescue under aseptic environment, is a valuable technique to provide new genetic variation to modern peach breeding programs aiming to get new early ripening varieties (Batlle et al., 2012; Devi et al., 2017; Sundouri et al., 2014). By using this methodology, the poor germination caused by incomplete embryo and seed development is solved using an aseptic and nutrient-rich environment (Liu et al., 2007; Sinclair & Byrne, 2003). Currently, the culture in Woody Plant Medium (WPM) supplemented with sucrose and plant growth regulators (McCown & Lloyd, 1981), following of an stratification at 4-5 ºC in dark conditions (Anderson & Byrne, 2002) is the most common practice to culture both small (5-10-mm-long) and large (>10 mm) embryos. To ensure the normal plant development, *in vitro* plantlets need to be transplanted to *ex vitro* conditions (Ko et al., 2018). Nevertheless, the acclimatization needs to be undertaken in a controlled plant growth environment since there are several external factors including humidity, temperature or light that seriously affect plant survival (Chandra et al., 2010; Maleki Asayesh et al., 2017).

Nowadays, the use of Plant Growth Promoting Microorganisms (PGPMs), as biological sources to stimulate plant growth, represents an ecological alternative in the implementation of a sustainable agriculture (Vejan et al., 2016). Plant Growth Promoting Rhizobacteria (PGPR) are non-pathogenic microorganisms, present in soil and rhizosphere, that improve host plant growth through a large number of mechanisms, all of them related with the close contact to the root system. This interaction encourage some adaptations involving the increase of root length or the development of lateral roots (Della Mónica et al., 2018; Zamioudis et al., 2013). Moreover, in *Arabidopsis thaliana,* has been previously reported that changes in root morphology are associated with the production of some plant-growth related phytohormones, including auxins and ethylene (Contesto et al., 2010; Iqbal & Hasnain, 2013). As a consequence, the morphological changes occurred in plants by these microorganisms increase the uptake of nutrients from soils (Calvo et al., 2014;

Collavino et al., 2010; Vessey, 2003), improvement of abiotic stress tolerance (Chu et al., 2019; Skz, 2018; Yang et al., 2009) and therefore, crop quality. Within the PGPR cluster, *Pseudomonas* represents one of the most common genus of bacteria widely involved in atmospheric nitrogen (N_2) fixation by legume-rhizobia symbioses (Vessey, 2003). Nevertheless, it has been recently shown that some species of this genus such as *Pseudomonas fluorescens* or *Pseudomonas nitroreducens* are involved in plant growth promotion by improving root development (Trinh et al., 2018; Zamioudis et al., 2013). Although most of the authors focus their attention on the implication of PGPR in plant growth, a significant group of Plant Growth Promoting Fungi (PGPF) including *Cladosporium sphaerospermum* and species belonging to *Phoma* genus also exists. In this case, the principal mechanisms through which these fungi increase plant growth include the production of hormones such as gibberellins and Volatile Organic Compounds (VOCs) (Hamayun et al., 2009; Hamayun et al., 2010; Naznin et al., 2013).

The work presented herein represents a first innovative approach that implies the introduction of microorganisms into an aseptic *in vitro* culture environment to stimulate woody plants growth and development. The aim of this research is to investigate whether three endophytic microorganisms (two fungi and one bacterium) isolated from *Pyrus* or *Prunus* immature embryos are able to improve the growth of *in vitro* plantlets obtained by *in vitro* embryo rescue. First, we applied these microorganisms to *in vitro* rooted pear plantlets to test their plant-growth promoting effect. Then, *in vitro* rescued nectarine embryos were inoculated with either one of the three microorganisms, and their impact on the percentage of germinated embryos, as well as on *in vitro* plantlet growth and the subsequent acclimatization of plants to greenhouse conditions were analysed.

2. Material and methods

2.1. *In vitro* plant material

Pear seedlings derived from directed crosses between *Pyrus communis* accessions belonging to the IRTA's pear breeding program, oriented to produce new varieties, were used in the study. In addition, seeds from three different crosses between nectarine (*P. persica* cv Nectarina) varieties, also belonging to IRTA's peach breeding program, were collected in different years and used for this study. Pear and nectarine seeds were extracted from cold stored fruits, harvested two weeks before commercial ripening stage in the IRTA's Experimental Field Station at Gimenells

(Lleida). Seeds extracted out of 10 fruits were placed in 50 mL Falcon tubes filled with distilled water, and washed twice before disinfection. This was done immersing and shaking the seeds for 15 min in a solution with 1% (w:v) NaOCl, followed by three 5-min-long rinses in sterile double distilled water. Embryos were dissected out of the seed teguments, at naked eye or with the help of a dissecting scope, under the flow hood. Afterwards, embryos were cultured in Woody Plant Medium (WPM) (McCown & Lloyd, 1981) supplemented with different hormonal combinations depending on embryo size and plant species. While nectarine embryos were cold stratified (3-5 ºC) under darkness and during 12 weeks, for seed germination embryo dormancy of pear embryos was broken with culture in GA_3 containing medium.

2.2. *In vitro* culture media

In this study, pear embryos were germinated in WPM supplemented with 3% sucrose (Duchefa Biochemie, Haarlem, The Netherlands) and 1µM of GA_3 (Duchefa Biochemie, Haarlem, The Netherlands), while nectarine embryos were cultured in the same media without hormones, but with vermiculite (50:40 v/v) (Dolcet-Sanjuan et al., 2017). The pH of the medium was adjusted to 5.7 using 1N NaOH prior the addition of 6 g L^{-1} gelling agar (Quimivita, Barcelona, Spain). Forty mL of medium was dispensed in each 38–mm-diameter tubes, with or without vermiculite. Media was then autoclaved at 121°C for 20 min, cooled down at room temperature, and stored at 14 ºC before culturing the embryos. Pear germinated embryos were transferred to tubes with WPM without hormones for plant development.

2.3. Preparation of microorganism's inocula

The two fungi and the one bacterium inocula used in the present experiment were originally isolated from contaminated *Prunus* and *Pyrus in vitro* cultured embryos. Their potential effect as PGPMs was suspected since the resulting plantlets from contaminated *in vitro* cultured embryos showed a greater shoot, leaf and root growth than the non-contaminated ones (data not shown). Samples of contaminated culture media were taken and cultured in nutrient yeast dextrose agar (NYDA: nutrient broth, 8 g L^{-1}; yeast extract, 5 g L^{-1}; dextrose, 10 g L^{-1}; and agar, 20 g L^{-1}) and potato-dextrose agar (PDA: potato 200 mL; glucose, 20 g L^{-1}; and agar, 20 g L^{-1}) to obtain pure cultures of the three microorganisms. Then, the selected strains, renamed as PGP01, PGP02 and PGP03, were identified as *P. oryzihabitans*, *C. ramotenellum* and *Phoma* spp. by the instrumental techniques laboratory from University of León (Spain) and they were included in the microorganisms' Postharvest Pathology Group

Collection of IRTA (Lleida, Catalonia, Spain). After isolation, bacterium and fungi were preserved on NYDA and PDA plates, respectively, and stored in 20% glycerol at -80 ºC. Both bacterial and fungi inoculants were prepared in solutions of 160 mL which contained the microorganism and sterile water. Plate dilution technique on solid PDA + 25 ppm of the antibiotic gentamycin (for fungi) and NYDA (in case of the bacterium) media was used to determine the real colony forming unit CFU mL^{-1}. Moreover, conidia were also determined for fungi by haemocytometer. Finally, the concentrations of the inocula were adjusted to $2x10^7$ espores per mL (sp mL^{-1}) for both fungi and $2x10^8$ CFU mL^{-1} in the case of the bacterium.

2.4. Inoculation of *in vitro* cultured embryos with microorganisms and growth conditions

In the case of the first nectarine cross, four hundred *in vitro* rescued embryos were used, grouped in four treatments: (1) control non inoculated embryos, (2) *P. oryzihabitans* strain PGP01, (3) *C. ramotenellum* strain PGP02 and (4) *Phoma* spp. strain PGP03-inoculated embryos. Each *in vitro* culture vessel containing one embryo was inoculated with 1 mL of the microorganism suspension, adding 1 mL of sterile water to those non-treated embryos (control). After inoculation, all embryos were submitted to a 12-week-long cold stratification period, in the dark and at 3 to 5 ºC (First stage). Seedlings starting germination were transferred to *in vitro* growth chambers, in which they were cultured for a 4-week-long period at 14 ºC and a 12h photoperiod (12h light / 12h dark), of cool white fluorescent lightening at 100-120 $\mu molm^{-2}s^{-1}$ photoactive radiation (PAR) (Second stage). At the final *in vitro* plant growth phase (Third stage), which was 8-week-long, the culture temperature was increased to 24 ºC, and the photoperiod to 16h light, of the same intensity as in the previous stage.

For the two following crosses, considering the results observed in the first trial, the inoculation with only the bacteria *P. oryzihabitans* PGP01 was taken into account. As a control treatment 100 embryos were used, and 100 embryos were inoculated with bacteria, following the same procedure described above.

2.5. Embryo germination, plant morphometric and photosynthetic parameters

At the end of the second embryo germination stage, the percentage of germinated seeds, accounting for those with open cotyledons showing the embryo

axis, was calculated. At the end of the third stage, the percentage of germinated seeds, with a growing shoot apex, was calculated.

At the final stage, pictures of the seedlings were taken and some morphological and photosynthetic characters were measured, including plant fresh weight (FW) in grams, total number of leaves, root volume (mL), stem length (cm), root length (cm) and photosystem II quantum yield (QY). Afterwards, plantlets were moved to the greenhouse for acclimatization into soil. Root volume was determined by volume of water displaced using a sectioned graded 25 mL cylinder. Root length was measured on the longest root from each plant. The QY parameter was determined using a PAR-FluorPen FP 100-MAX-LM (PSI spol. s.r.o., Drasov, Czech Republic), which reflects the maximum efficiency of photosystem II (PSII) or its quantum efficiency when all the centres are open (Maxwell & Johnson, 2000). Changes in this parameter provide information about the state of plants under stress conditions, being the values considered standard those ranged between 0.64-0.83.

2.6. Microorganisms population dynamics

At the end of the second and third embryo germination stages, cell population of the three inoculated microorganisms was determined in order to assess population dynamics in parallel to the growth of the embryos. Samples of *in vitro* culture media along with the contained cultured embryo were removed from the culture tubes and were placed together into sterile plastic filter bags (BagPage 400 mL, Interscience BagSystem, St Nom la Brètech, France) with 100 mL of water + Tween 80. Each bag was homogenized in a stomacher blender (Masticator Basic 400 mL, IUL SA, Torrent de l'Estadella, Barcelona, Catalonia, Spain) for 90 s at high speed. Serial ten-fold dilutions of the washings were made and plated on PDA + 25 ppm gentamycin plates for fungi and NYDA plates for bacterium. Plates were incubated at 25 ºC and population dynamics were collected as CFU mL^{-1}.

2.7. Acclimatization of plants to greenhouse conditions

Nectarine rooted plantlets derived from the 24-week-long *in vitro* culture process, were transferred to 200 mL pots filled with peat and vermiculite (2:1, v:v). Plants were acclimated in plastic tunnels, within a conventional greenhouse, designed to gradually and automatically decrease the relative humidity (RH) from 100% to 60%, in a 4-week-long period. Acclimatization tunnels had a soil temperature above 22/18 ºC (day/night) and a photoperiod of 16h light, supplemented with LED lights (SUP12100DC, AlternativaLED, Spain) to extend the day light hours, with 230 µmol $m^{-2}s^{-1}$ PAR at leaf level. At the end of this period, plantlet survival rate and growth,

measuring its new shoot length (cm), were used to determine the plantlet performance during the acclimatization process.

2.8. Statistical analysis

The experiment was set up as a completely randomized design (CRD), and the data was analysed by a one-way ANOVA. Statistical significance was judged at the level $P < 0.05$. When the analysis was statistically significant, Student's t-test was used for separation of means. Data analysis was performed using JMP Pro software (version 13.1.0, SAS Institute Inc., Cary, NC).

3. Results

3.1. Effect of the inoculation with three microorganisms in pear *in vitro* rooted seedlings

Pear *in vitro*-rooted plantlets inoculated with each of the three microorganisms showed some preliminary plant-growth promoting effect (Fig. 1A and B). All of the microorganisms tested in the study induced a significant increase in plant FW when compared to control (199, 173 and 207% for *P. oryzihabitans* PGP01, *Phoma* spp. PGP03 and *C. ramotenellum* PGP02, respectively) (Figure 1A). Moreover, a significant 125% rise in comparison to non-inoculated plantlets was recorded in stem length in those plantlets inoculated with *P. oryzihabitans* PGP01 (Figure 1A). Although an important increase in this parameter was also observed in *C. ramotenellum* PGP02-inoculated plantlets (50% regarding to control), the differences found were not statistically significant from the control (Figure 1A). On the other hand, whereas the application of *P. oryzihabitans* PGP01 and *Phoma* spp. PGP03 did not produce significant changes in root length, the inoculation of pear plantlets with *C. ramotenellum* PGP02 displayed a strong increase in root length (259% when compared with control plantlets) (Figure 1A). It is noteworthy to mention the thicker and lignified aspect of the roots in those plantlets inoculated with *P. oryzihabitans* PGP01 compared with the non-inoculated plantlets or those inoculated with either of the other two microorganisms (Figure 1B). Moreover, these roots had a darker colour as well, probably due to the close contact between root and bacterium.

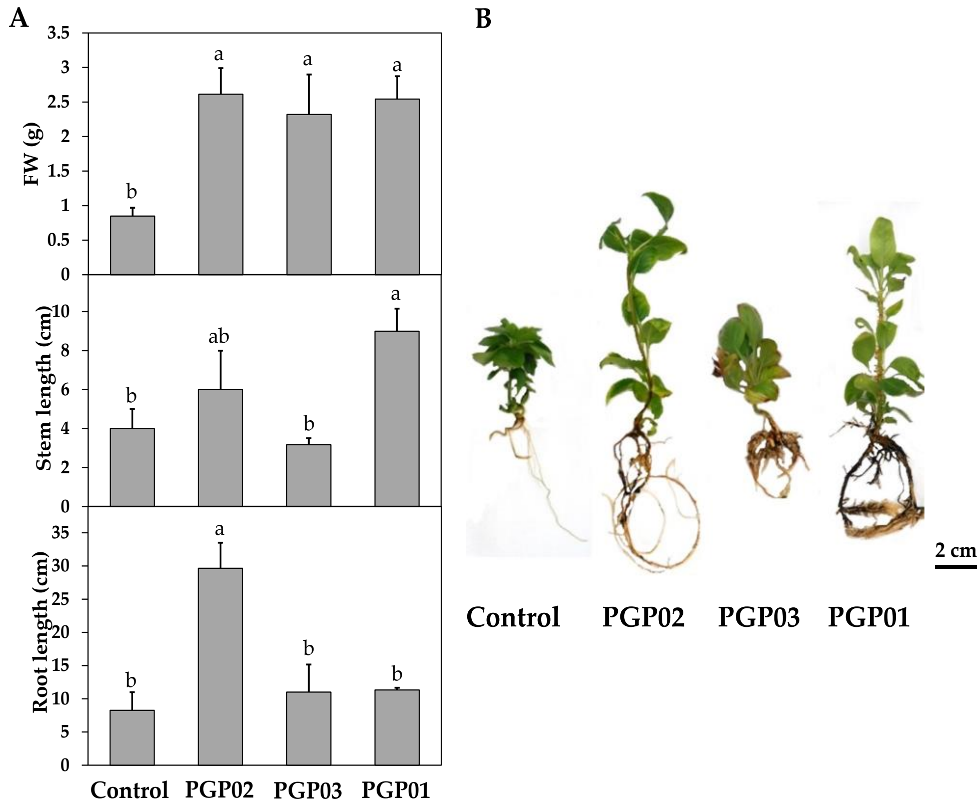

Figure 1. Effects of the application of three microorganisms (*C. ramotenellum* PGP02, *Phoma* spp. PGP03 and *P. oryzihabitans* PGP01) on growth parameters (A) and morphology (B) of pear rooted plantlets growing *in vitro*. Data represents the mean ± SE of at least three measurements. Different letters on bars of the same chard indicate significant differences according to the Student's t-Test ($P \leq 0.05$).

3.2. Effect of the inoculation with three microorganisms in nectarine embryos emergence

The percentage of germinated nectarine embryos (inoculated and non-inoculated) along with the population dynamics of the three microorganisms (*P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03) at the end of each culture period (12, 16 and 24 weeks after embryos inoculation) are shown in Figure 2. None of the three microorganisms induced an improvement in the germination of nectarine embryos, since for the crosses used, after the 24-week-long *in vitro* embryo rescue protocol, almost 100% of the control embryos were germinated (Fig. 2B). Indeed, both fungi (*C. ramotenellum* PGP02 and *Phoma* spp. PGP03) reduced the percentage of germinated embryos (Figure 2B). After 12 weeks of culture, at the

end of the first stage at 4 ºC, while control and *P. oryzihabitans* PGP01 inoculated embryos had exceeded the 50% of germination, similar to the control, none of the embryos treated with *C. ramotenellum* PGP02 had germinated, and those inoculated with *Phoma* spp. PGP03 had reached only 27% of germination. Related to the growth of the microorganisms in the culture media, it is worthy to mention that the stratification phase (12 weeks at 4 ºC and darkness) induced a decrease on the *P. oryzihabitans* PGP01 population, reaching values of almost 3.5 log (CFU mL^{-1}) (Figure 2A). However, the growth of this bacterium was highly recovered at the end of the second phase (14 ºC, 16-h photoperiod), obtaining values of 7.20 log (CFU mL^{-1}), which remains unchanged until the end of the *in vitro* culture process. Conversely, a time-depending increase in the populations of the two fungi was observed at 12 and 16 weeks. However, after 24 weeks of the *in vitro* culture process, *Phoma* spp. PGP03 population increased 0.86 log (CFU mL^{-1}) while *C. ramotenellum* PGP02 population showed a 0.44 log (CFU mL^{-1}) decrease with regard to the previous period.

Considering the results obtained in this first trial, only the bacterium *P. oryzihabitans* PGP01 was used in the next assays to inoculate the nectarine *in vitro* rescued embryos, and the same response was reported for both germination percentage and dynamics population (data not shown).

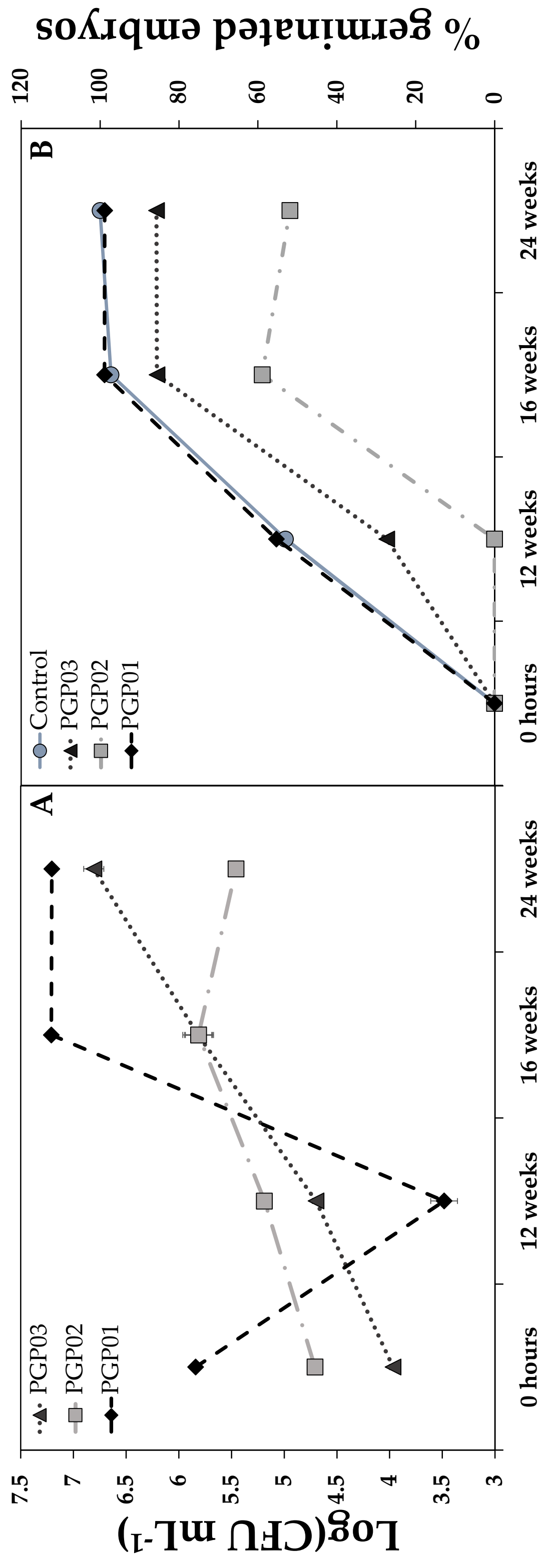

Figure 2. Microorganism population dynamics (A) and germination percentage of *in vitro* rescued nectarine embryos (B), following the three culture phases (12, 16 and 24 weeks) of *in vitro* culture. Inoculation was done at day 0 with three microorganisms (*C. ramotenellum* PGP02, *Phoma* spp. PGP03, *P. oryzihabitans* PGP01). Data of population dynamics represent the mean ± SE from at least three measurements.

3.3. Effect of the inoculation of embryos with three microorganisms on growth and development of resulting nectarine seedlings

Morphometric (FW, number of leaves, stem and root length and root volume), and photosynthetic (QY) parameters were measured in nectarine seedlings derived from immature embryos rescued in different years through a 24-week-long *in vitro* culture process (Figure 3).

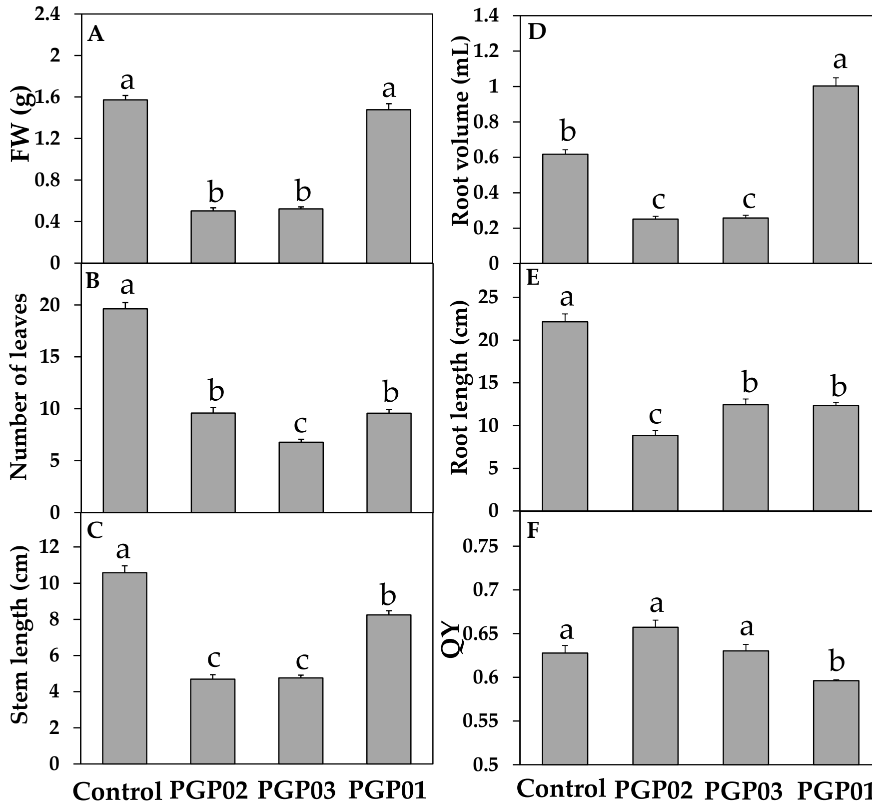

Figure 3. Effects of the *in vitro* co-culture of nectarine embryos with three microorganisms (*C. ramotenellum* PGP02, *Phoma* spp. PGP03 and *P. oryzihabitans* PGP01) on (A) total plantlet fresh weight (FW), (B) total number of leaves, (C) stem length, (D) total root volume, (E) maximum root length and (F) photosynthetic activity or QY, at the end of the 24-week-long *in vitro* culture process. Different letters on bars of the same chard denote significant differences according to the Student's t-Test ($P \leq 0.05$).

Compared with the control, no inoculated embryos, inoculation with the bacterium *P. oryzihabitans* PGP01 did not affect nectarine seedlings growth since effects on FW were not detected (Figure 3A). By contrast, plantlets from embryos inoculated with both fungi, *Phoma* spp. and *C. ramotenellum* PGP02, were drastically

affected, detecting reductions of almost 70% in both treatments (Figure 3A). The treatment with the three microorganisms resulted in plants with fewer number of leaves than non-inoculated (control) seedlings (Figure 3B). In this case, *Phoma* PGP03-treated plantlets showed the lowest values, detecting a decrease of 64% in comparison to control (Figure 3B). Regarding stem length, inoculation with the three microorganisms caused a decrease in this parameter, being less affected by the bacterium *P. oryzihabitans* PGP01 inoculated plants (22% lower than control) than for fungi inoculated plants (56% less than control plants for both fungi) (Figure 3C). The most prominent effect of the inoculation of embryos with the bacterium *P. oryzihabitans* PGP01, was that nectarine plantlets exhibited an increase of 61% in root volume when compared to control; while, the presence of fungi, *Phoma* spp. PGP03 or *C. ramotenellum* PGP02, resulted in seedlings with significant lower root volume (Figure 3D). All the inoculated seedlings displayed a statistically significant shorter and compact root system as shown by the measures of maximum root length (Figure 3E). The shortest roots were detected in *C. ramotenellum* PGP02-treated plantlets, which exhibited a decrease up to 60% compared to the control (Figure 3E). Significant morphological differences were observed on the produced nectarine plantlets, those plantlets derived from culture media inoculated with *P. oryzihabitans* PGP01 showed thicker roots than non-inoculated plantlets (Fig. 4), a similar effect to that observed in pear plantlets (Figure 1B). While no statistically significant differences in QY were found in *C. ramotenellum* PGP02 and *Phoma* spp. PGP03-inoculated seedlings, compared with the control, a slight significant decrease in this parameter was registered in *P. oryzihabitans* PGP01-treated plants (6% than control, approximately) (Figure 3F). These reduced values are in concordance with the fact that these plants had not as green leaves as control and *C. ramotenellum* PGP02-plants (Figure 4).

When inoculation of *in vitro* cultured embryos was done in two additional nectarine crosses, seedlings from embryos inoculated with *P. oryzihabitans* PGP01 displayed reduced values of all biometrical and photosynthetic parameters analysed except for root volume at the end of the *in vitro* embryo rescue process. Higher root volume values were observed in both crosses, being statistically significant in one of them (Figure 5B). Nevertheless, the *P. oryzihabitans*-inoculated plantlets of the two nectarine crosses displayed thicker and shorted roots than non-inoculated seedlings (Figure 5C and D), as previously observed (Figure 4), most likely due to the activity of the bacterium during the seedling root development.

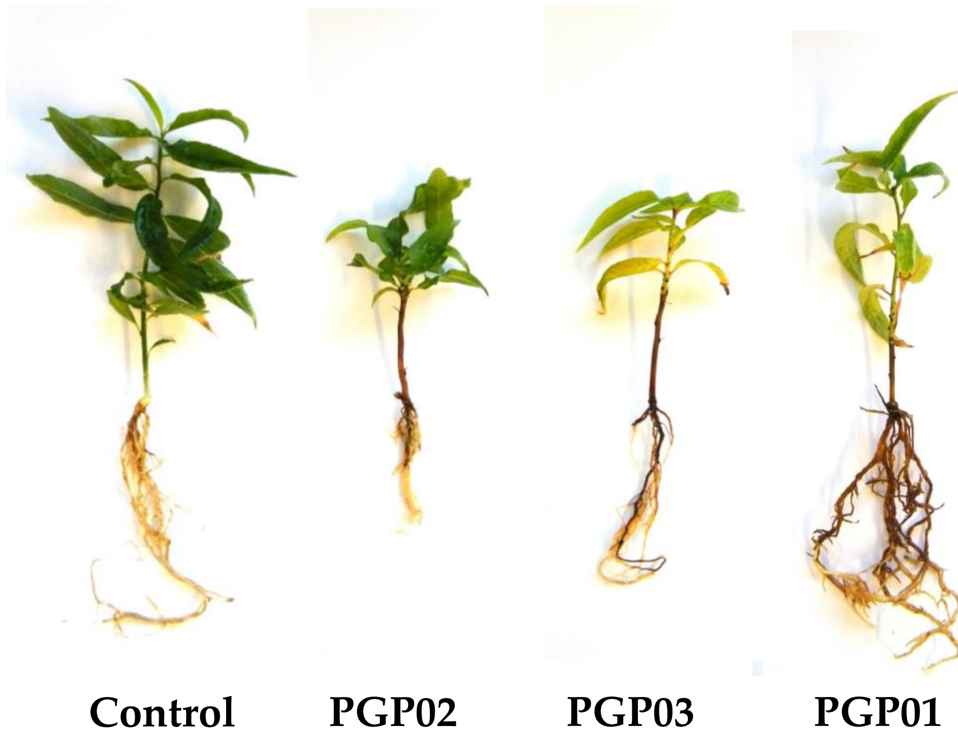

Figure 4. Morphological differences among nectarine plantlets derived from *in vitro* immature embryo rescue, after a 24-week-long *in vitro* co-culture process with three microorganisms (*C. ramotenellum* PGP02, *Phoma* spp. PGP03 and *P. oryzihabitans* PGP01).

3.4. Effect of the *in vitro* inoculation of nectarine embryos on the acclimatization of seedlings

In the first experiment using nectarine embryos, the inoculation with the three microorganisms resulted in plants more resistant to acclimatization in the greenhouse, displaying survival rates of 88, 85 and 87% for *C. ramotenellum* PGP02, *Phoma* spp. PGP03 and *P. oryzihabitans* PGP01, respectively, against the 71% recorded for the control plants (Figure 6A). These percentages were moderately significantly better in the case of *Phoma* spp. PGP03 (*P*–value = 0.020) and *C. ramotenellum* PGP02 (*P*–value = 0.013), and highly significantly for the *P. oryzihabitans* PGP01-inoculated plants (*P*–value = 0.005). Moreover, this improved survival rate did correlate with an increase of 92% on the new shoot length in the *P. oryzihabitans* PGP01 acclimated plants when compared with control (Figure 6B). An inhibition of the shoot growth under acclimatization conditions occurred in those plants from embryos inoculated with *C. ramotenellum* PGP02 and *Phoma* spp. PGP03, observing decreases of 55 and 70% regarding to control, respectively (Figure 6B). In the following trials with two

different nectarine genotypes, the survival rates after the 4-week-long acclimatization process were higher, but not statistically significant, for plantlets inoculated with *P. oryzihabitans* PGP01 than the control ones (data not shown). This could be explained by a plant genotype effect on the acclimatization survival rates. The differential response among microorganisms might be explained by the morphological changes occurred in plantlets from embryos inoculated with *P. oryzihabitans* PGP01 that provided a thicker root system related with a larger capacity to deal with *ex vitro* conditions, and therefore, a better resilience under stressful conditions.

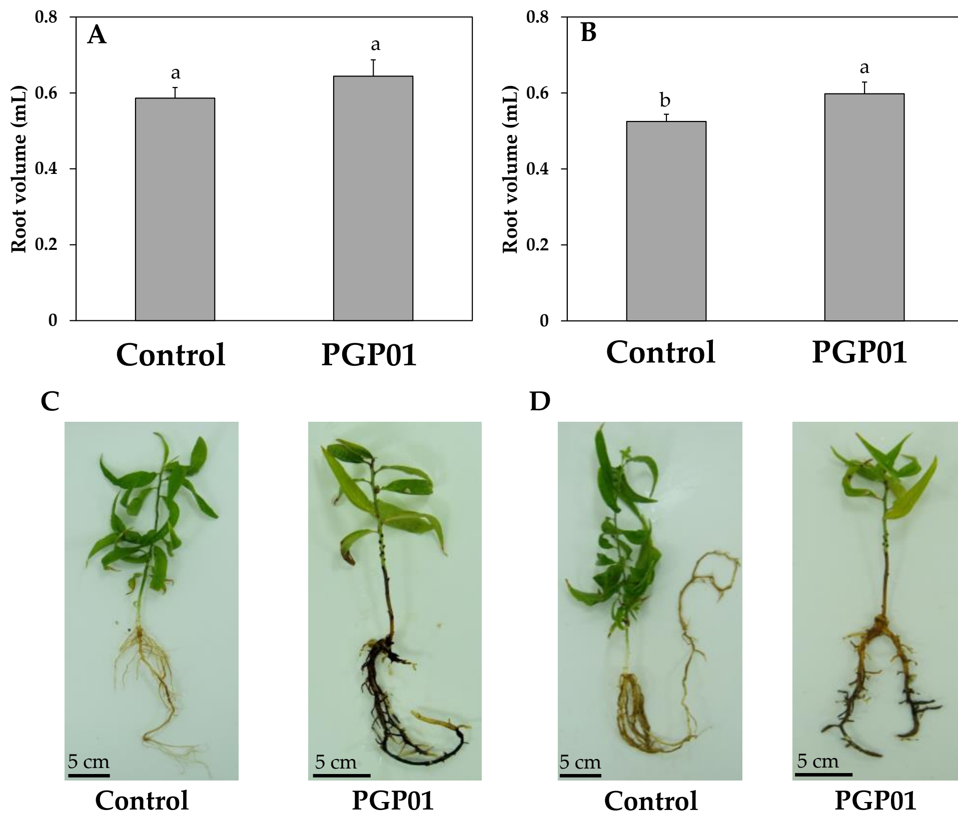

Figure 5. Root volume values (A,B) and morphological differences (C,D) of nectarine plantlets from two independent crosses, after a 24-week-long *in vitro* embryo rescue process, inoculated or not with the bacteria *P. oryzihabitans* PGP01.

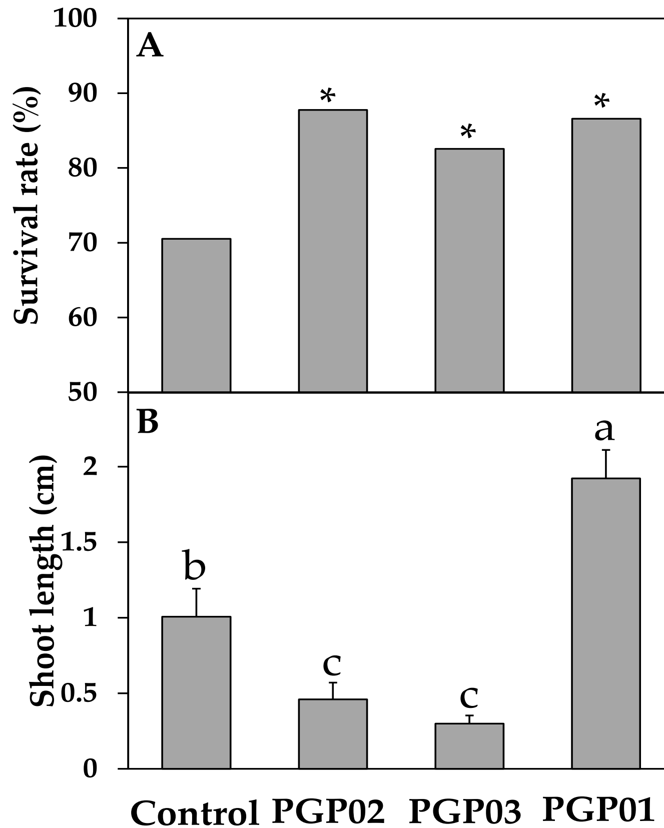

Figure 6. Percentage of survival rate and length of the new shoot formed of the resulting plants obtained from nectarine embryos inoculated with the three microorganisms (*C. ramotenellum* PGP02, *Phoma* spp. PGP03 and *P. oryzihabitans* PGP01) after 4 weeks on acclimation tunnels. Asterisk symbol (*) means significant differences between treatments and control according to the Fisher's Exact Test ($P \leq 0.05$). Different letters denote significant differences according to Student's t-Test ($P \leq 0.05$).

4. Discussion

The three microorganisms (two fungi and a bacterium) tested in the present study were obtained from immature *Pyrus* and *Prunus* embryos, rescued under *in vitro* conditions, following the described sterilization and culture process. Some of those *in vitro* contaminated embryos resulted in plantlets with a better growth than not contaminated embryos. After their posterior isolation and identification, the impact of these endophytic microorganisms on *in vitro* rooted pear plantlets was analysed, and the three microorganisms showed an important plant-growth promoting effect on this plant material improving one or two of the three parameters

analysed (fresh weight, stem and root length). The use of microorganisms with an endophytic origin to improve plant growth have been previously described in plants under greenhouse conditions (Dias et al., 2009). However, their application in an aseptic *in vitro* environment is being recently implemented (Perez-Rosales et al., 2018; Regalado et al., 2018), and the information found in the literature is scarce, even more among woody plants grown *in vitro*. For instance, Kavino and Manoranjitham (2018) and Rajamanickam et al. (2018) used endophytic bacterial strains isolated from different parts of banana field plants to induce resistance against pathogens in banana *in vitro* manipulated plantlets.

Given the potential effect on plant growth seen in the preliminary experiment with *Pyrus* rooted plants, the effects of these microorganisms on the *in vitro* embryo germination and plantlet growth from three different crosses of nectarine were studied. The *in vitro* rescue of embryos is being increasingly applied in peach breeding programmes to widen the diversity of commercially productive early ripening varieties with a higher commercial interest (Mancuso et al., 2002; Batlle et al., 2012). Herein, almost 100% of the rescued nectarine embryos, in the three crosses, sprouted at the end of the *in vitro* culture process, in contrast with the 70% of germination observed in interspecific hybrid embryos of peach and plum (Liu et al., 2007). This procedure allows to increase the germination of these immature embryos, that otherwise would degenerate, by culturing them in an aseptic and nutritionally enriched environment (Sinclair & Byrne, 2003). On the other hand, although there is much evidence that the *in vitro* application of microorganisms could influence the propagation or *in vitro* rooting (Kavino & Manoranjitham, 2018; Quambusch et al., 2014), the knowledge regarding the inoculation with beneficial microorganisms to enhance the *in vitro* germination of rescued embryos and posterior seedling development is currently rare. In our case, none of the three microorganisms applied in the first experiment improved nectarine embryos sprouting, mostly due to the high effectiveness of the *in vitro* rescue procedure mentioned above. Nevertheless, the most notable finding is that while *C. ramotenellum* PGP02 and *Phoma* spp. PGP03-inoculated embryos showed a negative effect on germination, no detrimental effect was reported in the case of *P. oryzihabitans*-inoculated embryos. The key difference of this behaviour lies in the growth of these microorganisms in the plant *in vitro* culture medium. Whereas the growth of the *P. oryzihabitans* PGP01 was highly recovered after 16 and 24 weeks of *in vitro* culture, the low temperatures (4 ºC) of the stratification phase dramatically affected the growth of this microorganism, which is quite logical because most of bacteria grow better at high temperatures (Dobrić & Bååth, 2018). By contrast, these conditions along with the important concentration of sugar present in the

culture medium favoured the development of both fungi, negatively affecting in most cases the embryo germination. This fact explained the results obtained at the end of the *in vitro* rescue in terms of morphological parameters, and helped us to discard the inoculation with fungi for the experiments with the following nectarine crosses. In these experiments, the same behaviour of *P. oryzihabitans* PGP01 in the culture medium was observed, with an important decrease of its growth after the stratification phase at 4 ºC, and a rapid recovery when the embryos were transferred to 14 ºC. Moreover, *P. oryzihabitans* PGP01 in the culture medium did not affect embryo germination percentage, suggesting that there is no interference of the microorganism with the embryo physiology during the stratification at 4 ºC.

At the end of the *in vitro* embryo rescue process, we found that the inoculation of nectarine embryos with *P. oryzihabitans* PGP01 produced shorter, thicker and more vigorous roots in all three crosses studied. Modifications in the root architecture system in response to the inoculation with PGPR represents one of the main mechanisms to induce plant growth (Mantelin et al., 2005; Wang et al., 2016). In other studies using soybean and tomato as plant material, the inoculation with *P. oryzihabitans* led to a higher root biomass that non-inoculated plants (Belimov et al., 2015; Kuzmicheva et al., 2017). However, most of the studies related to the effect of the application of bacteria on root development were conducted using the model *A. thaliana* plants cultured *in vivo*. The inoculation with *Bacillus megaterium* and some strains of *Pseudomonas* inhibited the elongation of primary roots, but stimulate the formation of lateral roots in wild-type plants (López-Bucio et al., 2007; Zamioudis et al., 2013). In contrast, the application of *Aeromonas punctata* stimulated the growth of primary root and lateral root density of Arabidopsis plants (Iqbal & Hasnain, 2013). Furthermore, in the present study, the plantlets inoculated with *P. oryzihabitans* PGP01 also showed reduced values of QY. The chlorophyll fluorescence parameters are a useful approach to predict changes in the status of the plant photosynthetic apparatus under different stress situations (Cantabella et al., 2017; Clemente-Moreno et al., 2015). The activity of the photosystem II (PSII) is strongly associated with root formation (Ślesak et al., 2017). Considering this, the alteration observed in QY for *P. oryzihabitans* PGP01-inoculated nectarine seedlings could be likely linked to the higher root development registered after 24-week-long *in vitro* culture process.

Regarding the acclimatization of seedlings from control and inoculated nectarine embryos to greenhouse conditions, the application of microorganisms produced more resistant plants with higher survival rate values than control plants. These results were most remarkable for the seedlings from embryos inoculated with

P. oryzihabitans PGP01 since a strong stimulation of the new shoot growth after 4 weeks of acclimatization was registered. This better performance of plants derived from *P. oryzihabitans* PGP01-inoculated embryos was not observed in two other nectarine genotypes, for which almost 100% of control plants survived to acclimatization in greenhouse conditions. This suggests that the genotype of the nectarine embryos interacts with *P. oryzihabitans* PGP01 on the survival and growth response during acclimatization, since this is a physiologically complex process, highly influenced by several environmental factors including humidity, temperature, light, CO_2 or nutrient levels (Chen, 2004; Hazarika, 2006; Tisarum et al., 2018). The awareness of using beneficial microorganisms on *in vitro* plants lies in overcoming the great losses of plant material recorded at the acclimatization stage (Orlikowska et al., 2017). Trivedi and Pandey (2007) demonstrated that the inoculation *Picrorhiza kurrooa* plantlets with three PGPR increased plant survival as well as growth parameters in a greenhouse environment. Similarly, banana hardened plants bacterized with two strains of *Bacillus subtilis* displayed a 100% of survival plants in comparison to 89% recorded for control treatment (Rajamanickam et al., 2018). Most studies using the term “biohardening” involve the application of microorganisms on acclimated plantlets to ensure plant establishment under acclimatization conditions (Harish et al., 2008; Yadav et al., 2013). In consideration with the above, the results presented in this work should be treated as an outcome of the *in vitro* inoculation and co-culture of embryos with this microorganism, *P. oryzihabitans* PGP01, and not as an independent effect of the inoculation with this bacterium on the acclimatization process. Moreover, plant defence mechanisms induced by PGPR could contribute to a success in plant endurance (Chandra et al., 2010). Nevertheless, in this study, the mechanisms of action of these microorganisms have not been studied, being this point the focus of a future research.

5. Conclusions

To sum up, among the three microorganisms (*P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03) with a plant growth promoting effect in pear *in vitro* rooted plantlets, *P. oryzihabitans* PGP01 had no detrimental effects on the *in vitro* embryo rescue efficiency of three early ripening nectarine crosses. Even so, the subsequent seedlings from the embryos inoculated with *P. oryzihabitans* PGP01 displayed highly significant modifications in root morphology that improved the acclimatization to *ex vitro* conditions of those genotypes with difficult adaptability to the acclimation process. This project could mean the first step of a significant change in woody plants breeding programmes favouring the adaptation of plants whose

endurance under greenhouse conditions resulted complex. However, further investigations in this regard will be required in order to understand the mechanisms underlying the plant growth promotion induced by the three microorganisms tested as well as the strong root development and better survival observed in plants inoculated with *P. oryzihabitans* PGP01.

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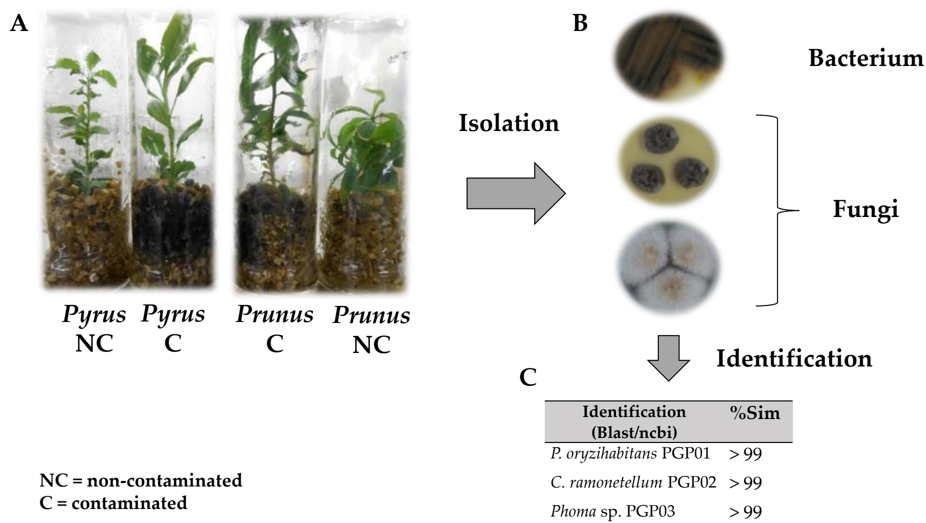
Supplementary data



Identification (Blast/ncbi)	%Sim
P. oryzihabitans PGP01	> 99
C. ramonetellum PGP02	> 99
Phoma sp. PGP03	> 99

Supplemental Figure S1. Presence of microbial contaminations (A), isolation (B) and identification (C) of microorganisms in *Prunus* and *Pyrus in vitro* cultures obtained by embryo rescue.

Chapter 2

Rhizosphere microorganisms enhance *in vitro* root and plantlet development of *Pyrus* and *Prunus* rootstocks

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Abstract

The rooting of fruit tree rootstocks is the most challenging step of the *in vitro* propagation process. The use of rhizosphere microorganisms to promote *in vitro* rooting and plant growth as an alternative to the addition of chemical hormones to culture media is proposed in the present study. Explants from two *Pyrus* (Py170 and Py12) rootstocks and the *Prunus* RP-20 rootstock were inoculated with *Pseudomonas oryzihabitans* PGP01, *Cladosporium ramotenellum* PGP02 and *Phoma* spp. PGP03 following two different methods to determine their effects on *in vitro* rooting and plantlet growth. The effects of the microorganisms on the growth of fully developed Py170 and RP-20 plantlets were also studied *in vitro*. All experiments were conducted using vermiculite to simulate a soil system *in vitro*. When applied to Py12 shoots, which is a hard-to-root plant material, both *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 fungi were able to increase the rooting percentage from 56.25% to 100% following auxin indole-3-butyric acid (IBA) treatment. Thus, the presence of these microorganisms clearly improved root development, inducing a higher number of roots and causing shorter roots. Better overall growth and improved stem growth of treated plants was observed when auxin treatment was replaced by co-culture with microorganisms. A root growth-promoting effect was observed on RP-20 plantlets after inoculation with *C. ramotenellum* PGP02, while *P. oryzihabitans* PGP01 increased root numbers for both Py170 and RP-20 and increased root growth over stem growth for RP-20. It was also shown that the three microorganisms *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 were able to naturally produce auxin, including indole-3-acetic acid (IAA), at different levels. Overall, our results demonstrate that the microorganisms *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 had beneficial effects on *in vitro* rooting and plantlet growth and could be applied to *in vitro* tissue culture as a substitute for IBA.

Keywords: Auxins; Fruit tree rootstocks; Micropropagation; Plant growth-promoting fungi; Plant growth-promoting rhizobacteria.

1. Introduction

Of the different steps of *in vitro* plant propagation, the rooting of micropropagated shoots of different fruit tree species belonging to the *Prunus* genus is not easy (Arab et al., 2018; Quambusch et al., 2016; Wiszniewska et al., 2016), and rooting enhancement is crucial to ensure plant production and survival in soil conditions. The main procedures used to stimulate the formation of roots in *in vitro* environments involve a reduction in the concentration of macronutrients, as well as the addition of exogenous auxin (Dobránszki & Teixeira da Silva, 2010; Goel et al., 2018; Iglesias et al., 2004; Lucchesini et al., 2019). The effectiveness of the process depends on several factors, including the genotype, the type of auxin and the dose of hormone applied (Magyar-Tábori et al., 2002; Ruzic & Vujovic, 2007).

In recent years, restrictions imposed by the European Commission concerning the use of chemicals in plant production (including auxin) have led to the development of new strategies to improve *in vitro* rooting using more ecological sources to avoid the application of exogenous auxin (Elmongy et al., 2018; Pacholczak et al., 2012). It is well known that some plant growth-promoting microorganisms (PGPMs) have an impact on *in vitro* plant development, increasing plant growth or the efficacy of the propagation and rooting of explants (Contesto et al., 2010; Trinh et al., 2018). Bacteria and fungi are able to produce hormones such as auxin or gibberellins (Iqbal & Hasnain, 2013; Meents et al., 2019; Waqas et al., 2012), making the use of microorganisms a promising alternative to the use of chemical compounds. In a study conducted by our research group, the plant growth-promoting effects of two fungi (*Cladosporium ramotenellum* PGP02 and *Phoma* spp. PGP03) and one bacterium (*Pseudomonas oryzihabitans* PGP01) isolated from *Pyrus* and *Prunus* endogenously contaminated *in vitro* cultures were reported (Cantabella et al., 2020).

On this basis, the aim of the present study involves the evaluation of the effects of these three microorganisms on root induction and development and on the growth of micropropagated plantlets from different rootstocks belonging to species of the *Pyrus* and *Prunus* genera. This application has generated universal interest in agricultural research as an instrument to increase abiotic stress tolerance or disease resistance (Asín et al., 2011; Elias-Roman et al., 2019; Riaz et al., 2019; Silva et al., 2019), and the use of *in vitro* tissue culture techniques serves as a way to study tolerance to abiotic stresses, such as tolerance to lime-induced chlorosis (Dolcet-Sanjuan et al., 2004a, 2008; Dolcet-Sanjuan et al., 1992; Donnini et al., 2009). To ensure better plant-microbe coexistence, an *in vitro* culture system using media comprising vermiculite

instead of the traditional approach in which semisolid agar-containing media are used was used in this study.

2. Material and methods

2.1. Plant materials and *in vitro* culture conditions

Rootstocks for which there is commercial interest for fruit production were used in this study. A *Prunus* rootstock marketed for commercial use named Rootpac® 20 (RP-20) (Agromillora Group, Barcelona, Spain) and two *Pyrus* rootstocks named "Py12" and "Py170", which are under agronomic evaluation by the IRTA, were propagated by axillary branching through *in vitro* shoot tip cultures. RP-20 is a natural hybrid between Myrobalan plum (*Prunus cerasifera* Ehr.) and almond (*Prunus dulcis*) and can be used as a rootstock for Japanese plum, peach, nectarine, almond and several apricot cultivars (Pinochet, 2010). Py170 is a hybrid between OH11 (Simard & Michelesi, 2002) and *Pyrus amygdaliformis*, while Py12 is derived from the open pollination of *Pyrus communis* cv. Williams. Both clones are in the last selection phase of an IRTA pear rootstock breeding programme that aims to obtain tolerance to both lime-induced chlorosis and reduced vigour.

Shoot-tip cultures of both *Pyrus* rootstocks (Py12 and Py170) and commercial *Prunus* (RP-20) rootstock were the source of shoot explants used to induce rooting and produce full plantlets. The three plant materials were propagated by axillary branching in MS media (Murashige & Skoog, 1962) supplemented with 5 μM benzylaminopurine (BAP), as described by Iglesias et al. (2004). For shoot elongation, 50 ml of liquid MS media without hormones was added to each flask after a 4-week-long culture period in 100 ml of semisolid multiplication media. Root induction on *in vitro* elongated shoots was conducted in ½-strength MS media supplemented with 10 μM indole-3-acetic acid (IAA), henceforth referred to as RIM-10IBA, for a 7-day-long period in darkness, followed by a transfer to ½-strength MS auxin-free media, henceforth referred to as root elongation media (REM). The *in vitro* coexistence of shoot explants or full plantlets with the microorganisms took place in ½-strength MS media without hormones but amended with vermiculite (50:40, v/v, vermiculite:medium), as described by Dolcet-Sanjuan et al. (2004b), henceforth referred to as root elongation media with vermiculite (REM-V). The pH of the different liquid media was adjusted to 5.7 using NaOH before the addition of gelling agar (8 g L^{-1} in RIM and REM and 6 g L^{-1} in REM-V). For semisolid agar-containing media, 15-mm-diameter tubes, each with 15 mL of media, were used. When REM-V

was used, 38-mm-diameter tubes, each containing 50 mL of vermiculite and 40 mL of semisolid media, were used. The media were autoclaved at 121°C for 20 min, and once they had cooled, they were stored at 14°C. All *in vitro* cultures, including those in co-culture with the microorganisms, were maintained in a culture chamber set at 24°C and providing 100-120 μmol m^{-2} s^{-1} cool-white fluorescent light under a 16-h-light photoperiod.

2.2. *In vitro* root induction and development in the presence of microorganisms

The effects of co-culture with microorganisms during root induction and development were evaluated using 3-cm-long elongated shoots of RP-20, Py12 and Py170. Two different protocols were followed to study the impact of the three microorganisms on *in vitro* rooting. In the first protocol (Method 1), elongated shoots were transferred to RIM-10IBA for 7 days in dark conditions. Afterward, the shoots were transferred to REM-V and immediately inoculated with 1 mL of the microorganism suspension. In the second protocol (Method 2), elongated shoots were immersed for 5 min in 1 mL of microorganism suspension and then placed in a sterile 2-cm-diameter well, which enabled immersion of 3 mm of the shoot base. Afterward, the shoots were cultured in tubes with REM for one week to ensure the microorganisms grew in direct contact with the shoot base. After this period, the shoots were transferred to REM-V to favour root development, as was done for method 1. For both methods, a total of fifteen shoots per treatment were used. Four treatments per experiment were used, namely, inoculation with PGP01 (*P. oryzihabitans*), inoculation with PGP02 (*C. ramotenellum*), inoculation with PGP03 (*Phoma* spp.) and inoculation with a control. The control treatment involved the addition of 1 ml of sterile water for method 1 or immersing the shoot base in 1 mL of sterile water for method 2. After 8 weeks of co-culture, the rooting percentage was calculated by dividing the number of rooted shoots by the total number of shoots. Morphometric and photosynthetic parameters, including the number of leaves, root number, stem length (cm), root length (cm), total plant fresh weight (FW, in g), root fresh weight (g) and stem fresh weight (g) were measured for each plantlet, as described by Cantabella et al. (2020).

2.3. *In vitro* plant development in the presence of microorganisms

To test whether *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 had plant growth-promoting effects, *in vitro* fully developed RP-20 and Py170 plantlets were used. In previous experiments (Cantabella et al., 2020), the three

microorganisms were able to increase the FW, stem length and root length of rooted pear seedlings derived from *in vitro* embryo rescue.

For these assays, a total of ten 3-cm-long micropropagated shoots per treatment were induced for rooting in RIM-10IBA for 7 days, followed by a 1- to 2-week-long culture period in REM to favour root elongation and development. After this period, the shoots that displayed visible roots were removed from the agar-containing semisolid media and transplanted into REM-V, followed by inoculation with 1 ml of each microorganism suspension or the same volume of sterile double distilled water for the control treatment. After 8 weeks of co-culture, morphometric and photosynthetic parameters, including the number of leaves, root number, stem length (cm), root length (cm), total plant fresh weight (FW, in g), root fresh weight (g) and stem fresh weight (g) were measured for each plantlet. The root fresh weight (g)/stem fresh weight (g) ratio was calculated for each plantlet and used as an indicator of differential plant growth distribution.

2.4. Preparation of microorganism inocula

In the present study, the bacterium *P. oryzihabitans* PGP01 and fungi *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 belonging to the IRTA postharvest programme PGPMs collection, were used to test their potential effects on the *in vitro* rooting and growth of *Prunus* and *Pyrus* rootstocks. These microorganisms were isolated from *Pyrus* and *Prunus* embryos germinated in an aseptic environment and preliminarily tested in *in vitro* rooted pear plantlets, the results of which showed beneficial effects (Cantabella et al., 2020). *P. oryzihabitans* PGP01 cultures preserved at -80°C were grown in nutrient yeast dextrose agar (NYDA: nutrient broth, 8 g L^{-1}; yeast extract, 5 g L^{-1}; anhydrous glucose, 10 g L^{-1}; and agar, 15 g L^{-1}) plates at 25°C, while *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 cultures were grown in potato dextrose agar (PDA: potato tissue, 200 mL; glucose, 20 g L^{-1}; and agar, 20 g L^{-1}) plates at the same temperature. Forty-eight-hour-old plates of bacteria and 14-d-old plates of both fungi were used to prepare the microorganism suspensions. *P. oryzihabitans* PGP01 cells were collected in phosphate buffer (70 mL KH_2PO_4 0.2 M; 30 mL K_2HPO_4 0.2 M; 300 mL of deionized water) and washed by centrifugation at 6164 × *g*. After suspension in phosphate buffer, the bacterial concentration was adjusted with sterile distilled water at $2x10^8$ CFU mL^{-1} by measuring the absorbance at 420 nm with a spectrophotometer (SP-2000 UV, Shanghai Spectrum Instruments Co., Ltd., Shanghai, China). The conidia of both *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 were measured using a haemocytometer, and the concentration was ultimately set to $2x10^6$

spores mL^{-1} with sterile distilled water (sp mL^{-1}). In both cases, the plate dilution technique on solid PDA and NYDA media was applied to calculate the true colony forming units (CFU mL^{-1}).

2.5. Determination of auxin production via spectrophotometry

The indole-3-acetic (IAA) production ability of *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 was tested spectrophotometrically according to the methods described by Gordon and Weber (1951). *P. oryzihabitans* PGP01 bacteria and *C. ramotenellum* PGP02 and Phoma spp. PGP03 fungi were grown in flasks containing 50 mL of tryptone soy broth (TSB) and potato dextrose broth (PDB) supplemented or not supplemented with 0.5 and 1 g L^{-1} tryptophan for 24 h and 168 h, respectively, and incubated at 25°C and 150 rpm. Bacterial and fungal cultures were pelleted by centrifugation for 10 min at 6164 × *g*, and 1 mL of the supernatants was tested for the presence of indole compounds using 2 mL of Salkowski reagent (1 mL of 0.5 M $FeCl_3$ in 50 mL of 35% $HClO_4$). After 25 min of incubation in darkness, the absorbance at 530 nm was measured with an SP-2000 UV spectrophotometer (Shanghai Spectrum Instruments Co., Ltd., Shanghai, China). The content of auxin was determined via a standard curve of synthetic IAA (Duchefa Biochemie, Haarlem, The Netherlands) at different concentrations (from 0 to 20 μg mL^{-1}) and treated in the same way as were the bacterial and fungal supernatants.

2.6. Data analysis

The different experiments were designed in accordance with a completely randomized design (CRD), and the data were analysed by one- or two-way factorial ANOVA using JMP Pro Software (version 13.1.0, SAS Institute, Cary, NC, USA). Different letters were used to denote significant differences according to Student's t-test ($P \leq 0.05$). Statistical significance was judged at the level $P < 0.05$, and Tukey's test was used to separate the means within one factor when the differences were statistically significant. Significant differences in *in vitro* rooting percentages between treatments and the control were analysed via Fisher's exact test ($P \leq 0.05$).

3. Results

3.1. Effects of the applications of three microorganisms on *in vitro* root induction, development and biometric parameters

To study the effect of the three microbes *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 on *in vitro* root induction and plantlet development, microorganisms were applied following two different methods: inoculation after root induction in the medium supplemented with 10 µM IBA (RIM-10IBA, Method 1), and immersion of the shoot basis in microbe suspensions without root induction with IBA (REM, Method 2). Using these methods, the effects of these microorganisms were tested in three rootstock genotypes: the easy-to-root *Prunus* RP-20, the easy-to-root *Pyrus* Py170 and the hard to root *Pyrus* Py12. To ensure the coexistence of plants and microorganisms, all the experiments were conducted in REM supplemented with vermiculite (REM-V). After 8 weeks of co-culture, the *in vitro* rooting percentage, as well as several parameters regarding root induction and development (number of roots and root length) and plantlet development (plant, shoot and root FW) were measured.

As expected, the plant material had an important effect on the rooting response. The control treatment of the shoots, with neither inoculation with microorganisms (Method 1) nor immersion into suspensions of microorganisms (Method 2), induced to root in RIM-10IBA (Method 1) or by a 7-day-long culture in REM (Method 2), followed by an 8-week-long culture in REM-V, induced 100% rooting on RP-20 and Py170, while it was only 56.3% for Py12, a hard-to-root *Pyrus* clone (Table 1).

Inoculation with *P. oryzihabitans* PGP01 or *C. ramotenellum* PGP02 through either methodology had no detrimental influence on rooting percentage, which remained at 100%, for the easy-rooting clones RP-20 or Py170 (Table 1). For Py12 in co-culture with *C. ramotenellum* PGP02 and *Phoma* spp. PGP03, the rooting percentage doubled, increasing to 100% when root elongation (Method 1) or root induction and elongation (Method 2) were performed in co-culture with the tested microorganisms (Table 1).

Table 1. Effect on the *in vitro* rooting percentage (%) of the inoculation with *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 in the *Prunus* rootstock RP-20 and the two *Pyrus* rootstocks Py170 and Py12 after root induction with 10 µM IBA (Method 1) or by immersion of the shoots in microorganism's suspensions without rooting induction (Method 2). Asterisk (*) symbol means significant difference between control and treatments according to the Fisher's Exact Test ($P \leq 0.05$).

% *in vitro* rooting						
	Method 1			**Method 2**		
	RP-20	**Py170**	**Py12**	**RP-20**	**Py170**	**Py12**
Control	100	100	56.25	100	100	100
***P. oryzihabitans* PGP01**	100	100	56.25	100	100	100
***C. ramotenellum* PGP02**	100	100	100*	100	100	100
***Phoma spp.* PGP03**	100	100	100*	100	86.7	100

The effect of the microorganisms on the number of developed roots ($P < 0.001$) and the length of rooted shoots of RP-20, Py170 and Py12 (Figure 1A) were highly influenced ($P < 0.001$) by the method of inoculation. After the first protocol, we observed that *P. oryzihabitans* PGP01 was able to induce a 35% significant increase in the number of roots in *Prunus* RP-20 shoots but not in either the *Pyrus* Py170 or Py12 rootstocks (Figures 1A and 2). However, this increase in the number of roots was not accompanied by a greater root length for either of the three tested plant genotypes compared with non-treated plantlets (Figures 1B and 2). Inoculation with *C. ramotenellum* PGP02 led to a higher number of roots for the three plant genotypes tested compared with their respective controls, with increases of 57, 54 and 312% for RP-20, Py170 and Py12, respectively (Figures 1A and 2). Together with the positive effect of inoculation with this microorganisms on the number of roots, a significant (135%) increase in root length was observed only in *Pyrus* rootstock Py170 after 8 weeks of co-culture (Figures 1B and 2). *Phoma* spp. PGP03 drastically affected this parameter in the hard-to-root Py12 rootstock compared with the non-treated plantlets; the root length increased 225% after 8 weeks of co-culture (Figures 1A and 2). However, a significant inhibition in root elongation was reported for the RP-20 and Py12 genotypes after inoculation with *Phoma* spp. PGP03 in comparison to that of the control plants (Figures 1B and 2).

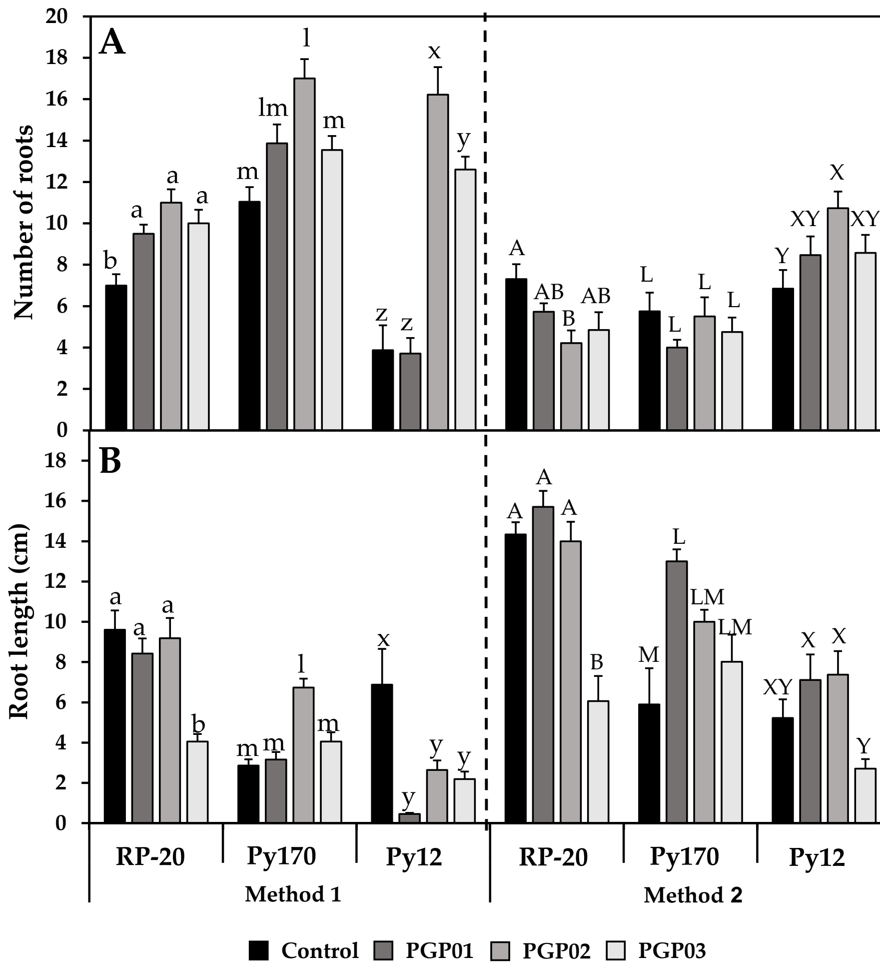

Figure 1. Effects of *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 on the number of roots (A) and root length (B) of *Prunus* RP-20 and *Pyrus* Py170 and Py12 plantlets after 8 weeks of co-culture and inoculation by two different methods. In method 1, the shoots were inoculated with microorganisms after root induction in 10 mM IBA, while in method 2, the shoots were immersed in microorganism suspensions without previous root induction with IBA. In all cases, the data represent the means ± SEs (standard errors) of at least fifteen shoots each. The means with different letters within each genotype are significantly different according to Tukey's test ($P < 0.05$).

Overall, the number of roots observed on RP-20 and Py170 shoots was similar or lower than that on the control plants when they were immersed in the microorganism suspensions (Method 2) with no previous exogenous auxin treatment (Figure 1A). However, with the Py12 clone, inoculation with *C. ramotenellum* PGP02 significantly increased the number of roots (almost 50% higher than those of the

control) (Figures 1A and 2). Regarding root length, inoculation by root immersion in the suspension of *P. oryzihabitans* PGP01 induced significantly longer roots (110% increase) from shoots of Py170 (Figures 1B and 2). As observed with Method 1, *Phoma* spp. PGP03 inhibited root elongation in RP-20 and Py12, and this reduction was significant only in RP-20 (58% lower than that of the control) (Figures 1B and 2).

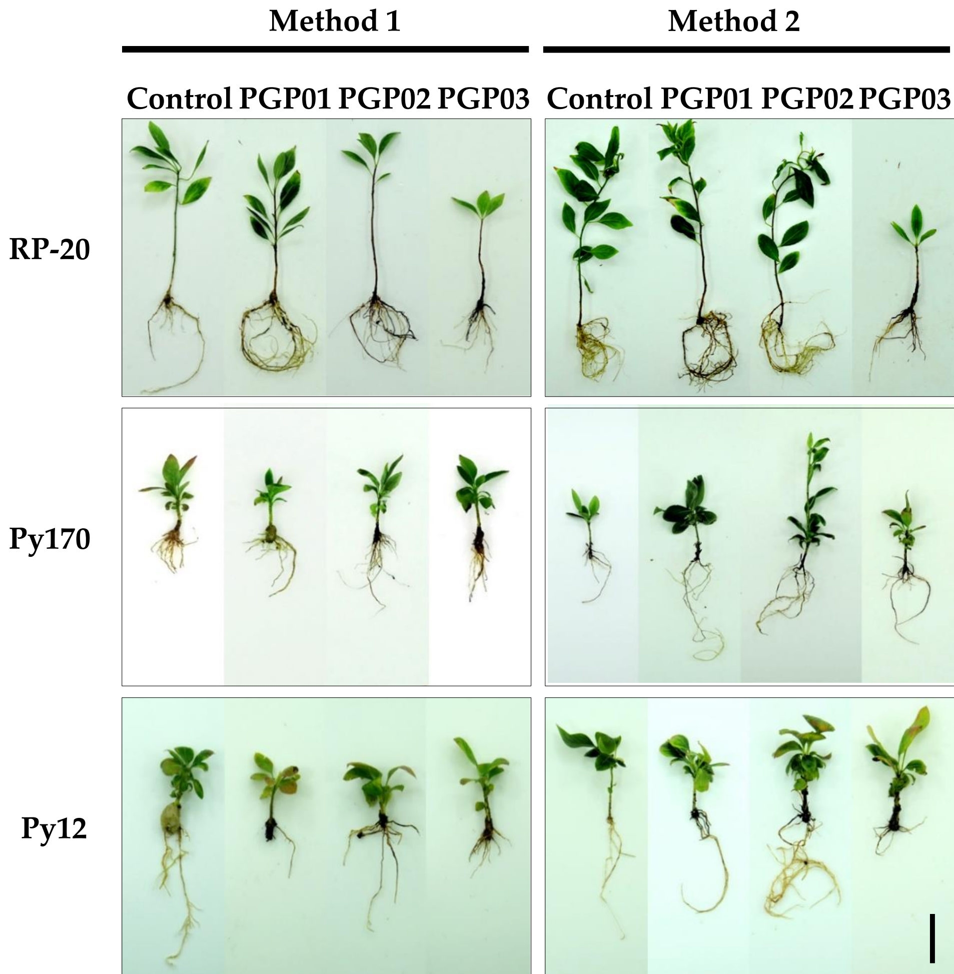

Figure 2. Plantlets of *Prunus* RP-20 and *Pyrus* Py170 and Py12 after an 8-week-long co-culture with *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03. The shoots were inoculated with microorganisms after root induction with 10 mM IBA (Method 1) or immersed in microorganism suspensions without previous IBA root induction (Method 2). The black bar is equivalent to 2 cm of length.

Regardless of the genotype, the effects of the three microorganisms on the total plant ($P < 0.001$), shoot ($P < 0.001$) and root FW ($P = 0.003$, $P = 0.004$ and $P < 0.001$ for RP-20, Py170 and Py12, respectively) were affected by the method used for their application (Figure 3A, B, C). After inoculation, during root elongation (Method 1) of RP-20 and Py170 with the suspension of *P. oryzihabitans* PGP01, while plant FW was not affected, an important reduction in this parameter was reported in the hard-to-root Py12 genotype (Figure 3A). The reduction in shoot FW occurred in the RP-20 and Py12 plantlets in response to inoculation with *P. oryzihabitans* PGP01 (48 and 67% lower than that of the control, respectively). Inoculation with *P. oryzihabitans* PGP01 following this method induced a nearly 70% increase in RP-20 root FW (Figure 3C). However, the plant, shoot and root FWs remained unchanged or were lower than those of the control plants after 8 weeks of co-culture with both *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 inoculated according to Method 1 (Figure 3A, B, C).

In Method 2, inoculation with *P. oryzihabitans* PGP01 significantly increased the plant and shoot FW of *Pyrus* Py170 rootstock (225% higher than that of the control) but not in RP-20 or Py12 rootstock (Figure 3A). When this bacterium was applied via this method, we reported an increase in root FW for all three genotypes: RP-20, Py170 and Py12 (68, 120 and 80% more than that of the non-treated plantlets) (Figure 3C). In this sense, it is important to highlight that this increase in root FW observed for RP-20 in response to inoculation with *P. oryzihabitans* PGP01 was quite similar to that obtained via Method 1.

Surprisingly, *C. ramotenellum* PGP02 significantly promoted plant, shoot and root FW of *Pyrus* rootstocks Py170 and Py12, whereas no effects on the *Prunus* RP-20 rootstock occurred after 8 weeks of co-culture with this microbe (Figure 3A, B, C). Finally, while a negative effect of the application of *Phoma* spp. PGP03 was observed on RP-20 shoots, the plant, shoot and root FW decreased (74, 86 and 171% lower than those of control, respectively), but no significant changes in these parameters were found in Py170 or Py12 (Figure 3A, B, C).

In general, inoculation with the three microorganisms following Method 1 stimulated root development, mainly increasing the number of roots of RP-20, Py170 and Py12 explants. On the other hand, increased growth of plantlets was observed when the inoculation was carried out by immersing the shoots in *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 suspensions, since better values of plant, shoot and root FW were observed in response to these microbes in the three genotypes tested in the study.

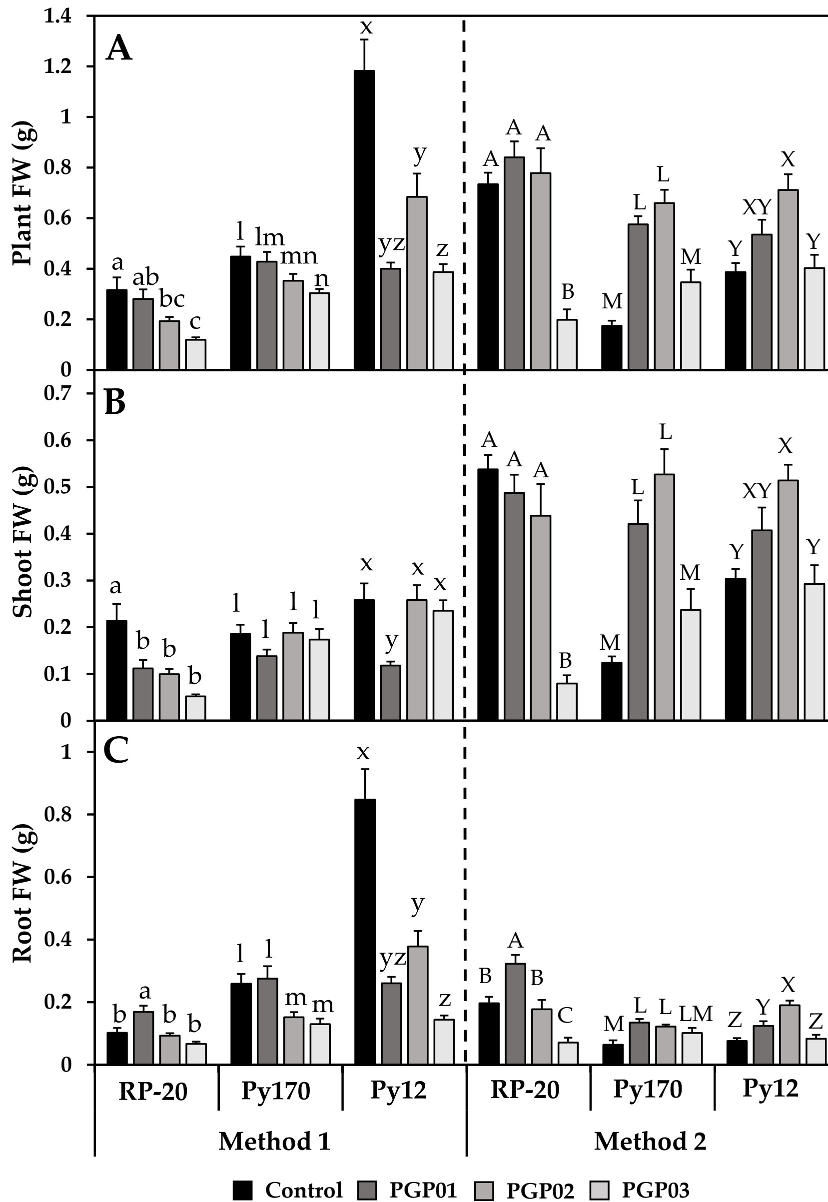

Figure 3. Effects of *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 on plant (A), stem (B) and root (C) FW of *Prunus* RP-20 and *Pyrus* Py170 and Py12 plantlets after 8 weeks of co-culture after inoculation through two different methods. In Method 1, the shoots were inoculated with microorganisms after root induction in 10 mM IBA, while in Method 2, the shoots were immersed in microorganism suspensions without previous root induction with IBA. In all cases, the data represent the means ± SEs of at least fifteen plants. The means with different letters within each genotype are significantly different according to Tukey's test ($P < 0.05$).

3.2. *In vitro* plantlet development in the presence of microorganisms

In this experiment, we focused our efforts in elucidating the effect of these three microorganisms *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 on *in vitro* plant development of the two rootstock genotypes *Prunus* RP-20 and *Pyrus* Py170. For that purpose, *in vitro* micropropagated explants were inoculated with the three microorganisms in REM-V after root induction and development in RIM-10IBA and REM, respectively. Again, several biometrical parameters including the number of roots, root and shoot FW and root/shoot FW ratio were measured after 8 weeks of co-culture with microbes.

Plant material had a significant effect on root and plantlet development after inoculation with microorganisms, and this interaction was significant for the number of roots ($P < 0.001$), shoot FW ($P = 0.002$), root FW ($P = 0.001$) and root/shoot FW ratio ($P = 0.002$). In this sense, we observed that *P. oryzihabitans* PGP01 positively affected all the parameters concerning root growth for the RP-20 plants, such as the number of roots, root FW and the root/shoot FW ratio compared with those of the non-treated plantlets (Figure 4A, B and D). This root-localized effect was also observed when the bacterium was applied to Py170 *in vitro* explants, since a significant increase in the number of roots was recorded after 8 weeks of co-culture (Figures 4A and 5). On the other hand, both plant genotypes behaved differently after inoculation with *C. ramotenellum* PGP02. The application of the fungus favoured the root growth of RP-20 *in vitro* plantlets, increasing their root FW (129% compared with that of the control plants). This fungus also increased shoot length compared with that of the non-treated plantlets (data not shown). Nevertheless, only shoot growth increased when the application was carried out on the *Pyrus* Py170 explants (Figures 4B, C and 5). In this sense, the shoot FW significantly increased by only 32% compared with that of the control plantlets. Again, no significant plant growth promotion was observed in response to inoculation with *Phoma* spp. PGP03, except for the higher number of roots observed for Py170 plantlets after 8 weeks of co-culture with this fungus (Figures 4A and 5). Other parameters, including root length and number of leaves, were not significantly affected by either of the three microbes (data not shown).

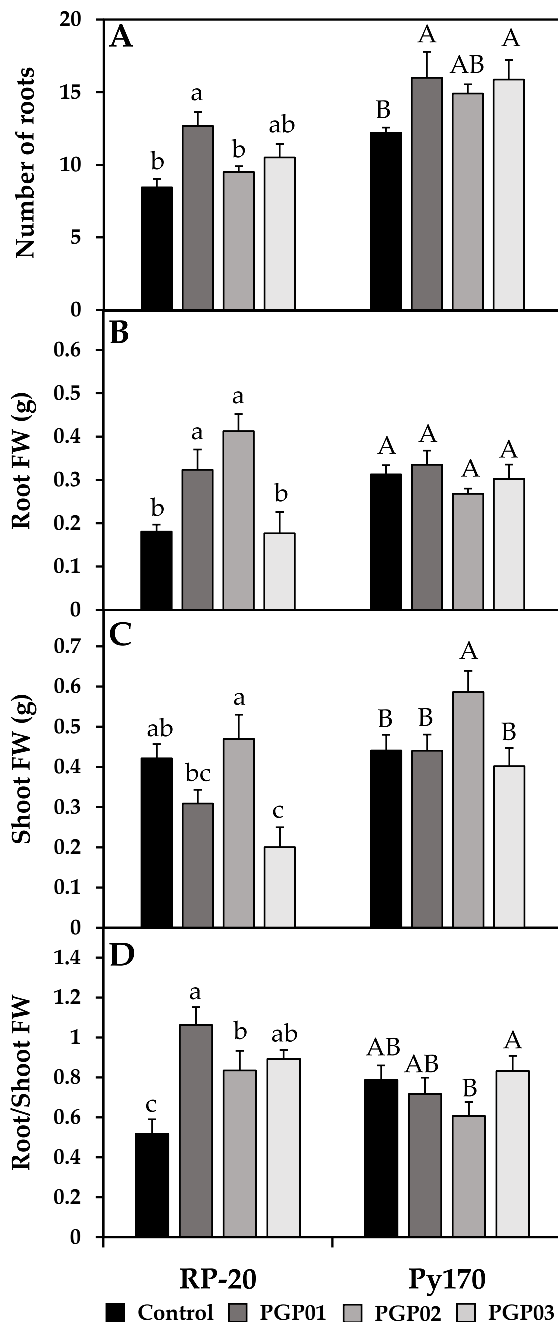

Figure 4. Effects of inoculation with *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 on the number of roots (A), root FW (B), shoot FW (C) and root/stem FW ratio (D) of *Prunus* RP-20 and *Pyrus* Py170 fully developed plantlets. The different lowercase and uppercase letters refer to significant differences between the control and treatments for RP-20 and Py170, respectively, according to Tukey's test ($P < 0.05$).

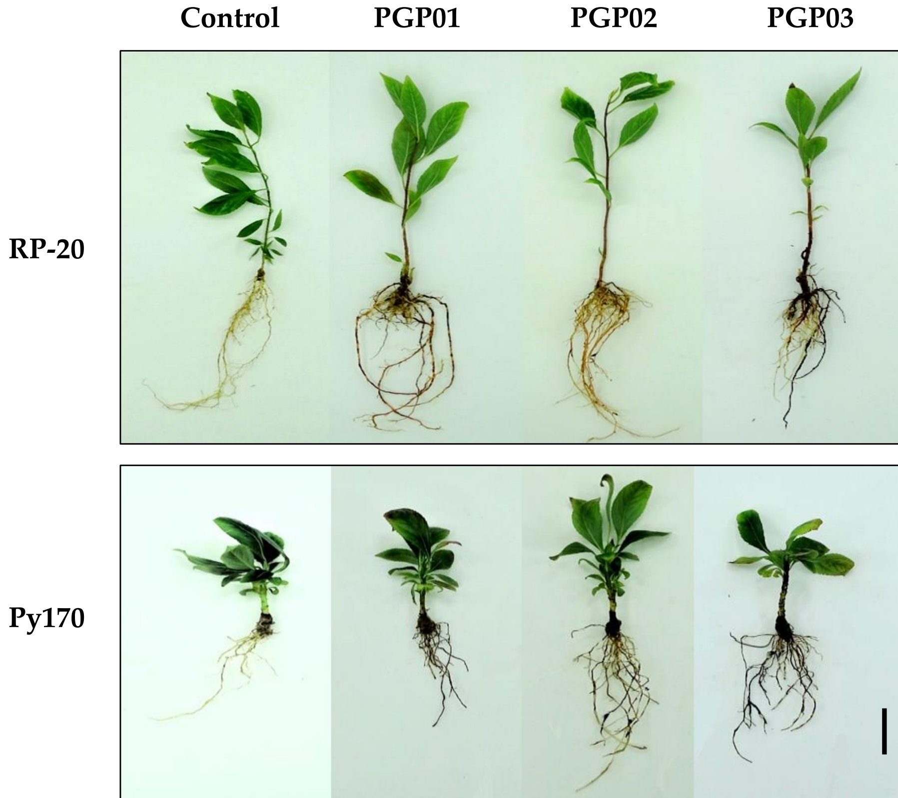

Figure 5. *Prunus* RP-20 and *Pyrus* Py170 plantlets after 8-week-long co-culture with *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03. The black bar is equivalent to 2 cm of length.

3.3. Determination of IAA in *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 cultures.

The content of IAA was determined in *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 cultures. The ability to produce auxins was suggested in the three microorganisms, as significant increases in the IAA content were detected when the different culture media were supplemented with Trp. In this context, it is noteworthy to mention that the concentration of IAA detected in *P. oryzihabitans* PGP01 cultures was higher than that observed for *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 In the case of *P. oryzihabitans* PGP01, an increase in the IAA content was observed when the tryptophan was added to the medium in a dose-dependent manner, registering a 4.35- and a 7.07-fold increase at 0.5 and 1 g L^{-1}, respectively (Figure 6). On the other hand, IAA was also found in *C. ramotenellum* PGP02 cultures when fungal cells were grown in PDB without Trp (Figure 6), and these levels

remained unchanged when the lowest concentration of tryptophan was tested. However, the addition of 1 g L^{-1} induced a 3.57-fold increase in IAA levels in comparison to those recorded in PDB without tryptophan (Figure 6). Regarding PGP03, the addition of tryptophan at 0.5 or 1 g L^{-1} to the PDB medium induced significant increases on IAA content in comparison to non-supplemented medium (3.2 and 3.7-fold), but, in that case, no differences between both doses of tryptophan were registered (Figure 6).

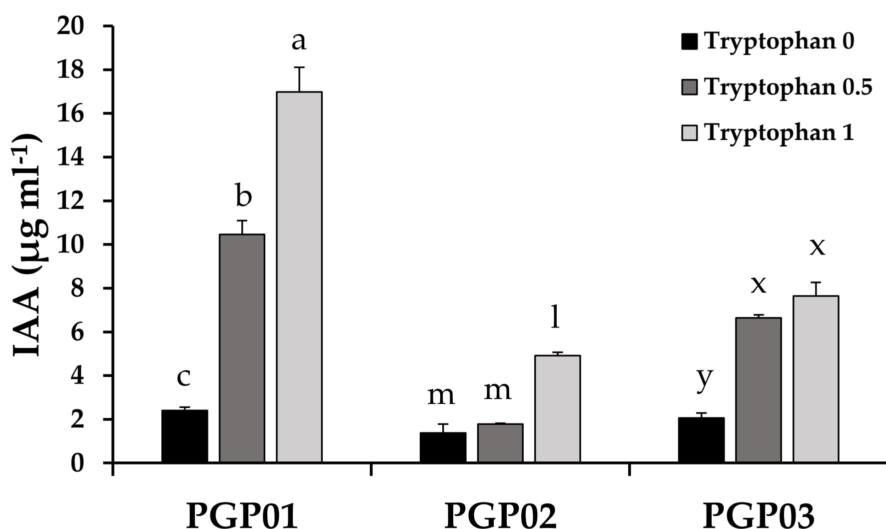

Figure 6. IAA production in *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 cultures. Bacteria and fungi were grown in TSB and PDB media, respectively, supplemented with 0.5 (dark grey bars) and 1 g L^{-1} tryptophan (light grey). Supernatants were collected after 24 and 168 h in the case of bacteria and fungi, respectively, and the absorbance at 530 nm was measured after 25 min of incubation with 2 mL of Salkowski reagent. The bars represent the means ± SEs of three replicates per treatment. The different lowercase and uppercase letters refer to significant differences between tryptophan-treated and non-treated media according to Tukey's test ($P < 0.05$).

4. Discussion

Rootstocks have important agronomic value in fruit tree crops since they are used for grafting commercial varieties, providing desirable fruit production and quality and tree tolerance to edaphic factors associated with abiotic and biotic stresses (Prodhomme et al., 2019). Some clones selected for their desirable agronomic characteristics, especially those clones that result in low vigour, have poor rooting efficiencies and root development, such as the following: apple rootstock M9 (*Malus*

x *domestica* Borkh) (Amiri & Elahinia, 2011; Sun & Bassuk, 1991); pear rootstocks OHxF 333, Brossier P.2274 and P.227 (Mielke & Turner, 2008; Necas & Kosina, 2008); and peach rootstocks Lovel and *Prunus americana* (Mayer et al., 2015). As a consequence, *in vitro* propagation is not feasible. Herein, owing to its tolerance to lime-induced chlorosis and reduced vigour (Asín et al., 2011), Py12, a pear rootstock clone selected in an IRTA breeding programme, also has low rooting efficiency compared to that of another *Pyrus* rootstock, Py170, or the commercial *Prunus* rootstock Rootpac® 20 (RP-20). When *in vitro* rooting is the main obstacle to obtaining complete viable plants in woody plant species (Wiszniewska et al., 2016), research to improve the efficiency of this step frequently emphasizes determining the hormone type and level (Dobránszki & Teixeira da Silva, 2010) to be added during *in vitro* growth, though the results are highly dependent on genotype. In three apple rootstocks (M.26, MM.106 and JTE-H) in which three different levels of IBA were used, different responses in terms of rooting percentage and root length were observed for the different genotypes (Magyar-Tábori et al., 2002).

Improvement in root development in peach and pear embryo-derived plantlets inoculated with the microorganisms used in the present work was reported in a previous work (Cantabella et al., 2020). Therefore, an *in vitro* model in which these three microorganisms (*P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03) were applied to *in vitro* clonally propagated *Prunus* RP-20 and *Pyrus* Py12 and Py170 rootstocks at different stages of *in vitro* development was constructed to determine their effects on root induction, root development, and whole-plantlet *in vitro* growth. Two independent experiments were conducted: first, to study the effects of the microorganisms on the *in vitro* rooting efficiency of shoots, with or without an IBA root induction treatment, and second, to study the microorganisms' effects on *in vitro* whole-plantlet growth. Inoculation of microorganisms following the two different methods has been previously reported in semiwoody olive microcuttings under nursery conditions (Montero-Calasanz et al., 2013). Herein, to improve the coexistence between shoots or plantlets and microorganisms, vermiculite was added to the culture media under *in vitro* conditions. In the first method, in which IBA was used to induce rooting, which is the standard protocol (Iglesias et al., 2004; Murashige & Skoog, 1962), *Pyrus* Py12 shoots showed 56.25% rooting, while in the presence of *C. ramotenellum* PGP02 and *Phoma* spp. PGP03, the *in vitro*-rooted shoots completely rooted (100%). In accordance with our results, other endophytic bacterial isolates demonstrated that inoculation of *in vitro* microshoots of two *Prunus avium* genotypes resulted in increased rooting percentage (Quambusch et al., 2014). Additionally, the results obtained with method 2, without IBA treatment of the shoots, confirmed that

the *P. oryzihabitans* PGP01 bacterium and both *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 fungi could act as a natural root-promoting source, likely due to their ability to produce IAA. In this context, it has been proven that the bacterium *P. oryzihabitans* PGP01 was able to produce a higher amount of IAA than both fungi. All inoculated and control shoots, including those of Py12, rooted after 8 weeks of culture, except those inoculated with *Phoma* spp. PGP03, which showed a decrease in this parameter. On the other hand, in the IBA-induced shoots (Method 1), invariably reduced growth was observed in all plantlets compared with those derived from Method 2. Nonetheless, the three microorganisms increased the number of roots per plantlet in the three different rootstocks in a genotype-dependent way, and these increases were statistically significant for *C. ramotenellum* PGP02 in all three tested rootstocks, *P. oryzihabitans* PGP01 in RP-20 and *Phoma* spp. PGP03 in RP-20 and Py12.

In the present work, the ability of both *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 to produce IAA could explain the increase in the number of roots. *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02, in the absence of IBA (Method 2), led to better root elongation and shoot growth – more significantly in the Py170- and Py12-treated shoots. Regarding *Phoma* spp. PGP03, no important effects on root length were observed in this method in spite that the ability to produce auxins was proven. Studies revealing the use of microorganisms in microshoots to induce rooting while avoiding the application of exogenous auxin are scarce, and some authors have reported positive effects of other natural compounds such as humic acid on improving the *in vitro* rooting efficiency (Elmongy et al., 2018). Herein, though an improvement in rooting percentage was proven only for Py12 shoots, better growth of the three rootstocks (Py170, Py12 and RP-20) was observed after exposure to *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02, and this technique is a promising alternative to the use of chemical hormones in plant production. Further assays employing clonal rootstocks with a compromised rooting ability under *in vitro* conditions, such as M9, OHxF 333, Brossier P.2274 and P.2276, Lovell or *P. americana,* will need to be carried out to corroborate and verify this effect. The performance of rhizobacteria in the *in vitro* rooting process is highly documented (Burygin et al., 2019; Kavino & Manoranjitham, 2018; Perez-Rosales et al., 2018; Quambusch et al., 2014); however, very little is known about the role of fungi in plant root development. It has been previously established that some beneficial fungi are able to produce and accumulate auxin in their mycelia, which could increase the levels of auxin as well as auxin-responsive plant gene expression in *Arabidopsis thaliana* (Meents et al., 2019). The present research constitutes the first work showing the auxin production ability of *C. ramotenellum*. In the case of *P. oryzihabitans*, Belimov et al. (2015) already reported

the ability of this bacterium to produce different indole compounds. In the same study, potato (*Solanum tuberosum*) plants showed increased root biomass when *P. oryzihabitans* was present in the rhizosphere compared to that of non-inoculated plants.

A different approach was conducted to analyse whether *P. oryzihabitans* PGP01 bacteria and the two *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 fungi are microorganisms with potential plant growth-promoting effects under *in vitro* conditions. For this purpose, fully developed Py170 and RP-20 plantlets were used to evaluate whether they were able to improve root and shoot growth. The effects in response to inoculation with *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 were somehow different depending on the genotype. In *Prunus* RP-20 rootstocks, a greater root FW was observed in response to *C. ramotenellum* PGP02, and a significantly higher number of roots was registered in Py170 after the inoculation with *Phoma* spp. PGP03. These results might be explained by the ability of both fungi to produce auxins. However, the clear effect of *C. ramotenellum* PGP02 on the growth of the aerial parts of Py170 and RP-20 suggested the role of other compounds produced by the fungus instead of auxin. Some fungi play a crucial role in plant growth and development, mostly due to the endophytic production of plant hormones such as gibberellins (GAs), which are involved in important physiological processes, including stem elongation (Calvo et al., 2014). Previous studies have demonstrated the ability of different isolates from rhizosphere fungi belonging to the *Cladosporium* and *Phoma* genera to produce GAs, which resulted in increased growth of soybean and cucumber seedlings (Hamayun et al., 2009, 2010). Plant growth promotion in *Atriplex gmelinii* by a gibberellin-producing strain of *Gliomastix murorum* was reported (Khan et al., 2009). Thus, considering the above reference, our results could suggest that GAs play a role in the enhancement of shoot growth induced by *C. ramotenellum* PGP02 in *Pyrus* Py170 rootstock. On the other hand, the bacterium *P. oryzihabitans* PGP01 tended to act more specifically in the roots, increasing the number of roots for both Py170 and RP-20 rootstocks, as well as the root FW and root/shoot FW ratio solely in RP-20. Reprogramming root system architecture in response to rhizobacteria belonging to the *Pseudomonas* and *Bacillus* genera is mediated by auxin signalling in *A. thaliana* (López-Bucio et al., 2007; Zamioudis et al., 2013). Pourjasem et al. (2020) recently reported that bacteria belonging to the *Pseudomonas* genus are able to release some elements into the culture media as a result of the decomposition of minerals present in vermiculite that might also favour root development. These results represent a step forward compared with those of a previous publication where the effects of the three microorganisms were described in

Prunus in vitro rescued embryos for the first time (Cantabella et al., 2020). However, more comprehensive studies are being undertaken to corroborate that this mechanism occurs in Py170 and RP-20 plants with the studied microorganisms.

5. Conclusions

In summary, the two different procedures concerning inoculation of *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 did not reduce the *in vitro* rooting percentage of the efficient-to-root *Pyrus* and *Prunus* rootstocks Py170 and RP-20. On the hard-to-root *Pyrus* Py12 rootstock, both fungi increased the rooting percentage from 56.25% to 100%. As a consequence of the auxin production ability by some of the microorganisms, a higher number of roots was observed with these microorganisms when co-cultured after IBA induction, and increased plantlet growth was observed when no synthetic auxin was used. Due to the strong potential of rootstocks in fruit tree production, this study constitutes a worthwhile approach to improve the rooting efficiency of difficult-to-root genotypes such as Py12. Furthermore, while *P. oryzihabitans* PGP01 primarily showed a root growth-promoting effect on both RP-20 and Py170 fully developed *in vitro* plantlets, a location-dependent effect in response to inoculation with *C. ramotenellum* PGP02, which positively affected the roots of RP-20 and shoots of Py170 rootstocks, was observed. The present results could suggest that auxin and gibberellins play a role, and the mechanisms underlying this plant growth promotion will be studied in the future.

6. Abbreviations

CRD, completely randomized design; NYDA, nutrient yeast dextrose agar; PDA, potato dextrose agar; PDB, potato dextrose broth; PGPMs, plant growth-promoting microorganisms; REM, root elongation media; REM-V, root elongation media with vermiculite; RIM, root induction media; TSB, tryptone soy broth.

7. Acknowledgements

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8. Author contribution statement

DC, NT and RDS contributed to the design of the study. All the experiments, as well as the data collection, were conducted by DC, MC, and GS. The data analysis and interpretation were performed by DC, NT, RT, and RDS. The writing and revision of the manuscript were performed by DC, NT, RT, and RDS. All the authors have read the article and made critical contributions to improve the quality of the manuscript.

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Chapter 3

Regulation of root architecture by *Pseudomonas oryzihabitans* is mediated by strigolactones and redox processes

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Abstract

Mechanisms that control root system architecture (RSA) are well characterised but little is known about how these processes respond to plant growth promoting rhizobacteria. Therefore, it was study how the presence of *Pseudomonas oryzihabitans* PGP01 altered wild type RSA and how these changes were modified in mutants that are defective antioxidant capacity (*vtc2-1*, *vtc2-2*, *pad2-1, cad2-1 and rax1-1*) or strigolactone (SL) synthesis (*max3-9* and *max4-1*) or signalling (*max2-3*). The presence of *P. oryzihabitans* PGP01 decreased the primary root and increased lateral root density in the wild type roots but not in the SL mutants. The presence of synthetic SL, GR24 in combination with *P. oryzihabitans* PGP01 significantly decreased the number and length of lateral roots in the WT, *max3-9* and *max4-1* but not *max2-3* seedlings. Lateral root density was increased in all genotypes in the presence of bacteria, but this effect was less pronounced in the ascorbate deficient *vtc2-1* and *vtc2-2* and glutathione –deficient seedlings than the wild type. Moreover, the number and length of lateral roots, was significantly decreased in the wild type seedlings in the presence of *P. oryzihabitans* but not in the GSH-deficient mutants (*pad2-1, cad2-1 and rax1-1*). Taken together, these results demonstrate the importance of SL-mediated signalling in root responses to growth promoting rhizobacteria, as well roles of cellular redox controls in these processes.

Keywords: antioxidants, ascorbate, glutathione, root system architecture, strigolactones, plant growth promoting rhizobacteria, *Pseudomonas oryzihabitans.*

Summary statement

The presence of the plant growth promoting rhizobacterium, *Pseudomonas oryzihabitans* alters root system architecture in *Arabidopsis thaliana* in a strigolactone- and glutathione- (but not ascorbate) dependent manner.

1. Introduction

Root system architecture (RSA), which is the term used to describe the organization of the primary (PR) and lateral roots (LRs) (and any accessory roots and root hairs on a microscale), is a key determinant of nutrient- and water-use efficiency in plants. Root architecture is defined by the distribution and size of the PR, adventitious roots, as well as the arrangement of secondary and tertiary roots (Shekhar et al., 2019). The first root structure that appears upon germination is the radicle, from which the PR develops. Taproots are characterized by the dominance of the PRs after germination and from which LRs develop. This class of root system, also known as allorhizic, is found in the model plant *Arabidopsis thaliana*. In contrast, fibrous or homorhizic root systems are found in monocotyledonous species, where post-embryonic secondary roots that develop adventitious roots, dominate root system architecture after germination. The molecular control of root branching that facilitates the elaboration of RSA is essential for the adaptation of the plant to the nutrients and microbiome of the local soil environment. While the regulation of RSA by phytohormones and nutrients is relatively well characterised, much less information is available on how soil microorganisms shape the root system. *A. thaliana* is frequently used as model for studies on root system architecture (Kellermeier et al., 2014) largely because of the simplicity of the root system, and the broad range of molecular tools that have been developed for this species (Shekhar et al., 2019). However, the genetic networks that control RSA in response to the microbiome remain largely unclear.

The successful detection and uptake of nutrients from the soil depends on the capacity of post-embryonic organogenesis and the plasticity of the PR. LRs are formed from xylem pools pericycle (XPP) founder cells on the PR. Eight distinctive stages of LRs development have been described. In the early stages (I to IV) XPP cells dedifferentiate and proliferate within the endodermis to form the lateral root primordia (LRP), which continually divide and grow through the cellular layers from the endodermis, emerging only at the latest stages of development (Péret et al., 2009). Pre-branch sites in which cells acquire competence to develop LRs are marked by an auxin-dependent signal in the transition zone between proliferating and elongating cells of the PR meristem (Möller et al., 2017). Both the initiation and development of LRs are complex processes that are controlled by a range of molecular pathways. For example, reactive oxygen species (ROS) play a crucial role in LR formation (Manzano et al., 2014). Genetic manipulation of ROS levels increases the number of pre-branch sites and facilitates the emergence of LR primordia (Orman-Ligeza et al., 2016). Other

redox-active compounds such as the glutathione (γ-glutamyl-cysteinyl-glycine; GSH), thioredoxins (TRX) and glutaredoxins (GRX) are important in the control of root development. Soil microorganisms, such as plant growth promoting rhizobacteria (PGPR) also modulate root development and RSA. For example, PGPR increase LR density and they also stimulate root hair (RH) elongation (Poitout et al., 2017; Verbon & Liberman, 2016).

Many microorganisms inhabit the rhizosphere and establish communication with the plant roots. It has been estimated that 10^6–10^9 bacteria, and 10^5–10^6 fungi per gram of soil compete for carbon-based metabolites derived from the root (Chuberre et al., 2018). However, the effect of microorganisms on the root system depends on the species that establishes communication and interactions with the root. Some interactions are beneficial to the plants and others are harmful. Pathogenic soil microorganisms such as *Fusarium oxysporum* and *Ralstonia solanacearum* cause severe soil borne-diseases in important crop species (Haas & Défago, 2005). Soil-borne diseases can be controlled by the microbiome status of the soil in what is known as disease-suppressive soil effects, which largely rely on competition between species of microorganisms for the uptake of plant nutrients from the rhizosphere (Schlatter et al., 2017). Conversely, PGPR, which are comprised of different orders of bacterial species, can establish mutualistic interactions with the plants in the rhizosphere, which positively affects the capacity of the root to explore the soil and uptake nutrients (Glick, 2012). The mechanisms by which PGPR induce positive changes in the root system remain poorly characterised, but they are considered to rely on either the modification of nutrients sources in the rhizosphere or by direct or indirect effects on phytohormones (Poitout et al., 2017). Bacteria such as *Rhizobium* spp. secrete nitrogenases that improve the fixation of nitrogen in anaerobic soils, as well as releasing organic acids to increase plant uptake of phosphorus (Yanni et al., 2001). They also induce changes in RSA by altering the levels of ethylene through the synthesis of rhizobitoxine, which inhibits the activity of 1-aminocyclopropane-1-carboxylate synthase and thus decreases the synthesis of ethylene (Yuhashi et al., 2000). Some PGPR species such as *Pseudomonas aeruginosa*, *Klebsiella* spp., *Rhizobium* spp., *Mesorhizobium* spp., also have the capacity to secrete phytohormones that alter RSA, such as the auxin indole-3-acetic acid (IAA), which is a major player in the control of the root system (Ahemad & Kibret, 2014).

Low molecular weight antioxidants, such as ascorbate (ASC) and glutathione (GSH) are involved in the regulation of signalling processes, which affect plant

growth and development (Kocsy et al., 2013). The characterisation of *A. thaliana* mutants that have low levels of GSH and ASC has impacted these antioxidants in the control of root development. While the GSH-deficient *root meristemless 1* cannot generate a root system, other weak mutants in the *GSH1* gene that accumulate 20-30% wild-type GSH are able to produce roots. For example, the phytoalexin-deficient2 (*pad2-1*), the cadmium-sensitive (*cad2-1*) and *rax1-1* show little impairment of the root system (Cobbett et al., 1998; Parisy et al., 2007; Schnaubelt et al., 2013). The ASC-deficient vitamin C2 (*vtc2*) mutants show altered growth that is linked to changes in the expression of genes encoding proteins involved in phytohormone pathways, particularly auxin, cytokinins, abscisic acid, brassinosterioids, ethylene and salicylic acid (Caviglia et al., 2018). The *vtc1* mutants contain between 30-60% of the ascorbate present in wild-type plants and it showed enhanced sensitivity to drought stress (Niu et al., 2013). The *Gossypium hirsutum* (Gh)VTC1 gene was reported to be a positive regulator of root cell elongation in *A. thaliana* mutants, a process that was under the control of ethylene (Song et al., 2019). However, there is little information in the literature about how ROS and antioxidants such as GSH and ASC influence root responses to PGPR. In this study, the responses of RSA to *P. oryzihabitans* were firstly compared in the wild type and mutants that are deficient in either strigolactone (SL) synthesis (*More Axillary Growth {max}3-9* and *max4-1*) or SL signalling (*max2-3*) to test the hypothesis that SL-dependent pathways are important in plant responses to PGPR. SLs play a key role in RSA through the inhibition of branching (Kapulnik et al., 2011; Rasmussen et al., 2012). They interact with other phtohormone, particularly auxins, to control root morphology (Agusti et al., 2011; Jong et al., 2014; Ruyter-Spira et al., 2011). They are also important signalling molecules in plant-microbe interactions attracting arbuscular mycorrhizal fungi or favouring the nodulation between bacteria *Rhizobium* spp. with legumes (López-Ráez et al., 2017). Secondly, we compared the root responses of the wild type *A. thaliana*, the ASC-deficient *vtc2-1* and *vtc2-2* mutants and the GSH-deficient *pad2-1, cad2-1 and rax1-1* mutants to *Pseudomonas oryzihabitans* strain PGP01. These studies show that plant responses to PGPR are changed in the SL mutants and the ASC-deficient mutants but not the GSH-deficient mutants relative to the wild type.

2. Material and methods

2.1. Growth on plates

Seeds of the *A. thaliana* Columbia-0 (Col-0) wild-type (WT), the SL-deficient mutants (*max2-3*, *max3-9* and *max4-1*), the ASC-deficient (*vtc2-1* and *vtc2-2*) mutants and the GSH-deficient (*pad2-1, cad2-1 and rax1-1*) mutants were surface sterilized for

5 min in 50% bleach, followed by three 5-min-washes with sterilized water. Sterile seeds were then cultured in 10 cm square petri dishes containing half-strength Murashige and Skoog medium (½ MS, pH 5.7), supplemented with 0.01% myo-inositol, 0.05% MES, 1% sucrose and 1% plant agar. Plates were stored at 4 ºC in a dark room between 2-4 days to synchronise germination, and then plated vertically in a plant growth cabinet with a 16-h photoperiod and 22 ºC of temperature during 6 days. In order to test the combined effect of the synthetic SL, GR24, and bacteria, 6-day-old Col-0, *max2-3*, *max3-9* and *max4-1* seedlings were transferred to new plates containing the same medium plus GR24 (2 μM) prior the inoculation with bacteria. For each experiment, at least three plates per treatment and genotype and 6 seeds per plate were used.

2.2. Inoculation of bacteria

P. oryzihabitans strain PGP01 was obtained from the IRTA Postharvest Plant Growth Promoting Microorganisms (PGPMs) Collection (Lleida, Catalonia, Spain). *P. oryzihabitans* PGP01 was grown in nutrient yeast dextrose agar (NYDA: nutrient broth, 8 g L^{-1}; yeast extract, 5 g L^{-1}; dextrose, 10 g L^{-1}; and agar, 20 g L^{-1}) medium for 48 h. The application of the bacteria to *Arabidopsis* seedlings was conducted following Zamioudis et al. (2013) protocol with some modifications. Bacteria were collected from plates in 10 mM $MgSO_4$, and washed by centrifugation at 5000 g during 5 min. After resuspension in 10 mM $MgSO_4$, bacteria concentration was adjusted to $1x10^6$ and $1x10^7$ CFU mL^{-1} by measuring turbidity at 600 nm. Fifty μL of both bacterial inocula were applied at 5-cm of root tip of 6-days-old Arabidopsis Col-0 seedlings to test their effects in root architecture. For experiments involving mutants seedlings, only $1x10^6$ CFU mL^{-1} was used.

2.3. Root architecture

After 7 days of co-culture with bacteria, pictures of control and bacteria-treated plates were taken, and different parameters such as primary root length (PRL), number of visible lateral roots (NLR) and length of lateral roots (LLR) were measured using ImageJ software ver. 1.52p (National Institutes of Health). Lateral root density (LRD) was calculated by dividing the NLR by the PRL for each root analysed.

2.4. Statistical analysis

All the experiments were repeated at least three times. Data from the experiments using Col-0 and bacteria were analysed by one-way ANOVA, and the analysis of data from SLs, ASC and GSH mutants experiments was performed by a

two-way ANOVA. Statistical significance was judged at the level $P < 0.05$, and the Duncan's test was used for the means separation when the differences were significant using IBM SPSS statistics 25 program.

3. Results

3.1. Root responses in Col-0 seedlings in the presence of *P. oryzihabitans* PGP01

The presence of *P. oryzihabitans* PGP01 reduced the length of the WT Arabidopsis PR by about 36 and 28%, at bacterial concentrations of 1×10^6 and 1×10^7 CFU mL^{-1} respectively, after 7 days of co-culture (Figure 1A). Col-0 seedlings had a significantly higher NLR at the lower (1×10^6 CFU mL^{-1}) but not at the higher bacterial concentration (Figure 1B). The LLR was significantly changed in response to the presence of *P. oryzihabitans* PGP01 at either bacterial concentration (Figure 1C).

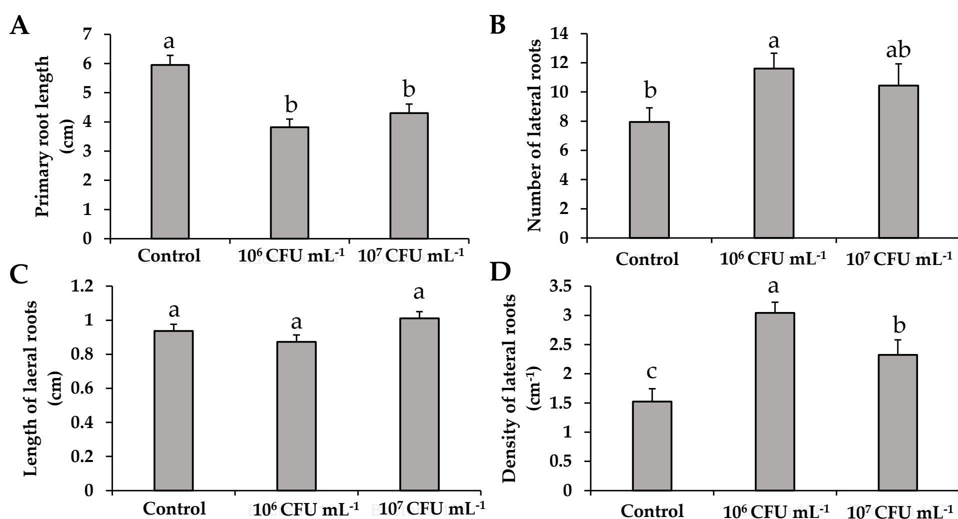

Figure 1. Effect of the application of two different doses of *P. oryzihabitans* PGP01 (1×10^6 CFU mL^{-1} and 1×10^7 CFU mL^{-1}) on primary root length (A), number of lateral roots (B), length of lateral roots (C) and lateral root density (D) of *A. thaliana* Col-0 seedlings. Six-days-old seedlings were inoculated with this bacterium at 5 cm of the root tip, and the measurements of the different parameters were carried out 7 days after the inoculation. Data show mean ± standard error (SE) of at least three independent biological replications. In each graph, different letters indicate significant differences between control and treatments ($P < 0.05$; analysis of variances and Duncan's *post hoc* test).

As a consequence of the decreased PRL and the higher NLR, lateral root density was increased in the presence of both concentrations (1×10^6 and 1×10^7 CFU

mL^{-1}) of *P. oryzihabitans* PGP01, being this effect more evident at the 1x10^{6} CFU mL^{-1} concentration (100% increase compared to the WT) (Figure 1D). Based on these results, we chose to use the 1x10^{6} CFU mL^{-1} concentration in all the following experiments with defective mutants.

3.2. Root responses in strigolactones (SLs) deficient mutants

In the absence of bacteria, the *max2-3*, *max3-9* and *max4-1* seedlings had a shorter PR than the WT seedlings, with decreases of 32, 18 and 42%, respectively (Figure 2A).

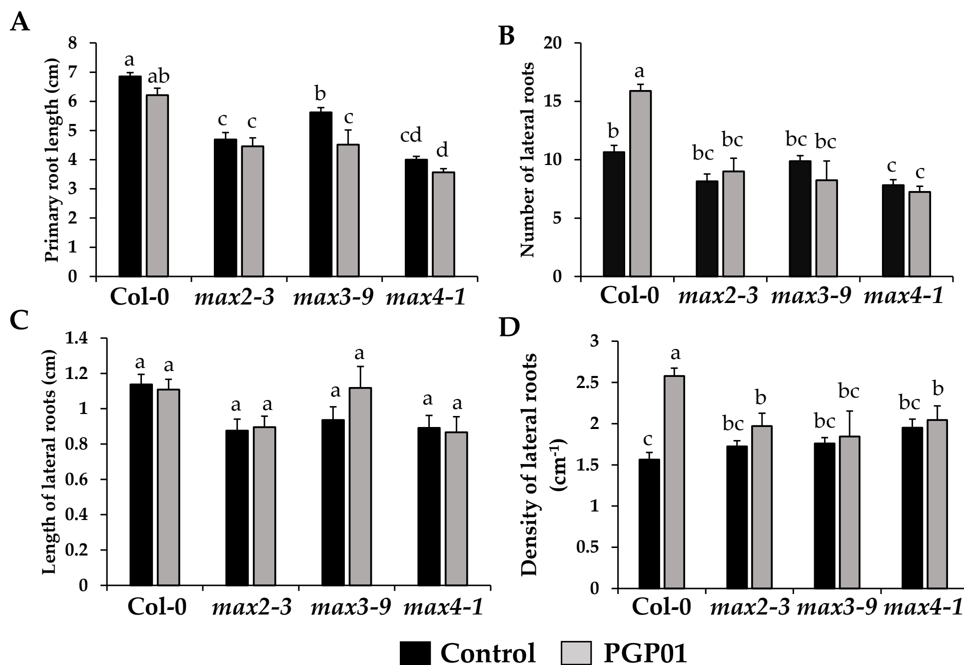

Figure 2. Effect of the application of *P. oryzihabitans* PGP01 at 1x10^{6} CFU mL^{-1} on primary root length (A), number of lateral roots (B), length of the lateral roots (C) and lateral root density (D) of SLs defective mutants *max2-3*, *max3-9* and *max4-1* seedlings. Six-days-old seedlings were inoculated with this bacterium at 5 cm of the root tip, and the measurements of the different parameters were carried out 7 days after the inoculation. Data show mean ± standard error (SE) of at least three independent biological replications. In each graph, different letters indicate significant differences between control and treatments ($P < 0.05$; analysis of variances and Duncan's *post hoc* test).

However, only the NLR was not greatly changed in the mutants compared to the WT seedlings (Figure 2B). No significant differences were detected for the LLR in

absence of bacteria in either the WT or *max2-3*, *max3-9* and *max4-1* seedlings (Figure 2C). Lateral root density was increased by 12, 12 and 25% in these lines respectively, relative to the wild type but these changes were not significant (Figure 2D). While the presence of *P. oryzihabitans* PGP01 decreased the PRL in the WT, with a higher NLR and a greater lateral root density after 7 days of co-culture (Figure 2), these bacterium-induced changes were largely absent from the SLs mutants. Only *max3-9* showed a significant 20% reduction in PRL after the presence of *P. oryzihabitans* PGP01 (Figure 2A).

The culture of Col-0 seedlings in the presence of GR24 2 μM significantly reduced the PRL in a 14% respect to non-treated seedlings, and this decrease was more evident in the presence of GR24 and PGP01 (almost a 30% lower than control) (Figure 3A). In *max2-3* and *max3-9* seedlings, only the combined application of GR24 and *P. oryzihabitans* PGP01 led to a lower PRL than control, being these reductions of 44 and 32%, respectively. The culture of Col-0 seedlings with GR24 2 μM in the presence and absence of *P. oryzihabitans* PGP01 also reduced the NLR in comparison to non-GR24 treated (Figure 3B). However, when *P. oryzihabitans* PGP01 was present, the observed decrease was lower than the registered when only GR24 2 μM was applied (32% lower than control). In addition, only *max2-3* and *max4-1* seedlings had a lower NLR than control in the presence of GR24 and *P. oryzihabitans* PGP01 (Figure 3B). The application of GR24 alone or combined with *P. oryzihabitans* PGP01 did not produce changes in LLR (Figure 3C). GR24 2 μM significantly reduced the LRD in Col-0 seedlings in comparison to non-treated seedlings, while in combination with *P. oryzihabitans* PGP01, a 17% in this parameter was registered after 7 days of culture (Figure 3D). In *max2-3* and *max3-9* seedlings, LRD was not affected by the presence of GR24 neither GR24 nor *P. oryzihabitans* PGP01. However, *max4-1* seedlings showed a lower LRD than control in GR24 and GR24 and *P. oryzihabitans* PGP01 (Figure 3D).

After 7 days growth in media supplemented with the SL analogue GR24 in combination with *P. oryzihabitans* PGP01, a significant decrease in PRL was observed in the mutant seedlings relative to the WT (Figure 3A). The presence of GR24 in combination with *P. oryzihabitans* PGP01 significantly decreased the NLR in the WT, *max3-9* and *max4-1* seedlings but not *max2-3* seedlings (Figure 3B). While the presence of GR24 and *P. oryzihabitans* PGP01 had no consistent effect on the LLR in the WT and *max2-3* seedlings, LRs were significantly shorter in *max3-9* and *max4-1* seedlings that the WT under these conditions (Figure 3C). Similarly, the lateral root densities of the *max3-9* and *max4-1* seedlings were lower than the other genotypes under these conditions (Figure 3D).

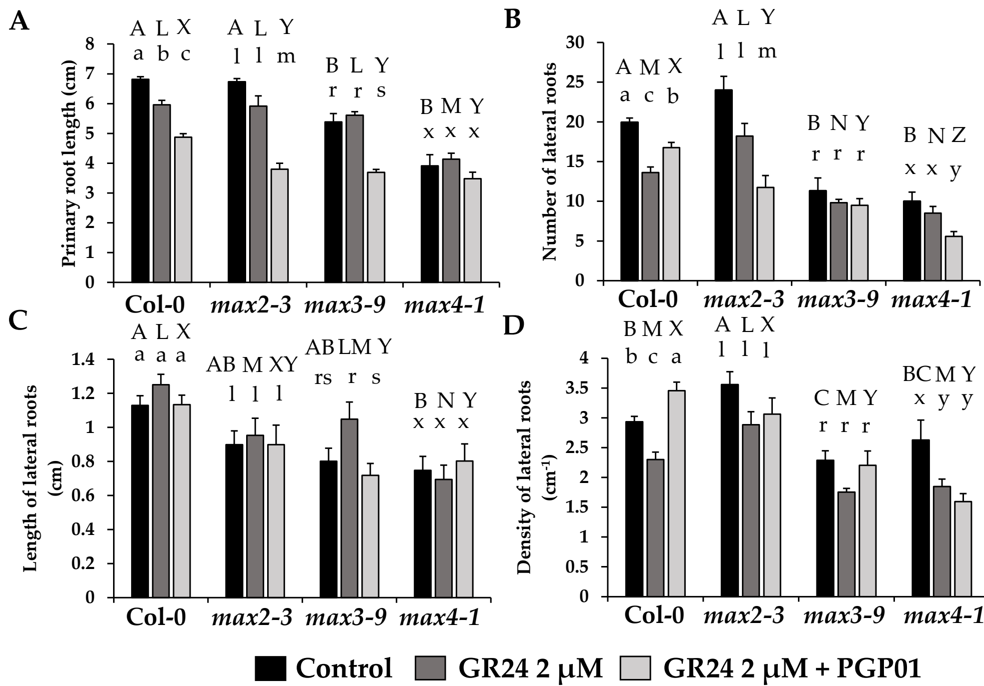

Figure 3. Effect of the application of the synthetic SLs GR24 2 μM and the bacterium *P. oryzihabitans* PGP01 at 1x10^{6} CFU mL^{-1} on primary root length (A), number of lateral roots (B), length of lateral roots (C) and lateral root density (D) of SLs defective mutants *max2-3*, *max3-9* and *max4-1* seedlings. Six-days-old seedlings were inoculated with this bacterium at 5 cm of the root tip, and the measurements of the different parameters were carried out 7 days after the inoculation. Data shown mean ± standard error (SE) of the mean of three independent biological replications. For each genotype (WT and mutants), lowercase letters indicate differences among treatments (Control, GR24 2 μM and GR24 2 μM + PGP01) according to Duncan's post hoc test (P < 0.05). For each treatment, uppercase letters denote significant differences among genotypes according to Duncan's post hoc test (P < 0.05).

3.3. Ascorbate-deficient mutants

The PRs were significantly shorter in the *vtc2-1* and *vtc2-1* mutants than the WT in the absence of bacteria (Figure 4A). However, the NLR, the LLR and lateral root density were similar in all genotypes (Figure 4B, C, D). The presence of *P. oryzihabitans* PGP01 produced a reduction in the PRL of WT seedlings in comparison to the absence of this bacterium (Figure 4A). However, in this case, this decrease was not significant. The application of *P. oryzihabitans* PGP01 to the mutant *vtc2-1* led to a significant 16% reduction of this parameter compared to non-treated

vtc2-1 seedlings (Figure 4A). The NLR was significantly increased in the wild type seedlings in the presence of *P. oryzihabitans* PGP01 but this effect was less pronounced in the ASC-deficient mutants, being only significant for the mutant *vtc2-2* (Figure 4B). The presence of *P. oryzihabitans* PGP01 significantly reduced the LLR in the vtc2-1 mutant in comparison to the absence of bacterium (Figure 4C). However, the opposite response was observed in the *vtc2-2* mutant, observing a significant 16% increase in this parameter regarding the absence of *P. oryzihabitans* PGP01 (Figure 4C). Lateral root density was increased in all genotypes in the presence of bacteria, but this effect was less pronounced in the *vtc2-1* and *vtc2-2* seedlings than the WT (Figure 4D).

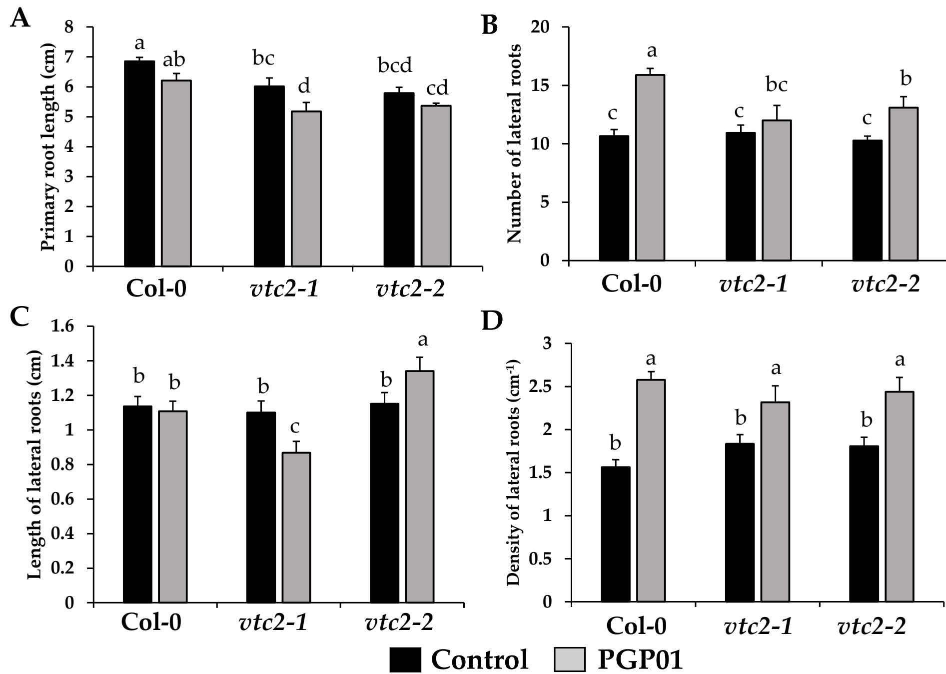

Figure 4. Effect of the application of *P. oryzihabitans* PGP01 at 1x10^6 CFU mL^{-1} on primary root length (A), number of lateral roots (B), length of lateral roots (C), and lateral root density (D) of ASC defective mutants *vtc2-1* and *vtc2-2* seedlings. Six-days-old seedlings were inoculated with this bacterium at 5 cm of the root tip, and the measurements of the different parameters were carried out 7 days after the inoculation. Data show mean ± standard error (SE) of at least three independent biological replications. In each graph, different letters indicate significant differences between control and treatments ($P < 0.05$; analysis of variances and Duncan's *post hoc* test).

3.4. Glutathione-deficient mutants

In the absence of *P. oryzihabitans* PGP01, there were no differences in the PRL in the GSH-deficient mutant *cad2-1* compared to the WT (Figure 5A). Nevertheless, while *pad2-1* showed a slight but significant increase in the PRL (8% over WT), a 9% decrease in this parameter was observed in *rax1-1* in the absence of bacteria (Figure 5A). The *cad2-1*, *pad2-1* and *rax1-1* mutants had fewer NLR than the WT seedlings in the absence of bacteria (Figure 5B). LRs of the mutant *cad2-1* in the absence of *P. oryzihabitans* PGP01 were a 19% shorter than WT (Figure 5C).

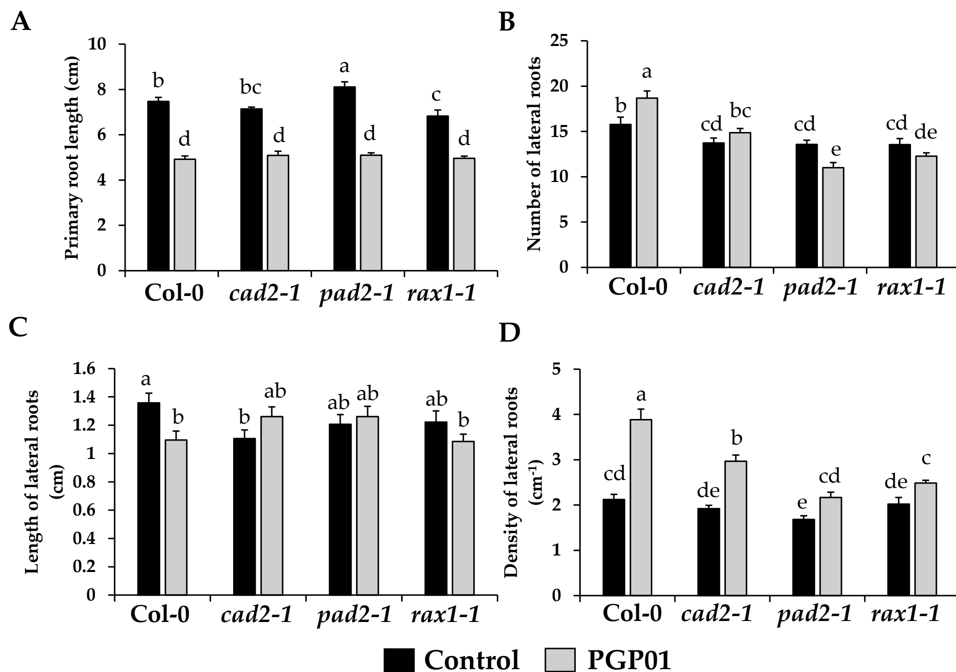

Figure 5. Effect of the application of *P. oryzihabitans* PGP01 at $1x10^6$ CFU mL^{-1} on primary root length (A), number of lateral roots (B), length of lateral roots (C), and lateral root density (D) of GSH defective mutants *cad2-1, pad2-1 and rax1-1* seedlings. Six-days-old seedlings were inoculated with this bacterium at 5 cm of the root tip, and the measurements of the different parameters were carried out 7 days after the inoculation. Data show mean ± standard error (SE) of at least three independent biological replications. In each graph, different letters indicate significant differences between control and treatments ($P < 0.05$; analysis of variances and Duncan's *post hoc* test).

Only the *pad2-1* mutants had a lower lateral root density than the WT in the absence of *P. oryzihabitans* PGP01 (Figure 5D). In the presence of *P. oryzihabitans* PGP01, the length of the PRL was significantly decreased in the presence of in all genotypes (Figure 5A). While the presence of *P. oryzihabitans* PGP01 led to a significant increase of this parameter in the WT seedlings, no significant changes were observed for the *cad2-1* and *rax1-1* mutants (Figure 5B). Only *pad2-1* showed a reduced NLR in the presence of *P. oryzihabitans* PGP01 in comparison to the absence of *P. oryzihabitans* PGP01. The LLR was significantly lower in the WT seedlings, but not in the GSH-deficient mutants, in the presence of *P. oryzihabitans* PGP01 (Figure 5C). Lateral root density was significantly increased in all the genotypes in the presence of bacteria. However, while, this increase was about 83% in the WT compared to seedlings grown in the absence of bacteria, the bacteria-induced effects on this parameter were less pronounced in the GSH-deficient mutants (Figure 5D).

4. Discussion

In the present research, the effect of the bacterium *P. oryzihabitans* PGP01 in the RSA of *A. thaliana* Col-0 seedlings was studied. This bacterium have previously shown potential effects in the improvement of *in vitro* plant growth by promoting of root development (Cantabella et al., 2020, 2021). Similarly, other studies have also reported the effects of bacteria belonging to *Pseudomonas* genus in root development events have in *A. thaliana*. For instance, Zamioudis et al. (2013) demonstrated that the strain *Pseudomonas fluorescens* was able to promote the growth of *A. thaliana* seedlings, increasing the NLR. A similar effect was observed by Ortiz-Castro et al., (2020) using *Pseudomonas fluorescens* and *Pseudomonas putida*. The PGPR *P. simiae* WCS417r was found to stimulate lateral root formation in *A. thaliana*, a response that was partly regulated by the production of volatile organic compounds by the bacterium. Although nearly all Arabidopsis accessions examined were found to respond positively to WCS417r, there was a large variation between accessions in terms of the extra NLR formed, and the effect on PRL (Wintermans et al., 2016). However, the influence of *P. oryzihabitans* in *A. thaliana* root system has not been previously studied. In this study, it was demonstrated that the presence of this bacterium resulted in a strong response of the WT *A. thaliana* root system, leading to a decrease in PRL and an increased lateral root density. Although mechanisms regulating RSA are well known, very little has been explored about these processes in the presence of rhizobacteria. For that reason, *A. thaliana* mutants defective in plant hormones such as SLs (*max2-3*, *max3-9* and *max4-1*), and redox molecules including ASC (*vtc2-1* an *vtc2-2*) and GSH (*cad2-1*, *pad2-1* and *rax1-1*) were proved for their root responses in

the presence of *P. oryzihabitans* PGP01 to determine their role in the regulation of root changes induced by this bacterium.

SLs regulate many important processes in plants including shoot branching, root growth, secondary growth and tolerance to cold and drought stresses (Cooper et al., 2018; Foo & Reid, 2013; Jong et al., 2014; Quain et al., 2014). They are also important regulators of the *Rhizobium*-legume symbiosis and other plant–microbe interactions in the rhizosphere, such as the symbiosis with arbuscular mycorrhizal fungi, particularly under conditions of the limitation of nutrients such as phosphorus and nitrogen (Aliche et al., 2020). The synthetic SL analogue GR24 stimulating swarming motility of the alfalfa symbiont *Sinorhizobium meliloti* (Peláez-Vico et al., 2016). The data presented here show that SL are crucial for the appropriate responses of roots to the plant growth promoting rhizobacterium *P. oryzihabitans* PGP01. The results obtained in *max2-3*, *max3-9* and *max4-1* seedlings presented here clearly implicate SL in the root responses of *A. thaliana* to *P. oryzihabitans* PGP01. In these mutants, the different parameters of root development measured remained mostly unchanged in the presence of this bacterium. These changes in the root systems of the SL mutants relative to wild type *A. thaliana* seedlings in the absence of bacteria are consistent with previous reports (Ruyter-Spira et al., 2011). These workers also demonstrated that the SLs are able to modulate auxins levels, suggesting a relationship between these two hormones. The role of auxin signalling in the root modifications induced by PGPR in *A. thaliana* have been previously described (Contesto et al., 2010). This increase in the auxin response could be triggered by the auxin activity of compounds produced by some PGPR (Ortiz-Castro et al., 2020). In the case of *P. oryzihabitans* PGP01, it has been demonstrated that this bacterium is able to IAA *in vitro* (Cantabella et al., 2021). Therefore, it may be suggested that SLs are involved in the root development processes induced by *P. oryzihabitans* PGP01 in a SLs-dependent manner. The results obtained in WT seedlings in the presence of GR24 together with *P. oryzihabitans* PGP01 might support this hypothesis since an increase in the LRD was observed under the culture in these conditions.

In addition, the results also suggested the role of redox processes in the control of root responses induced by *P. oryzihabitans* PGP01. In this regard, it is known that redox processes are also important in the control of root growth and development (Kocsy et al., 2013), but little attention has been paid thus far to how ROS-processing molecules such as ASC and GSH contribute to plant responses to PGPR. In this study, it was observed that *cad2-1*, *pad2-1* and *rax1-1* mutants had fewer visible LR than the WT seedlings in the absence of bacterium. Moreover, the presence of *P. oryzihabitans*

PGP01 led to a significant increase in the NLR in the WT seedlings but not in the GSH deficient mutants, showing a lower root response in those mutants in the presence of *P. oryzihabitans* PGP01. Hence, low GSH levels inhibit the processes that regulate lateral root formation in response to this bacterium. This result is perhaps not surprising given that there is strong literature evidence showing that GSH is required for root development and is involved in the control of root growth and architecture. For instance, mutants lacking glutathione peroxidases (GPX) have an altered root phenotype (Passaia et al., 2014) and glutaredoxins such as GRXS8 are considered to be major regulators of RSA (Ehrary et al., 2020). The presented results might suggest that an appropriate cellular redox balance is required to promote appropriate root responses. However, the role of GSH in the control of root development might not be an independent finding, since it is known that a substantial cross-talk between auxin, SLs and GSH exists. For instance, GSH is required for IBA conversion to IAA, suggesting an important role for GSH-dependent regulation of the auxin pathway in root development (Trujillo-Hernandez et al., 2020). In addition, it was recently shown that GSH deficiency inhibited the ubiquitination-dependent degradation of Aux/IAA proteins, as well as limiting the expression of early auxin-responsive genes, such as DR5 (Pasternak et al., 2020). Overall, the results obtained in the present study might suggest that the complex link between SLs, auxins and GSH might regulate root development in the presence of *P. oryzihabitans* PGP01, as previously described by (Márquez-García et al., 2014). However, further investigations in this regard will be required to fully understand this interaction.

In ASC deficient mutants *vtc2-1* and *vtc2-2*, the same responses in terms of root development than the WT seedlings were observed in the presence of *P. oryzihabitans* PGP01 produced the same response in terms of than the wild type, observing a higher number of visible LRs and therefore, an increased lateral root density. At first sight, these results might suggest that ASC is not needed for the control of root responses induced by *P. oryzihabitans* PGP01 in *A. thaliana* seedlings. However, it has been reported a role of ASC in root development mediated by the auxin-regulated S-nitrosylation/ denitrosylation of the ascorbate peroxidase 1 (APX1) (Correa-Aragunde et al., 2013). Considering the above, a more in-depth study would provide valuable information about the possible role of ASC in the control of root development in the presence of *P. oryzihabitans* PGP01.

Taken together, these data show that SL and GSH are key components in the root responses of *A. thaliana* root to *P. oryzihabitans* PGP01. They confirm that the pathways of signalling by SLs, auxin and GSH are interlinked in the regulation of root

architecture in response to rhizosphere signals. It is tempting to suggest therefore that a bacterium-induced oxidative burst in the plasma membrane of roots cells is a primary signal of recognition by the plant that redox changes thereafter trigger phytohormones pathways that mediate appropriate responses in root architecture.

5. Author contributions

Daniel Cantabella, Christine H. Foyer, Ramon Dolcet and Neus Teixidó developed the project; Daniel Cantabella, Christine H. Foyer and Barbara Karpinska designed and performed experiments; Daniel Cantabella and Barbara Karpinska measured root parameters; Daniel Cantabella and Christine H. Foyer analysed data; Daniel Cantabella and Christine H. Foyer wrote the article, and all other authors read and contributed to previous versions and approved the final version.

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Chapter 4

GreenTray® TIS bioreactor as an effective *in vitro* culture system for the micropropagation of *Prunus* spp. rootstocks and analysis of the plant-PGPMs interactions

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Abstract

The use of the GreenTray® TIS bioreactor for the *in vitro* analysis of the interaction between plantlets and two Plant Growth-Promoting Microorganisms (PGPMs) (*Pseudomonas oryzihabitans* PGP01 and *Cladosporium ramotenellum* PGP02) is reported herein. This *in vitro* culture system improved micropropagation of *Prunus* spp. Rootpac 20® rootstock, showing a significant increase in the shoot length and fresh weight, 49 and 28%, respectively, compared with culture in semisolid agar-containing medium. Plant responses in co-culture with the PGPMs, their ability to control endophytes growth in the culture media and hormonal changes associated to plant growth were studied in the GreenTray® culture system. Inoculation with *P. oryzihabitans* PGP01 had no significant effects, instead *C. ramotenellum* PGP02 considerably reduced endophytes population after 5 days post inoculation, although the fungi overgrew the plants roots seriously reducing their growth. However, these results suggested the effect of the medium pH as a factor to control microbial endophytic growth in the culture medium. For that reason, a higher *P. oryzihabitans* PGP01 concentration was inoculated in culture media previously adjusted to pH 5.7 or 7, observing an increased number of roots when plantlets were co-cultured for 15 days with the bacterium in medium adjusted to pH 5.7. This was associated with changes in the levels of IAA in the culture medium, and higher leaf chlorophyll contents than in the control non-inoculated cultured plantlets. However, *P. oryzihabitans* PGP01 was not able to reduce endophytes growth in either of both media pHs. Altogether, the GreenTray® bioreactor was shown as an efficient system to *in vitro* micropropagate RP-20 explants as well as to monitor its interaction with PGPMs.

Keywords: GreenTray® Bioreactor; Liquid MS medium; PGPMs; *In vitro* biological control; Low pH; Auxins.

1. Introduction

In vitro micropropagation has become a worldwide implemented technique in the plant clonal production as it allows obtaining a large quantity of free-disease plant material all-year round (Dobránszki & Teixeira da Silva, 2010). Conventional micropropagation methods involving the use of small flasks with semi-solid media represents the most extended procedure (Pereira-Lima et al., 2012). Nevertheless, the intense manipulation of these cultures has made of *in vitro* micropropagation a very time-consuming technique. In this sense, further progress to improve the shoot propagation rates, its growth and quality, reducing the cost of the process have already been intended with the implementation of liquid cultures in bioreactors. Innovative approaches conducted during the 90s demonstrated the effectiveness of this system over the traditional micropropagation methods using agar-containing media (Alvard et al., 1993; Escalona et al., 1999). However, it has been reported that direct liquid immersion cause vitrification or hyperhydricity of plant material, leading to a disruption of *in vitro* growth and development (Akdemir et al., 2014). To avoid this issue, bioreactors with Temporary Immersion Systems (TIS) improves the benefits of liquid cultures to ensure the *in vitro* performance of explants (Godoy et al., 2017). In TIS bioreactors, the soaking process of *in vitro* explants with the culture medium only occurs during shorts periods of time, sufficient for plants to uptake nutrients and plant growth regulators (Georgiev et al., 2014). In addition, the forced aeration within the explants vessel provided the explants with an environment with a high gas exchange, resulting in a better shoot or plantlet growth and development (Pereira-Lima et al., 2012). In the last years, TIS bioreactors have been successfully implemented for the micropropagation of several plant species including pistachio (Akdemir et al., 2014), cherry (Godoy et al., 2017) or myrtle (Aka Kaçar et al., 2020).

On the other hand, microorganisms and plants coexisting in their natural environment establish mutualistic or commensals relations with their hosts (Senthilkumar et al., 2011). In *in vitro* cultures, the occurrence of contaminant microorganisms (especially bacteria) has been frequently associated to an inappropriate explant manipulation, as *in vitro* tissues must be maintained sterile (Orlikowska et al., 2017). Nonetheless, it has been assumed since many years that, in spite of the surface sterilization process, *in vitro* cultures are unexpectedly contaminated by microorganisms that colonize the internal tissues (Quambusch et al., 2016). Those microorganisms are commonly known as endophytes. Several authors have defined the term "endophyte" as referring to microorganisms including bacteria or fungi that are capable of living within plants without expressing disease symptoms

(Petrini, 1991; Wilson, 1995). In this regard, Cassells and Tahmatsidou (1997) also referred to endophytes as non-pathogenic bacterial strains with the ability to colonise plant tissue through the natural openings available on plant surface. Although not pathogenic, microorganisms in *in vitro* cultures may alter the behavior of tissues, negatively affecting propagation, growth and development (Cassells, 2012; Leifert & Cassells, 2001; Tsao et al., 2000). Some authors proposed the addition of antimicrobial compounds to the culture medium as strategy to eliminate endophytes and thereby obtain clean plant *in vitro* cultures (Khan et al., 2018; Lotfi et al., 2020; Shehata et al., 2010). However, some endophytic bacteria may reappear after transference to a medium without antibiotic (Bunn & Tan, 2002). No reports concerning the use of plant growth-promoting microorganisms (PGPMs) to suppress the growth of *in vitro* endophytes, while promoting plant growth and development, are available in literature. It is well established that PGPMs are able to suppress the growth of antagonistic microorganisms through multiple mechanisms (Morales-Cedeño et al., 2021), and their introduction to control endophytes growth in plant *in vitro* cultures might represent a more sustainable approach to mitigate the high losses of plant material associated to this issue. On the other hand, other mechanisms used by PGPMs to promote plant growth have been described such as the production of phytohormones that could be used for plants to complete their growth and functionality (Calvo et al., 2014). In this context, auxins, cytokinins (CKs) and gibberellins (GAs), among others, could be synthetized by PGPMs (Arkhipova et al., 2005; Hamayun et al., 2010; Zamioudis et al., 2013), altering the hormone status of plants and favouring numerous physiological processes including cell division, root initiation, root elongation, senescence or shoot elongation (Lugtenberg & Kamilova, 2009; Vejan et al., 2016).

Although much evidences concerning the benefits of the application of PGPMs in plants have been provided in the last few years, very little of them have been conducted using *in vitro* culture systems. The TIS bioreactor used in the study, patented (Patent No. ES201831164) and registered as GreenTray® (Dolcet-Sanjuan & Mendoza, 2018), is a mid-sized unit bioreactor, from 1 to 4L capacity, with the distinction in the fact that, firstly, transforms glass, transparent or other commercial flasks into a bioreactor. Other advantages of the GreenTray® bioreactor are: (1) the high illumination of the plant material, since the lid is not shading it; (2) the elimination of all the liquid medium and condensed water in the recipient walls closer to the leaves, since the flask surface is curved and the liquid moves to the bottom from where it returns by gravity to its external original container; (3) atmospheric air renewal in the recipient holding the plant material each time there is a movement of

the liquid media, or alternatively when needed though and independent circuit, and (4) it needs small volumes of media (150 to 250 mL) to temporally immerse the cultured explants or plantlets. Such GreenTray® particular characteristics enhance leaf development, shoot elongation, and avoids vitrification or hyperhydricity observed with the plant materials cultured in flasks with semisolid media o in other TIS bioreactors. Herein, the present study shows the first application of a TIS bioreactor as a niche for the study of the *in vitro* plant-microbe interactions between two microorganisms (*Pseudomonas oryzihabitans* PGP01 and *Cladosporium ramotenellum* PGP02) with plant growth promoting ability and the *Prunus* commercial rootstock Rootpac 20® (RP-20). In this interaction, our attempts were destined to study if these microorganisms were able to control the growth of endophytic contaminants existing in plants without negatively affecting growth. In addition, their effect on the hormonal status was also considered in order to elucidate the mechanism of action of these microorganisms.

2. Material and methods

2.1. *In vitro* plant material

The experiments of the study were conducted using shoot-tip cultures of the *Prunus* rootstock Rootpac®20 (RP-20) (Agromillora Group, Spain). This rootstock constitutes a natural hybrid between the "Myrobalan" plum (*Prunus cerasifera* Ehr.) and an almond (*Prunus dulcis*). RP-20 rootstock was selected for this study due to the presence of natural endophytic contaminants. In the present research, RP-20 rootstock *in vitro* micropropagated explants were destined to two independent experiments: a study on the use of GreeTray® bioreactor for the micropropagation of RP-20 explants, as well as its use as a system to monitor the interaction between two PGPMs (*P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02) and RP-20 plantlets. In this experiment, the evolution of the two PGPMs in this micropropagation system, and how their co-culture influenced the development of RP-20 plantlets were studied. In addition, these two PGPMs were proven for its ability to control the growth of endophytic contaminants. Finally, the hormonal changes in the culture medium mediated by the inoculation with the two microorganisms were considered.

2.2. Micropropagation of RP-20 in GreenTray® bioreactor

In this experiment, the main goal was to compare the efficiency of the GreenTray® bioreactor with the conventional micropropagation method in agar-containing medium. For that reason, micropropagation in flasks with semisolid medium were used as the standard culture conditions described earlier (Iglesias et al., 2004). Murashige and Skoog (MS) (Murashige & Skoog, 1962) supplemented with 3% sucrose and 5 μM 6-Benzylaminopurine (BAP), pH to 5.7, agar (7 g L^{-1}) and autoclaved at 121 °C for 20 minutes was used for flasks experiments. In the GreenTray bioreactor culture system, the same MS + 5BAP medium, but without agar, was used. In both culture systems, explants were kept during 3 weeks in MS + 5BAP medium, and then in MS without BAP medium, to promote shoot elongation, for an additional 3-week-long period.

Two-cm-long RP-20 nodal segments, derived from shoot tip cultures in the multiplication phase, using semisolid medium, were used as explants to initiate new cultures in the GreenTray® bioreactor or in glass flasks. Seventy explants per bioreactor and 10 explants per flask, with three replicates per treatment were used. All cultures were kept at 24 ± 1 ºC under a photoperiod of 16h of cool-white fluorescent light (140 μmol $m^{-2} \cdot s^{-1}$), and 8h darkness. The GreenTray® bioreactor was set at an immersion frequency of 2 minutes every 6 hours. Three weeks later, the culture medium in the bioreactor was replaced with fresh medium of the same composition but without BAP. In the case of flasks, 50 mL of the same medium was dispensed to each flask, containing the shoot growing in 100 mL semisolid medium. For shoot multiplication evaluation, in the case of flasks with semisolid medium, shoot clumps were moved out with the help of forceps, and in the case of the GT bioreactor, were all moved at once by pooling out the tray holding the plant material. Individual shoots were separated from the clumps with the help of scalpel and forceps. The number of new shoots from each initial explant, their length in cm, fresh weight in grams, and multiplication rate were determined for each container.

2.3. GreenTray® bioreactor as a system for the plant-PGPMs interaction study

As previously said the GreenTray® bioreactor, based on the principle of TIS, was used in this research as a system to evaluate the biological control activity of two fruit tree PGPMs, *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02. Both microorganisms were selected for their ability to promote *in vitro* plantlet growth and

development of different *in vitro* fruit tree plant material (). On the other hand, since in the GreenTray® bioreactor, micropropagated explants and culture medium are placed in separated vessels, this system allowed an easy sampling process. Firstly, RP-20 micropropagated explants in MS+5BAP semisolid medium were transferred to the same medium without BAP to promote shoot elongation. In this case, the experiment was performed using *in vitro* rooted plantlets as some phytohormones such as auxins and CKs are involved in root events (Sokolova et al., 2011; Zamioudis et al., 2013). For this reason, 3-cm-long shoots were cultured in ½ MS medium supplemented with 10-μM indole-3-butyric acid (IBA) for one week in darkness for root induction. Before initiating root elongation, RP-20 IBA-treated shoots, with root primordia initiated in the shoot basis, were randomly transferred to each GreenTray® bioreactor 48 h prior microbial inoculation. Cultures in the bioreactor were kept 48 h with the presence of only MS medium, with 3% sucrose and pH adjusted to 5.7, to ensure the release of endophytes to the culture medium. After 48 h of culture, microbial inoculation took place following two independent repetitions. In a first trial, the inoculation of GreenTray® bioreactors containing RP-20 explants took place by adding 3 mL of *P. oryzihabitans* PGP01 or *C. ramotenellum* PGP02 suspensions at $1x10^3$ CFU mL^{-1} and $1x10^5$ sp mL^{-1}, respectively. On the other hand, in a second trial, GreenTray® bioreactors were only inoculated with *P. oryzihabitans* PGP01 at $1x10^6$ CFU mL^{-1}. However, in the latter case, MS media at pH 5.7 (MS 5.7) and 7 (MS 7) were used to study how this factor affects to the interaction of *P. oryzihabitans* PGP01 and RP-20 *in vitro* plantlets. The pH of the medium was adjusted to 5.7 or 7 before autoclaving at 121 ºC during 20 min. In the latter case the pH was buffered with the addition of 0.06% 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer. All media were then autoclaved at 121 ºC during 20 min, and cooled down before use. Three bioreactors per treatment were set up containing fifteen RP-20 shoots each, and the co-culture in the presence of the two PGPMs was maintained during 15 days.

2.4. Inocula preparation

Two microorganisms, *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 in the first trial, and *P. oryzihabitans* PGP01 in the second trial were used to inoculate the bioreactors containing RP-20 shoots. Bacterial inoculum was prepared from 48 h-old *P. oryzihabitans* PGP01 plates grown in nutrient yeast dextrose agar (NYDA: nutrient broth, 8 g L^{-1}; yeast extract, 5 g L^{-1}; anhydrous glucose, 10 g L^{-1}; and agar, 15 g L^{-1}). *P. oryzihabitans* PGP01 cells were collected in phosphate buffer (70 mL KH_2PO_4 0.2 M; 30 mL K_2HPO_4 0.2 M; 300 mL of deionized water). Bacterial cell concentration was estimated by measuring the absorbance at 420 nm, and the final concentration was set

up with sterile distilled water at $1x10^3$ colony forming unit per mL (CFU mL^{-1}) in the first trial, and $1x10^6$ CFU mL^{-1} in the second trial. For *C. ramotenellum* PGP02, a concentrated suspension from 14 d-old plates grown on potato dextrose agar (PDA: potato tissue, 200 mL; glucose, 20 g L^{-1}; and agar, 20 g L^{-1}) was used for the preparation of the fungal inoculum used in the first trial. Conidia of *C. ramotenellum* PGP02 of this suspension were measured using a haemocytometer, and the concentration was ultimately set to $1x10^5$ spores per mL (sp mL^{-1}) with sterile distilled water. The inoculation of GreenTray® bioreactors took place by the application of 3 mL of microbial inoculum to each bioreactor.

2.5. Population dinamics of the microorganisms

As in GreenTray® bioreactor, explants and culture medium are placed in two separated compartments, the evolution of the microorganisms *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02, as well as endophytic contaminants naturally existing in RP-20 explants could be easily monitored. Regarding sampling, 2 mL were taken from the culture medium vessel at 0, 1, 2, 5, 7, 9, 12, 15 days post-inoculation (dpi), and the CFU mL^{-1} for each microorganism was determined using dilution plate technique in NYDA medium. In addition, the endophytic microbe population was calculated in microbe-treated and non-treated bioreactors for the two trials.

2.6. Gas content, biometrical measurements and chlorophyll content

In parallel to the determination of the evolution of microorganisms, the CO_2 and O_2 curves were monitored in control and PGPM-treated bioreactors for the different trials, by air sampling through one of the two ventilation ducts, protected from external contamination by a 0.22 μm filter unit. Measures were taken every two hours during 12 h using the portable gases analyser Dansensor® CheckPoint3 (AMETEK Instruments, Barcelona, Spain). On the other hand, after 15 days of co-culture, RP-20 plants from control and inoculated bioreactors following the two trials were removed for the measures of plant fresh weight (FW) in g, and the number of roots, which were measured as previously described in Cantabella et al (2020). For chlorophyll determination, 0.04 g of leaves were extracted from RP-20 *in vitro* explants cultured 15 days in the absence or presence of the two microorganisms in the case of the first trial, as well as in the presence of *P. oryzihabitans* PGP01 in MS medium at pH 5.7 or 7, in the second trial, and incubated in 10 mL of 80% acetone (v/v) during 24 h under darkness. After 24 h of incubation, absorbance at 645 and 663 nm was measured using the spectrophotometer SP-2000UV (Shangai Spectrum Instruments Co., Ltd,

Shangai, China), and the contents of total chlorophyll (Chlt), chlorophyll a (Chla) and chlorophyll b (Chlb) were determined according to Arnon (1949).

2.7. Determination of the phytohormone content in liquid culture medium

Thoroughly homogenized samples of 3 mL culture medium samples were measured into 15 mL Falcon centrifuge tubes and spiked with 5 ng of each internal standard, homogenized and frozen at -80°C prior to lyophilizing. The lyophilized samples were re-dissolved with 0.5 mL of water/acetonitrile (98+2) 0.1% formic acid and filtered through 0.22 μm hydrophilic PTFE filter before UHPLC-MS/MS analysis. The standards of phytohormones such as abscisic acid (ABA), indole-3-acetic acid (IAA), N^6-isopentenyladenine (iP) and dihydrozeatin (DHZ) and their stable isotopically labeled counterparts [$^2H_6$]-ABA, [$^2H_5$]-IAA, [$^2H_6$]-iP and [$^2H_3$]-DHZ used as internal standards were purchased from OlchemIm Ltd. (Olomouc, Czech Republic). Stock solutions (100 μg mL^{-1}) and working solutions (10 μg mL^{-1}) of phytohormones and internal standards were separately prepared in methanol (Fisher Scientific, Madrid, Spain). All standard solutions were stored at -80 °C. Thus, a solution of the mixture of phytohormones and another mixture of internal standards at 1 μg mL^{-1} in water/acetonitrile (Fisher Scientific, Madrid, Spain) (98+2) 0.1% formic acid (Fisher Scientific, Madrid, Spain) were prepared. A six-point calibration curve was obtained in water/acetonitrile (98 + 2) 0.1% formic acid to cover 0.5 – 50 ng mL^{-1} range with 10 ng mL^{-1} of internal standards. Ultrapure Milli-Q water (H_2O) was obtained by Synergy UV (Merck KGaA, Darmstadt, Germany). UHPLC-MS/MS (ultra-high performance liquid chromatography tandem mass spectrometry) analysis was performed on Waters Acquity UPLC binary system coupled to a Xevo TQ-S triple-quadrupole mass spectrometer equipped with an ESI source (Waters, Milford, MA, USA). A Waters Acquity UPLC® HSS T3 1.8 μm 2.1 × 100 mm column (Milford, MA, USA) was used for chromatographic separation. A water/acetonitrile (98+2) 0.1% formic acid solutions was used as the mobile phase A and acetonitrile was used as the mobile phase B with gradient elution (Table S1). The flow rate was 0.250 mL min^{-1}. The sample injection volume was 2.5 μL and the column oven temperature was kept at 40°C. The sample was separated for 10 min. The tandem mass spectrometer (MS/MS) was operated in ESI positive/negative mode and data were acquired in the multiple reaction monitoring (MRM) mode (Table S2). The MS/MS parameters were set as follows: Capillary voltage 3000V/-2.500V, source temperature 150°C, desolvation temperature 350°C, desolvation gas (nitrogen) flow 800 L h^{-1}, cone gas (nitrogen) flow 150L h^{-1} and collision gas (argon) flow 0.15 mL min^{-1}. Acquired data

were processed by MassLynx™ MS Software with TargetLynx™ program version 4.1 (Waters, Milford, MA, USA).

2.8. Statistical analysis

All the experiments were repeated at least three times for confirmation of the results. The experiment was designed considering a completely random design (CRD), and data analysis were carried out by using JMP Pro Software (version 13.1.0, SAS Institute Inc., Cary, NC). Statistical significance was judged at $P < 0.05$, and the Tukey test was used to separate means when the differences were statistically significant.

3. Results

3.1. Shoot proliferation in the GreenTray® bioreactor versus conventional cultures in flasks

Comparing the multiplication rates of RP-20 in the GreenTray® temporal immersion system bioreactor (Figure 1A) with the culture system in flasks with semisolid agar-containing medium, although differences in the multiplication rates were observed, after the 6-week-long culture process (Figure 2A), no statistically significant differences were found for RP-20. As shown in Figure 1B and C, shoot development after culture in the two culture systems were comparable. However, no hyperhydration and better leaf development was observed in the shoots produced with GreenTray® (Figure 1C) than conventional culture in agar containing medium (Figure 1B). Moreover, significant statistical differences were observed on the shoot length and the fresh weight per shoot between both culture systems (Figure 2B, C). RP-20 rootstocks were almost 50% longer when growing in the GreenTray® bioreactor than in flasks with semisolid medium (Figure 2B), which was in concordance with a 28% increase in the shoot fresh weight per new shoot (Figure 2C).

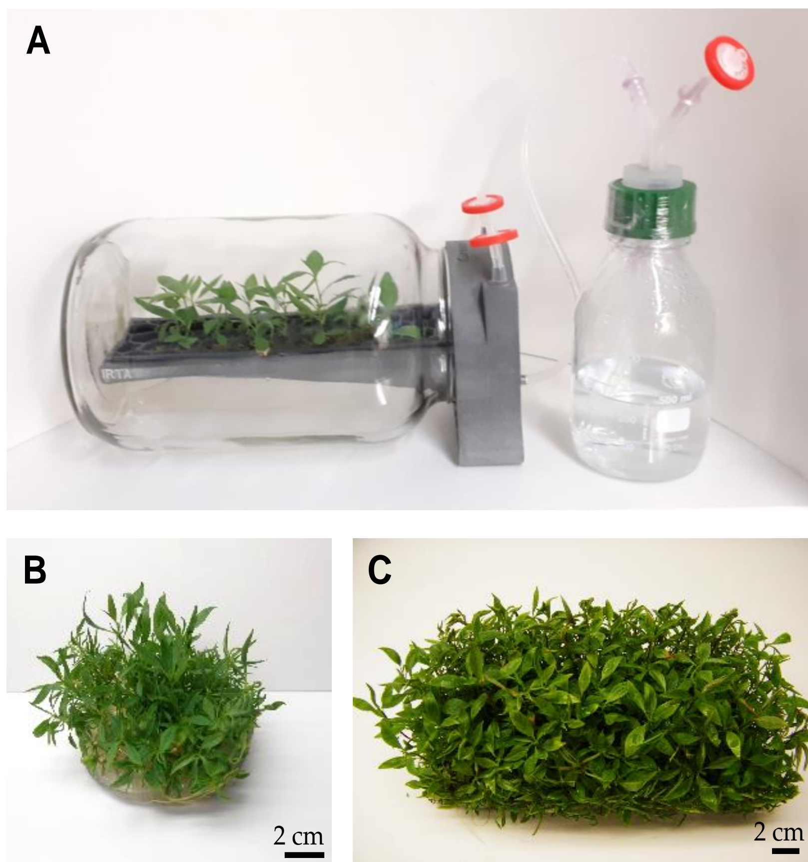

Figure 1. Use of GreenTray® TIS bioreactor for *in vitro* micropropagation RP-20 (A), and explants appearance in comparison to conventional culture using semi-solid medium with agar (B, C).

3.2. Interaction of RP-20 explants and PGPMs in GreenTray® bioreactor

In this experiment, a TIS bioreactor was used for the first time as a system to evaluate the interaction between *in vitro* micropropagated plantlets and PGPMs. In this experiment, the main goal was to monitor the interaction between *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 and the *in vitro* cultured commercial rootstock RP-20. In addition, the ability of these two PGPMs to control or suppress the growth of endophytes contamination naturally present in RP-20 shoots without disturbing plant growth was evaluated. For that purpose, two independent trials were conducted. In the first one, both *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 were inoculated at $1x10^3$ CFU mL^{-1} and $1x10^5$ sp mL^{-1}, respectively, to IBA-treated RP-20 shoots. In the

second trial, only *P. oryzihabitans* PGP01 was applied, increasing inoculum concentration at 1x10^6 CFU mL^{-1}. In addition, RP-20 explants cultured in GreenTray® bioreactors using a culture medium with a more favorable pH for the growth of the bacterium (pH 7) were added and inoculated with *P. oryzihabitans* PGP01. In both experiments, population dynamics of the PGPMs as well as endophytes was monitored during the whole process in parallel to the evolution of CO_2 and O_2 content. After 15 days of co-culture, the effect of the inoculation on plantlet growth parameters, chlorophyll content and hormonal status in the culture medium were determined.

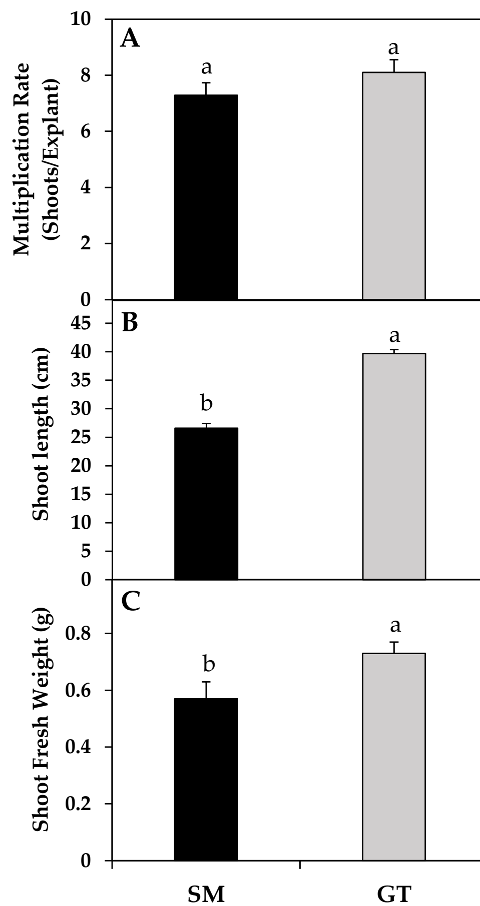

Figure 2. Effects in the multiplication rate (A), shoot length (B), and shoot FW (C) of RP-20 *in vitro* explants compared to the conventional culture in semi-solid medium (SM) with agar. Data represents the mean ± SE of the shoots obtained from the 25 and 10 explants initially cultured. Different letters denote significant differences according to Student t test ($P < 0.05$).

3.2.1. Growth of *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and endophytic contaminations in GreenTray® bioreactor

The evolution of the PGPMs under study (*P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02) in the first trial is shown in Figure 3A.

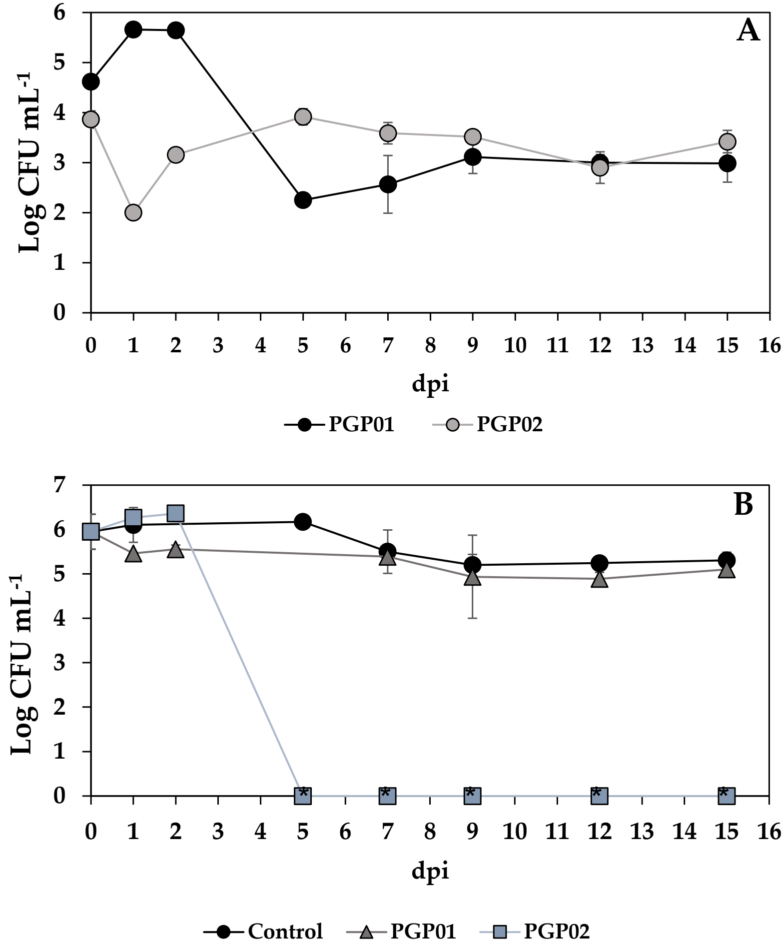

Figure 3. Dynamics population of *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 (A) and bacterial endophytes population (B) in GreenTray® TIS bioreactors inoculated with *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 with MS medium at pH 5.7. Data represents the mean ± SE of at least three replicates. Asterisks (*) symbol indicate significant differences between control and treatments according to Student t test ($P < 0.05$).

In the first experiment, where both *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 were used to inoculate GreenTray® bioreactors containing RP-20 explants, we could observe that the bacterium *P. oryzihabitans* PGP01 peaked its growth after 1 dpi, achieving a 5.66 log CFU mL^{-1} (Figure 3A). After this period, bacterial growth decreased, observing the lowest growth after 5 dpi (2.25 log CFU mL^{-1}). Bacterial cell population remained practically unchanged from 5 dpi until the end of the process, ranging from 2.56 to 2.98 log CFU mL^{-1} at 7 and 15 dpi, respectively (Figure 3A). Regarding *C. ramotenellum* PGP02, although a 1.86 log CFU mL^{-1} reduction of growth was observed after 1 dpi, fungal population rapidly recovered and reached a maximum of 3.95 log CFU mL^{-1} (Figure 3A). *C. ramotenellum* PGP02 stabilized after this period, and at the end of the process, 3.41 log CFU mL^{-1} was observed (Figure 3A). On the other hand, populations dynamics of endophytes in GreenTray® bioreactors non-treated and treated with *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 at $1x10^3$ CFU mL^{-1} and $1x10^5$ sp mL^{-1}, respectively, were registered and represented in Figure 3B. The co-culture of RP-20 explants with *P. oryzihabitans* PGP01 in GreenTray® bioreactors did not reduce the endophytes population as significant differences in Log CFU mL^{-1} were not found between both treatments (Figure 3B). However, the inoculation with the fungal isolate *C. ramotenellum* PGP02 drastically reduced contaminant population, and no endophytes were counted after 5 dpi (Figure 3B).

3.2.2. Effects of *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 in the content of CO_2 and O_2 in the vessel of the GreenTray® bioreactor

Along with the population dynamics of PGPMs and endophytes, the evolution of the CO_2 and O_2 within the plant material vessel of the control and PGPM-treated GreenTray® bioreactors was registered during the whole process. In this experiment, it is noteworthy to mention that while the content of both CO_2 and O_2 was not altered in the presence of *P. oryzihabitans* PGP01 at $1x10^3$ CFU mL^{-1}, a huge production of CO_2 in the presence of *C. ramotenellum* PGP02 at $1x10^5$ sp mL^{-1} was registered from 2 dpi to the end of the co-culture process, being the highest level after 6 dpi (Figure 4A). This large production of CO_2 was accompanied by a consumption of O_2 in the same periods of time (Figure 4B).

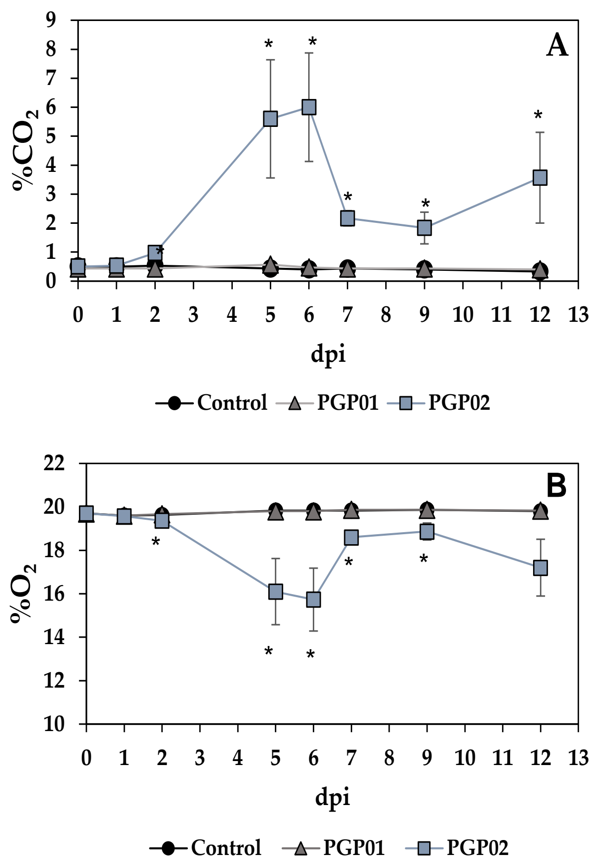

Figure 4. CO_2 (A) and O_2 (B) evolution in the plant culture vessel of GreenTray® TIS bioreactors inoculated with *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 during 15 days. Data represents the mean ± SE of at least three replicates. Asterisks (*) symbol indicate significant differences between control and treatments according to Student t test ($P < 0.05$).

3.2.3. Effects of *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 in RP-20 *in vitro* plant growth and chlorophyll content

After 15 days of co-culture with *P. oryzihabitans* PGP01 no significant changes in the plantlets FW of RP-20 were observed in the first trial (Figure 5A). In contrast, *C. ramotenellum* PGP02 negatively affected RP-20 growth, observing a decrease of about a 26% in this parameter (Figure 5A). Although no effects on plant growth were observed in RP-20 plant FW in response to the inoculation with *P. oryzihabitans* PGP01, a 29% decrease in the number of roots was recorded after 15 days of co-culture

(Figure 5B), being this decrease even more pronounced in *C. ramotenellum* PGP02-treated RP-20 plants (Figure 5B).

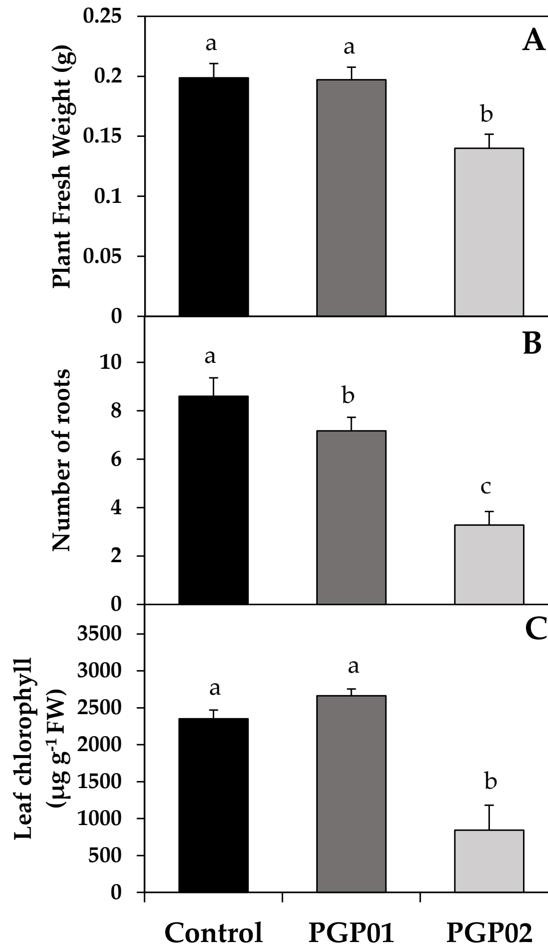

Figure 5. Effects on *in vitro* plant FW (A), number of roots (B) and chlorophyll content in leaves (C) of RP-20 explants after 15 days of co-culture. Data of plant FW and number of roots represents the mean ± SE of at least ten plants, and chlorophyll content data represents the mean ± SE of three independent replicates. In all cases, different letters denote significant differences between control and treatments according to Student t test ($P < 0.05$).

Concerning the chlorophyll content observed in RP-20 leaves cultured in GreenTray® bioreactor treated or not with PGPMs, significant changes in the content of Chlt (Figure 5C), Chla and Chlb (data not shown) were not registered in response to the inoculation with *P. oryzihabitans* PGP01 at $1x10^3$ CFU mL^{-1} when compared to

non-treated RP-20 plants (Figure 5C). The negative effects of *C. ramotenellum* PGP02 observed in plant growth were also correlated with an important decrease in the content of Chlt, Chla and Chlb (64, 67 and 53%, respectively) in comparison to control GreenTray® bioreactors after 15 days of co-culture (Figure 5C).

3.2.4. Growth of *P. oryzihabitans* PGP01 and endophytic contaminations in GreenTray® bioreactor using MS medium at different pHs

Effects of inoculating GreenTray® with *P. oryzihabitans* PGP01 at 1x10^6 CFU mL^{-1} depended on the pH of the MS medium. In general, bacterial growth was favored in the medium MS pH 7 as significantly higher values of bacterial growth were observed at several points of the co-culture process (Figure 6A). During the most active bacterial growth period, thus mean, between 0 and 2 dpi, 7.45 and 7.31 log CFU mL^{-1} were reached at 1 and 2 dpi, respectively, when *P. oryzihabitans* PGP01 was cultured in the medium MS at pH 7 (Figure 6A). It is important to remark that a characteristic curve showing bacterial growth was obtained when *P. oryzihabitans* PGP01 was cultured at pH 7. On the contrary, at the same period, significantly lower values of *P. oryzihabitans* PGP01 (5.92 and 5.69 log CFU mL^{-1}) were observed at pH 5.7 (Figure 6A). Furthermore, endophytic population existing in RP-20 rootstocks cultured in GreenTray® bioreactors in the presence or absence of *P. oryzihabitans* PGP01 at 1x10^6 CFU mL^{-1} also displayed a different response when the pH of the MS medium was adjusted to 5.7 (Figure 6B) or 7 (Figure 6C). In this sense, a very slight reduction of the endophytic population in the presence of *P. oryzihabitans* PGP01 in GreenTray® bioreactor with medium MS 5.7 occurred; however, these reduced values were only significant after one and 7 dpi (Figure 6B). Conversely, the inoculation of GreenTray® bioreactors with the medium MS pH 7 with *P. oryzihabitans* PGP01 at 1x10^6 CFU mL^{-1} somewhat promoted endophytes development in comparison to non-treated GreenTray® bioreactors although no statistically significant differences were found at any sampling period (Figure 6C).

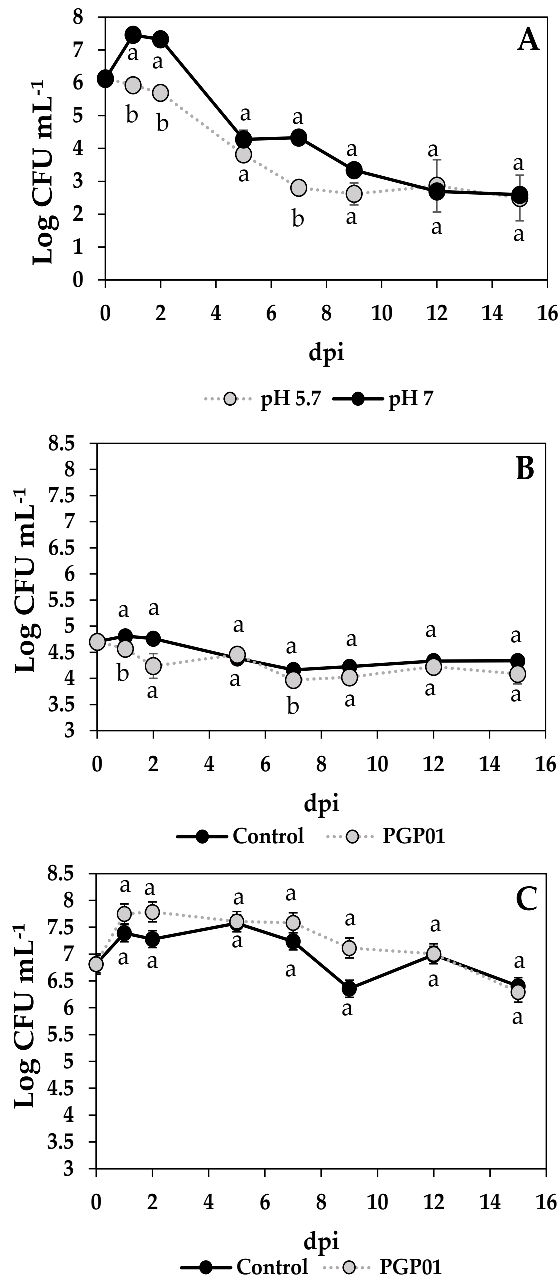

Figure 6. Dynamics population of *P. oryzihabitans* PGP01 in GreenTray® bioreactors with MS medium at pH 5.7 and 7 (A), and bacterial endophytes population in GreenTray® bioreactors at pH 5.7 (B) and 7 (C). Data represents the mean ± SE of at least three replicates. Different letters represents significant differences between control and treatments according to Student t test ($P < 0.05$).

3.2.5. Effects of *P. oryzihabitans* PGP01 cultured at different pHs in the content of CO_2 and O_2 in the vessel of the GreenTray® bioreactor

When only *P. oryzihabitans* PGP01 at $1x10^6$ CFU mL^{-1} was used for the inoculation of GreenTray® bioreactors, the pH of the MS medium differently affected the content of CO_2 and O_2.in the vessel atmosphere containing the plantlets (Figure 1A). The inoculation of GreenTray® bioreactors containing RP-20 explants with *P. oryzihabitans* PGP01 at $1x10^6$ CFU mL^{-1} did not alter the CO_2 and O_2 content when the medium pH was 5.7 (Figure 7A, B). Nevertheless, the response was quite different when the inoculation of this bacterium took place using the medium adjusted at pH 7 (Figure 7C, D). In this case, a significant production of CO_2 compared to non-treated GT bioreactors was observed during the first 48 h of bacterial growth (Figure 7C). On the other hand, during the same period, a significant reduction on the O_2 content was also observed (Figure 7D).

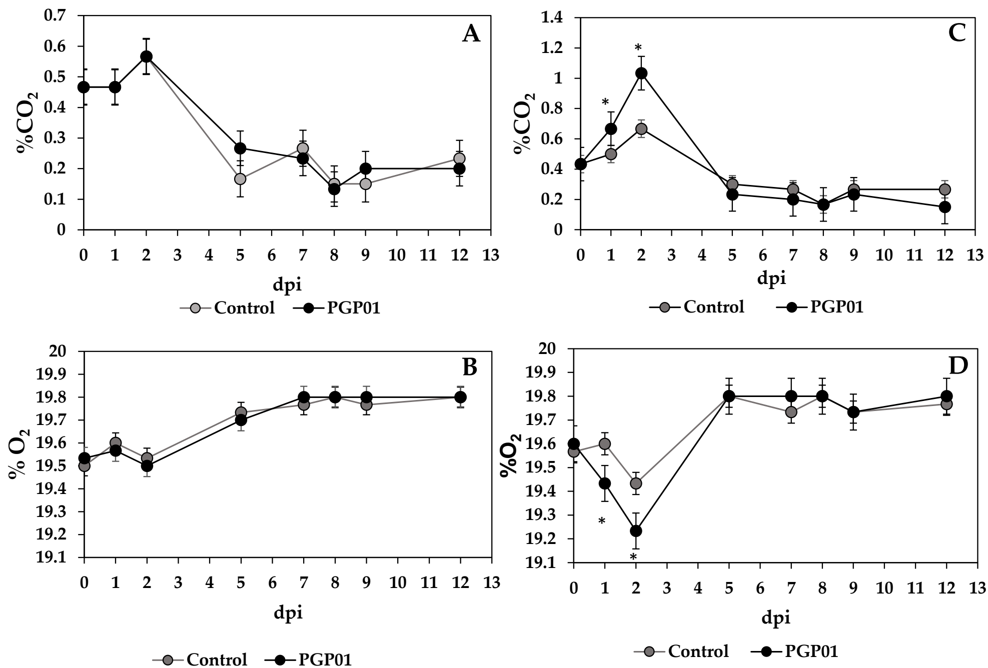

Figure 7. Effects on the evolution of the $\%CO_2$ and $\%O_2$ in GT bioreactors after the inoculation with *P. oryzihabitans* PGP01 in the GreenTray® bioreactor with MS medium at pH 5.7 (A, B) and 7 (C, D). Data represents the mean ± SE of at least three replicates. Asterisks (*) symbol represents indicate significant differences between control and treatments according to Student t test ($P < 0.05$).

3.2.6. Effects of *P. oryzihabitans* PGP01 on plantlet growth and chlorophyll content of RP-20 plantlets cultured at different pHs

It is important to remark that, in the absence of the bacterium *P. oryzihabitans* PGP01, the culture of RP-20 *in vitro* plantlets in the medium MS at pH 7 negatively affected plant growth, leading to significant reductions of several growth parameters such as plant shoot and root FW, number of leaves, and root length (data not shown). When *P. oryzihabitans* PGP01 at $1x10^6$ CFU mL^{-1} was co-cultured for 15 days with RP-20 in the medium MS at pH 5.7, no significant changes in plantlet FW occurred (Figure 8A). However, RP-20 plantlets from GreenTray® bioreactors inoculated with *P. oryzihabitans* PGP01 displayed a 53% increase in the number of roots after 15 days of co-culture (Figure 9B). When the co-culture took place in the medium MS at pH 7, the co-culture with *P. oryzihabitans* PGP01 at $1x10^6$ CFU mL^{-1} did not promote changes neither in the plant FW nor in the number of roots of RP-20 *in vitro* plantlets (Figure 8C, D).

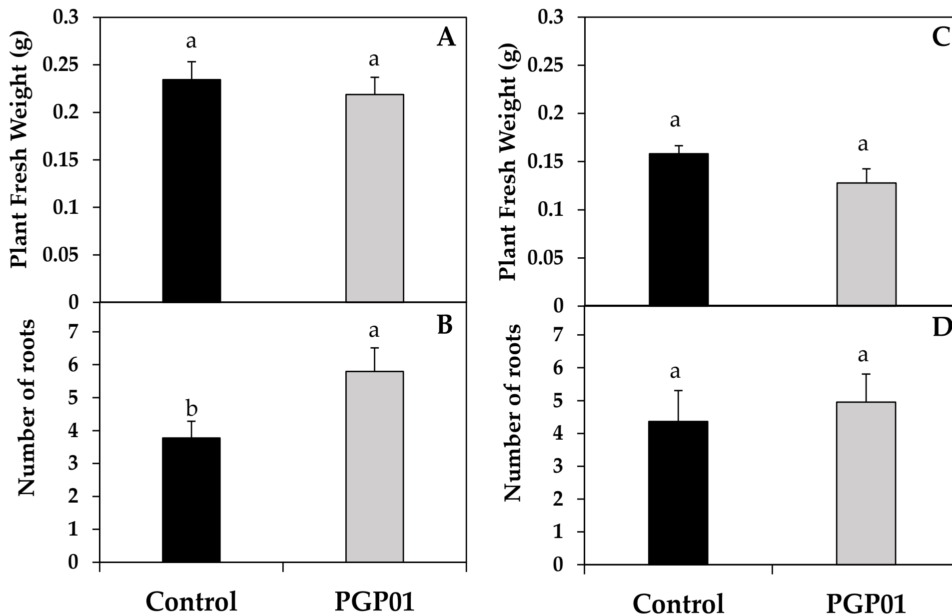

Figure 8. Effects on *in vitro* plant FW and number of roots of RP-20 plants after 15 days of co-culture in GreenTray® bioreactor with *P. oryzihabitans* PGP01 in MS medium at pH 5.7 (A, B) and pH 7 (C, D). Data represents the mean ± SE of at least ten plants. Different letters denote significant differences between control and treatments according to Student t test ($P < 0.05$).

Conversely, the culture of RP-20 *in vitro* plantlets with *P. oryzihabitans* PGP01 at 1x10^{6} CFU mL^{-1} induced a slight but significant increase in the content of Chlt and Chla (11 and 12%, respectively), but not Chlb (Figure 7A) when the liquid MS medium was adjusted at pH 5.7 (Figure 9A). The inoculation of GT bioreactors with medium MS at pH 7 with *P. oryzihabitans* PGP01 did not significantly change the content of Chlt, Chla and Chlb (Figure 9B).

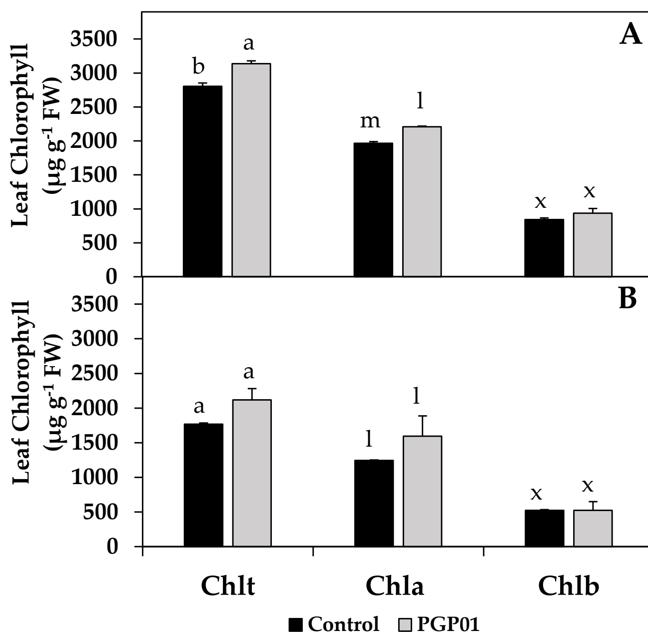

Figure 9. Effects on the content of total chlorophyll (Chl t), chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) in leaves of *in vitro* RP-20 after 15 days of co-culture with *P. oryzihabitans* PGP01 in the GreenTray® bioreactor at pH 5.7 (A) and 7 (B). Data represents the mean ± SE of at least three replicates. Different letters denote significant differences between control and treatments according to Student t test ($P < 0.05$).

3.2.7. Effects of *P. oryzihabitans* PGP01 and pH on the hormonal content of the culture media

The inoculation of GreenTray® bioreactors with PGPMs also promoted changes in the content of iP, DHZ, ABA and IAA in the culture medium. In the presence of 1x10^{3} CFU mL^{-1} of *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 at pH 5.7, hormones levels in the culture medium were so low that could not be detected (data not shown). A higher inoculum concentration of *P. oryzihabitans* PGP01 in GreenTray® bioreactors did not produce significant differences in the levels of DHZ

and ABA at pH 5.7 compared to control (data not shown). In fact, DHZ was not detected for neither control nor *P. oryzihabitans* PGP01-treated bioreactors under these conditions (data not shown). However, the inoculation with *P. oryzihabitans* PGP01 at $1x10^6$ CFU mL^{-1} and pH 5.7 reduced (3-fold decrease) the IAA levels compared to non-treated bioreactors (Figure 10). Furthermore, the behavior of *P. oryzihabitans* PGP01 in GreenTray® bioreactors at pH 7 altered the levels of iP in the culture medium, observing a significant 1.47-fold increase compared to non-treated GT bioreactors (Figure 10). The CK DHZ was not detected in the culture medium inoculated *P. oryzihabitans* PGP01 at pH 7 (data not shown). IAA levels were not significantly changed in the presence of the bacterium *P. oryzihabitans* PGP01 (Figure 10).

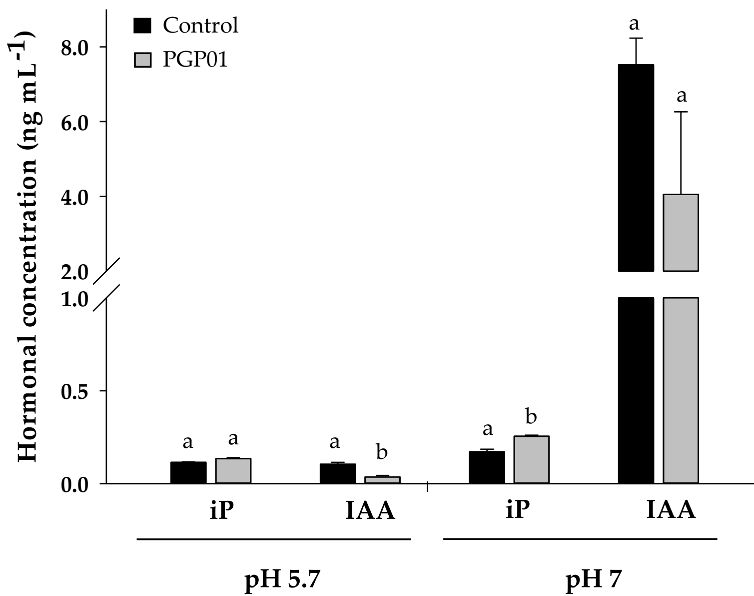

Figure 10. Effects on the content of acid 3-indoleacetic acid (IAA) in the culture medium of GreenTray® bioreactors in the presence of *P. oryzihabitans* PGP01 inoculated in the MS medium at pH 5.7 and 7. Data represents the mean ± SE of at least three replicates. Different letters denote significant differences between control and treatments according to Student t test ($P < 0.05$).

4. Discussion

The results from this research demonstrated the feasibility of the recently patented GreenTray® bioreactor for the *in vitro* micropropagation plants and monitoring of the interaction between RP-20 plantlets and the two PGPMs

P. oryzihabitans PGP01 and *C. ramotenellum* PGP02. So far, studies concerning the application of beneficial microorganisms to improve *in vitro* plant performance have been conducted by directly inoculating the base of the explant (Larraburu et al., 2010; Perez-Rosales et al., 2018) or by incubation of *in vitro* shoots in microbial suspensions (Della Mónica et al., 2018; Quambusch et al., 2014). In all the above cases, *in vitro* plantlets as well as beneficial microorganisms were developed in the semisolid agar media commonly used in conventional micropropagation methods. Therefore, this investigation represents a breakthrough in the field of plant-microbe interactions exploiting a plant propagation system based on liquid culture principles as a matrix to host microorganisms with potential effects on plant development. Nowadays, there are available different TIS bioreactors conformations used in commercial and research propagation including RITA® (Teisson et al., 1996), SETIS® (Bello-Bello et al., 2019), TIB® (Escalona et al., 1999) and MATIS® (Etienne et al., 2013), being SETIS® and RITA® the most widely implemented. In the case of our study, several reasons led us to select the GreenTray® bioreactor, developed by Dolcet-Sanjuan and Mendoza (2018), to analyze the evolution of the interaction between RP-20 *in vitro* plantlets and both, *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02. Firstly, this bioreactor has proven to be effective in improving the performance of *in vitro* fruit tree RP-20 rootstocks micropropagation as longer shoots were obtained, which would also explain the increase in shoot FW observed after the culture on this system. The multiplication rate, although slightly higher, was not significantly enhanced by the cultivation of RP-20 shoots in GreenTray® bioreactors. The main advantage of the implementation of TIS bioreactors in plant production is aimed at reducing the costs of the *in vitro* micropropagation process, equaling or even surpassing the micropropagation rate obtained in the semisolid systems (Ramos-Castellá et al., 2014; Scherer et al., 2013). Hand manipulation costs reduction associated to GreenTray® is based on the fact that all explants or plantlets can be moved out at once of the vessel, without forceps, by pulling the tray holding all the plant material, which is attached to the lid. In line with this approach, a similar response was reported by Welander et al. (2014) when cultured three ornamental species (*Digitalis*, *Echinacea* and *Rubus*) in a TIS bioreactor. In this experiment, multiplication rate was not increased for either of the three plant species after the culture in a TIS bioreactor. Nevertheless, *Echinacea* and *Rubus* shoots cultured in TIS bioreactor showed higher FW than those cultured in agar medium. The proven effectiveness of the TIS bioreactors in increasing the throughput of the *in vitro* micropropagation process had led to many authors focusing on *in vitro* woody plant species to develop profitable micropropagation protocols. For instance, Bello-Bello et al. (2019) reported that the culture of *in vitro* banana (*Musa*

spp.) explants in TIS bioreactors led to an increased multiplication rate in comparison to the culture in semi-solid agar medium. These authors also completed the micropropagation protocol concluding that banana micropropagated plantlets showed a higher survival in acclimatization conditions. On the other hand, the GreenTray® bioreactor was also selected to perform the study due to its practicity and ease to assembly. The multiples advantages of the GT bioreactor including its horizontal placement as well as the absence of solid medium made possible to easily observe how *in vitro* plantlets evolve together with the microorganisms. Moreover, the presence of liquid medium, physically separated from the plant culture vessel, also allowed the evolution of microbial populations to be monitored. For all the aforementioned, a complete experiment where the interaction between individual *in vitro* RP-20 shoots, prior rooting elongation, and the two PGPMs *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 was studied using GreenTray® bioreactors. In this study, the three major objectives explored were (1) the performance of the two PGPMs and RP-20 plantlets in a TIS bioreactor, (2) the ability of these two PGPMs to control the growth of endophytic contaminations naturally habiting RP-20 explants, and (3) the study of the effect of the application of the two PGPMs in the hormonal status of the culture medium. Two independent experiments were conducted, modifying the culture conditions or the microbial inoculum in order to meet all these objectives.

Regarding the performance of PGPMs and RP-20 *in vitro* plantlets in the presence of *C. ramotenellum* PGP02 and *P. oryzihabitans* PGP01, it was observed that, in general, *P. oryzihabitans* PGP01 did not importantly altered RP-20 growth, possibly attributed to the low inoculum concentration. In contrast, the conditions given in the plant culture vessel of the GreenTray® bioreactors inoculated with *C. ramotenellum* PGP02 negatively affected *in vitro* RP-20 plant development as a considerably reduced plant FW was registered after 15 days of co-culture. In these bioreactors, an uncontrolled production of CO_2 was observed in the plant culture vessel in response to the inoculation with *C. ramotenellum* PGP02 after 2 dpi together with a concomitant decrease in the O_2 content. These conditions were associated with a *C. ramotenellum* PGP02 overgrowth that disrupted *in vitro* plantlet development. However, these changes in the content of these two gases might be caused by the use of the sucrose present in the culture medium as a substrate for fungal metabolism as previously reported by many authors (Brannon, 1923; Mason & Righelato, 1976). It is widely known that CO_2 has an important role in *in vitro* culture, increasing the rate of photosynthetic carbon fixation rates and improving the performance of plants on the acclimatization stage (Pérez-Jiménez et al., 2015). In fact, the enrichment of the environment with CO_2 constitutes the basis for the establishment of a

photoautotrophic culture leading to the removal of sugars from the culture medium (Xiao et al., 2011). However, CO_2 concentration present in the culture vessel must be strictly controlled as high levels of CO_2 lead to growth disturbances in some plant genotypes (Tisarum et al., 2018). In this study, this uncontrolled production of CO_2 within the culture vessel could also explain the reduced values of the chlorophyll content observed in the leaves of RP-20 plantlets at the end of the culture process. Long term exposure to high concentrations of CO_2 may seriously affect the photosynthesis process by a negative adaptation of the enzyme RUBISCO, resulting in a reduction in the chlorophyll content (Arigita et al., 2002). On the other hand, endophytic contaminant population was determined in GreenTray® bioreactors together with the growth of *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02. Endophytic contaminations in *in vitro* cultures are often responsible of high losses of plant material due to that bacterial growth may overrun the cultures reducing micropropagation rates (Leifert & Cassells, 2001). Most of the studies published in literature have reported the efficiency of antibiotics such as gentamicin, tetracycline or tobramycin in the successful elimination of endophytes growth (Fang & Hsu, 2012; Khan et al., 2018). Moreover, other authors have proposed the application of the Plant Preservative Mixture™ (PPM) in the suppression of the growth of some bacterial endophytes species belonging to *Sphingomonas* genus (Lotfi et al., 2020; Miyazaki et al., 2010). In this study, the results obtained in this research may suggest the role of the pH of the culture medium in the modulation of the biological control activity of the two tested PGPMs. In the presence of *C. ramotenellum* PGP02 drastically suppressed the growth of endophytic contaminants after 5 dpi, but associated to the detriment of plantlet growth. After 7 days of co-culture, *C. ramotenellum* PGP02 decreased the pH of the MS medium up to approximately 2.6, making difficult the growth of bacterial contaminants. In contrast, the low inoculum concentration of *P. oryzihabitans* PGP01 used in this first trial did not promote important changes in the endophytes populations as no important changes in the pH of the MS medium were recorded regarding non-treated bioreactors.

As the initial pH of the culture medium has been shown to be a key factor to ensure bacterial growth, in a subsequent experiment, GreenTray® bioreactors were inoculated solely with *P. oryzihabitans* PGP01 at pH 5.7 and 7, which is the optimum for bacteria growth, to analyse if a better bacterial growth induced the effects in roots reported in previous studies (Cantabella et al., 2020, 2021). As expected, a better growth of *P. oryzihabitans* PGP01 was registered during the whole process when co-culturing with RP-20 plants at pH 7. These results are also supported by the decrease of O_2 and the increase of CO_2 observed during the most active growth of this

bacterium, revealing a higher activity at pH 7. Nevertheless, the greater performance of *P. oryzihabitans* PGP01 did not result in an improvement of RP-20 growth explants after 15 days of co-culture, mostly due to the negative effect of pH 7 on plantlet growth. This factor is crucial not only for bacterial growth but also for plantlet development as it is known that the optimum pH for *in vivo* plants ranges between 4.2 and 6.5 (Leifert et al., 1992). Surprisingly, the best results in terms of plant development were obtained when RP-20 explants and *P. oryzihabitans* PGP01 coexist in the medium MS at 5.7 as a stimulation of root development occurred to by increasing the number of roots as previously reported (Cantabella et al., 2021). In scientific literature, there are recent evidences available that reports the effect of PGPMs in the chlorophyll pigments (Khanghahi et al., 2019), most of them using this parameter as an indicator of both biotic and abiotic stress alleviation (Arkhipova et al., 2020; Jain et al., 2020; Kour et al., 2020). It is widely demonstrated that in green plants, chlorophyll represents the major pigments involved in the photosynthesis process, being Chl *a* and Chl *b* the main constituents of the photosynthetic apparatus (Tanaka & Tanaka, 2011; Zhao et al., 2020). In this process, chlorophyll do not act alone in this photosynthesis process as CO_2 is also involved through its fixation by the RUBISCO enzyme (Parry et al., 2013). In the present study, the inoculation with *P. oryzihabitans* PGP01 induced positive changes in the photosynthetic pigments content, observing an increase in the amount of Chl t mainly provoked by an increase in the Chl *a* content. Thus, the results herein presented might suggest that *P. oryzihabitans* PGP01, as a PGPM, could stimulate the photosynthesis process. However, in the GreenTray® bioreactors inoculated with this bacterium, similar evolution of the percentage of CO_2 was recorded in comparison to those non-treated bioreactors. Therefore, if the higher chlorophyll content observed in RP-20 leaves after 15 days of co-culture in the presence of *P. oryzihabitans* PGP01 is related to a better photosynthetic performance remains unclear, requiring further investigations to fully understand this relationship. For instance, Paradiso et al. (2017) reported a higher photosynthetic rate in plants inoculated with a mixture of PGPMs attributed to the improvement of leaf anatomical attributes. In this study, authors did not report differences in the levels of leaf chlorophyll in the presence of this PGPMs mixture. Higher inoculum concentration of *P. oryzihabitans* PGP01 at the same pH 5.7 either produced an evident control of endophytes growth. In addition, the increase of the initial pH of the MS medium up to 7, more suitable for *P. oryzihabitans* PGP01 growth, also favoured endophytes performance, slightly surpassing *P. oryzihabitans* PGP01 growth. Considering these results, endophytic bacteria population existing in RP-20 *in vitro* displayed a dynamic behaviour, being the pH the key factor modulating their

population. Endophytes in micropropagated plants have been shown as dynamic microorganisms, adapted to *in vitro* conditions and displaying fluctuations in populations over the years of culture (Quambusch et al., 2016). In the case of our study, future experiments will be needed to corroborate if these two PGPMs are able to control the growth of endophytic contaminations in RP-20 *in vitro* cultures.

It is widely reported that root colonization and the subsequent plant growth promotion mediated by PGPMs is commonly associated to changes in the hormonal homeostasis (Tsukanova et al., 2017). For that reason, in the present study, we decided to analyse how the inoculation of GreenTray® bioreactors with the PGPMs *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 may modify the hormonal status of the culture medium. In this case, it has been demonstrated that the inoculation of *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 co-cultured *in vitro* with RP-20 plantlets under different conditions affected the pattern of the different hormones analysed. In general, in the first trial, the application of either the two microorganisms did not promote plant growth of RP-20 *in vitro* plantlets after 15 days of co-culture. Furthermore, the results obtained in terms of plant FW and number of roots in RP-20 plantlets in GreenTray® bioreactors treated with both microorganisms in comparison to those non-inoculated might suggest an induction of a senescence process, as it was revealed by the increase on ABA content. This hormone has been widely associated with the stimulation of senescence signalling (Xie et al., 2004). In contrast, in the second trial, we found that the inoculation with *P. oryzihabitans* PGP01 at pH 5.7 only produced a significant decrease in the IAA content in MS medium after 15 days of co-culture with RP-20. Lower auxin levels in the culture medium might help root elongation and root number, and consequently plantlets cultured under these conditions showed higher number of roots than non-treated plantlets. For unravelling the mechanisms underlying *in vitro* plant growth promotion, many authors have paid particular attention in auxins as the main hormones involved in root development (Asghar et al., 2002; Dias et al., 2009; Iqbal & Hasnain, 2013a). In a previous study, Cantabella et al. (2021) suggested the IAA-producing ability of *P. oryzihabitans* PGP01 as the responsible of root modifications occurred in RP-20 *in vitro* micropropagated explants after 8 weeks of co-culture. On the other hand, some bacteria belonging to *Pseudomonas* spp. are able to produce auxin-like compounds that triggers auxin signalling stimulating root development (Ortiz-Castro et al., 2020; Zamioudis et al., 2013). In this study, auxins produced during the most active growth period of *P. oryzihabitans* PGP01 could have promoted root induction, explaining the increase in the number of roots observed in RP-20 *in vitro* plantlets. As a result, the higher root surface in RP-20 likely increased auxins uptake throughout the rest of the co-culture

process, explaining the lower levels of IAA present in MS medium of *P. oryzihabitans* PGP01-treated bioreactors after 15 days of co-culture. Moreover a lower IAA content in the culture medium could have facilitated root elongation. The negative effects of the increase on the initial medium pH on plant FW could in the absence of *P. oryzihabitans* PGP01 be corroborated by the higher contents of ABA observed in this GreenTray® bioreactors. Under these conditions, RP-20 senescence could be promoted by the presence of *P. oryzihabitans* PGP01 as higher values of this hormone were found in MS medium after 15 days of co-culture.

5. Conclusions

To conclude, this study demonstrated the feasibility of the GreenTray® bioreactor for an efficient *in vitro* micropropagation of the *Prunus* rootstock RP-20, increasing the FW and length of the produced explants. In addition, this system has also been effective for the study of the interaction between RP-20 plantlets and PGPMs, especially *P. oryzihabitans* PGP01, inducing a stimulation of root development by an increase in the number of roots. Both PGPMs tested in this study were not able to control endophytes growth; however, the results obtained in this regard suggested the determining effect of low pHs (acidic) in the removal of endophytes in the culture medium. Further experiments will be conducted to study in depth how the pH might modify endophytes population without affecting *in vitro* micropropagation in the absence of microorganisms.

6. Declaration of Competing Interest

Authors declare no conflict of interest.

7. Acknowledgements

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Chapter 5

Acidification of the culture medium as a successful strategy to control endophytic contaminations in plant micropropagation

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Abstract

Overgrowth of endophytes in some *in vitro* cultures may disrupt the normal shoot tip growth and proliferation, being necessary to obtain endophytes-free cultures to achieve a normal plant micropropagation process. To remove these contaminations from the culture medium, antibiotics are commonly added to the culture medium. However, its use in plant production must be urgently reduced because of the current restrictions imposed by the European Union. For that purpose, the effect of acidic (pH 3) and neutral (pH 7) pH was tested in the GreenTray® TIS bioreactor as an alternative to control endophytes growth without affecting the micropropagation of the *Prunus* rootstock RP-20 explants. The results demonstrated that culture at pH 3 did not affect the number of shoots, shoot FW, shoot length and the amount of chlorophyll pigments, but significantly reduced endophytes population. The identification also revealed that *Roseomonas mucosa*, *Microbacterium oxydans*, *Bacillus subtilis* and *Luteibacter yeojuensis* were the bacterial isolates responsible of those contaminations. These results might suppose a real breakthrough in the *in vitro* tissue cultures field, although more research is required to meet the pH requirements for the different plant species and other endophytic microorganisms.

Keywords: *In vitro* micropropagation; GreenTray®, *Prunus* rootstock; Inhibition of endophytes growth; Acidification; Chlorophyll content.

1. Introduction

Endophytes have been defined as microorganisms with the ability to colonize inner plant tissues without expressing disease symptoms (Petrini, 1991; Wilson, 1995). In *in vitro* cultures, most are bacteria belonging to *Methylobacterium* and *Curtobacterium* genera (Panicker et al., 2007; Pohjanen et al., 2014). Although, in their natural environment they do not induce harmful effects in plants, it is known that their presence in *in vitro* cultures might somehow modify explants behaviour. In some cases, endophytes in *in vitro* cultured plants led to a plant growth promotion and the improvement of *in vitro* processes such as multiplication or rooting of recalcitrant genotypes (Cantabella et al., 2021; Quambusch et al., 2014; Zawadzka et al., 2014). Nevertheless, in other plant species, endophytes may disturb *in vitro* explants performance, seriously affecting shoot micropropagation and leading to high losses of plant material (Cheong et al., 2020) and commercial plant micropropagation. In those cases, it is of crucial importance to establish a protocol for their removal from the culture medium. In the last years, the use of antibiotics or the Plant Preservative Mixture (PPM™) to obtain endophytes-free cultures has been reported as an effective procedure (Khan et al., 2018; Lotfi et al., 2020). However, these approaches should be abandoned due to the restrictions imposed by the European Commission concerning the addition of chemicals to the culture medium for plant production (Elmongy et al., 2018; Wiszniewska et al., 2016). In this context, more sustainable alternatives to achieve this goal are required. In a previous research, the ability of the two plant growth-promoting microorganisms (PGPMs) *Pseudomonas oryzihabitans* PGP01 and *Cladosporium ramotenellum* PGP02 to control endophytic contaminants was evaluated using the GreenTray® TIS bioreactor (Cantabella et al., unpublished data). In this study, although an effective biological control of these contaminations in the presence of both microorganisms did not occur, it was suggested that the effect of the pH might represent a crucial factor for endophytes control. For this reason, the present study has been designed to evaluate whether culture medium adjusted to more acidic (pH 3) or more basic (pH 7) pH values, compared with the optimal pH 5.7 used in plant growth,are able to control endophytes without affecting *in vitro* micropropagation of the commercial *Prunus* rootstock Rootpac® 20.

2. Material and methods

2.1. *In vitro* plant material

Explants of the *Prunus* commercial rootstock Rootpac® 20 (RP-20) (Agromillora, Barcelona, Spain) were used for the study. Twenty 2-cm-long shoots

were transferred from glass flasks to each GreenTray® bioreactor (Dolcet-Sanjuan & Mendoza, 2018) after 3 weeks of culture. Flasks contained Murashige and Skoog (MS) (Murashige & Skoog, 1962) supplemented with 3% sucrose and 5 μM 6-Benzylaminopurine (BAP), pH to 5.7, agar (7 g L^{-1}) and autoclaved at 121 °C for 20 minutes. Shoot explants were apparently clean, with no endophytic growth in the culture media at the shoot clump base.

2.2. Experimental conditions

RP-20 micropropagated explants in GreenTray® bioreactors were cultured using Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) at pH 3 and pH 7 using a buffer solution based on different proportions of citric acid 0.1 M and Na_2HPO_4 0.2 M following the indications of Buffer Reference Center (Sigma Aldrich). As standard, MS medium adjusted to pH 5.7 with 0.1 N of NaOH was used. Media at pH 3 and 7 were sterilised by filtration using a 0.22 µm filter, while media at pH 5.7 was sterilized by autoclaving at 120 ºC during 20 min, following the standard protocol. Three GreenTray® bioreactors for each treatment were set up.

2.3. Evaluation of *in vitro* micropropagation and dynamics of endophytes population

After 8 days of culture, the effects of pHs in RP-20 *in vitro* micropropagation was determined by measuring the number of shoots, shoot length, shoot FW, as well as the content of total chlorophyll (Chl t), chlorophyll a (Chl *a*) and chlorophyll b (Chl *b*). In addition, a representative number of colonies with different morphological aspect were isolated and identified by DNA sequencing of the 16S rRNA gene and MALDI-TOF by the Laboratory of Instrumental Techniques, University of León (Spain). The sequencing of the 16S rDNA gene represents a universal molecular tool whereby the identification of microorganisms takes place by amplifying the intervening variable regions of that gene. The analysis of these variable portions provide a specific identification of bacterial species in the samples (James, 2010). In contrast, identification of microbial species by MALDI-TOF is done by comparison of the peptide mass fingerprint of the microorganisms in the samples with the peptide mass fingerprint in the database (Singhal et al., 2015). During the *in vitro* culture process, samples of culture medium were taken to monitor the population dynamics of total endophytes.

2.4. Statistical analysis

The experiment was design considering a completely random design (CRD), and data analysis was carried out by using JMP Pro Software (version 13.1.0, SAS Institute Inc., Cary, NC). Statistical significance was judged at $P < 0.05$, and the Tukey test was used to separate the means when the differences were statistically significant.

3. Results

After 8 days of culture in GreenTray® bioreactors, the micropropagation of RP-20 explants was not negatively affected by pH 3, since no differences were found in the number of shoots produced, shoot FW and shoot length in comparison to when the micropropagation is carried out at optimal pH 5.7 (Figure 1A, B and C). In contrast, when the pH was adjusted to 7 in RP-20 micropropagation, drastically decreased the number of shoots produced (86.5%) and shoot FW (83.9%) after 8 days of culture (Figure 1A, B and C).

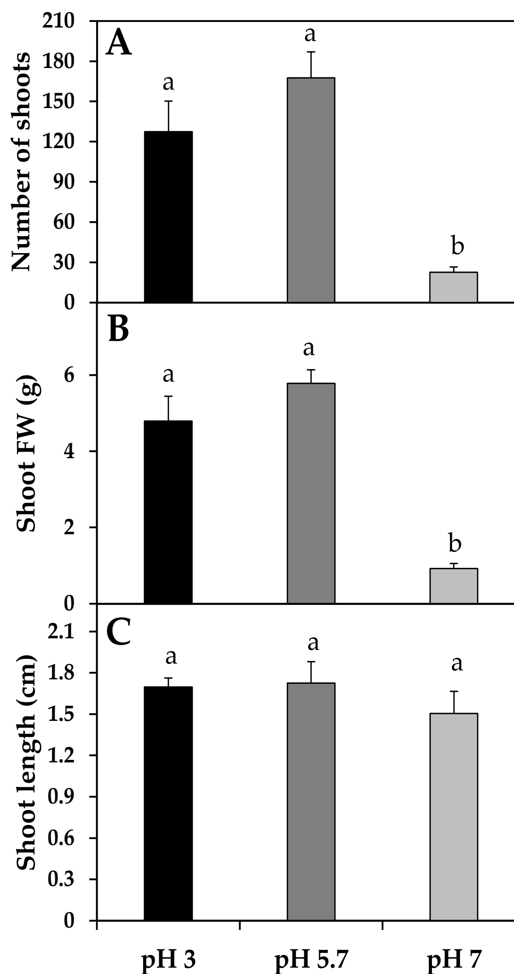

Figure 1. Effect of the culture of RP-20 *in vitro* explants in GT bioreactors at pH 3, pH 5.7 and pH 7 in the number of shoots (A), shoot FW and shoot length (C) after 8 days of culture. Data represents the mean ± standard error (SE) of the measures taken in three bioreactors per treatment. Different letters indicate significant differences among treatments according to Tukey HSD test ($P < 0.05$).

Regarding endophytes population, it was clearly shown that the micropropagation at pH 3 controlled the growth of bacterial endophytes in RP-20 shoot cultures, observing reductions of 3.01, 2.23 and 2.43 log CFU mL^{-1} after 1, 3 and 6 days of culture, respectively (Figure 2). Under this acidic pH, endophytes drastically decreased and were not detected in the culture medium after 8 days of *in vitro* culture. Conversely, endophytes in RP-20 cultured in MS medium at pH 7 displayed significant increases on their population of 1.5, 2.66, 3.73 and 3.38 log CFU mL^{-1} after 1, 3, 6 and 8 days of culture (Figure 2).

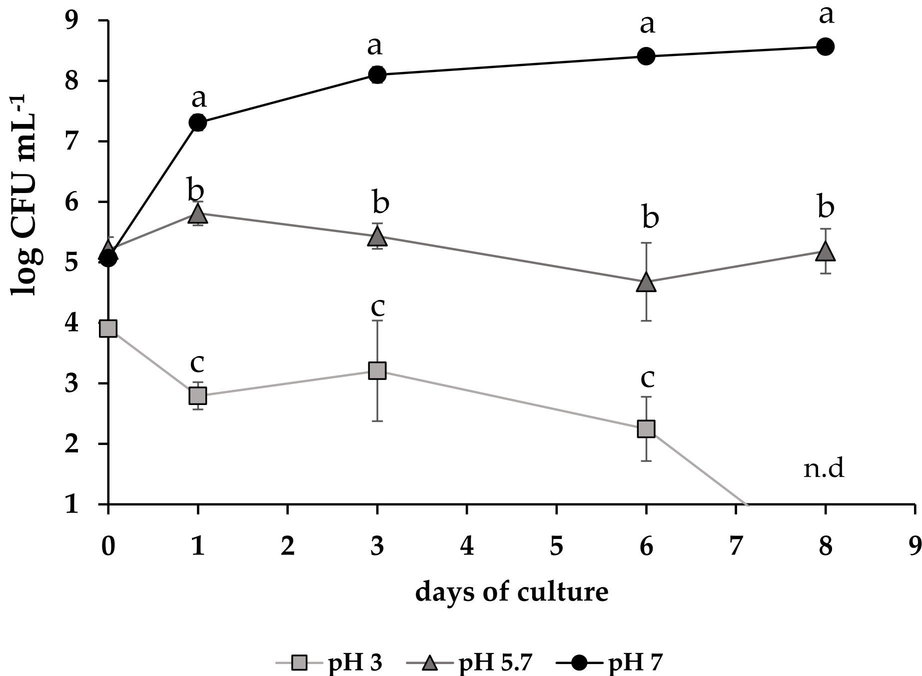

Figure 2. Effect of the culture of RP-20 *in vitro* explants in GT bioreactors at pH 3, pH 5.7 and pH 7 in the population dynamics of endophytes. The showed values for each treatment represents the mean ± SE of samples taken in three bioreactors Different letters indicate significant differences among treatments according to Tukey HSD test ($P < 0.05$).

After 8 days, the culture of RP-20 at pH 3 did not negatively affect the content of Chl t, Chl *a* and Chl *b* compared to the culture at pH 5.7 (Figure 3A). In addition, RP-20 plantlets cultured at both pHs showed green and fully expanded leaves after 8 days of culture (Figure 2B, C). Nevertheless, a 90 and 96% significant decrease in the amount of Chl t and Chl *a* was recorded in RP-20 leaves cultured in MS medium at pH 7 (Figure 3A). Although not significant, it was also registered an almost 72% decrease in the amount of Chl *b* in RP-20 leaves cultured under this pH compared to

the culture at pH 5.7 (Figure 3A). Under pH 7, RP-20 *in vitro* shoots displayed a stressed appearance with shrunken, yellowish or brownish leaves (Figure 3D) than those cultured at pH 5.7 or 3 (Figure 3C).

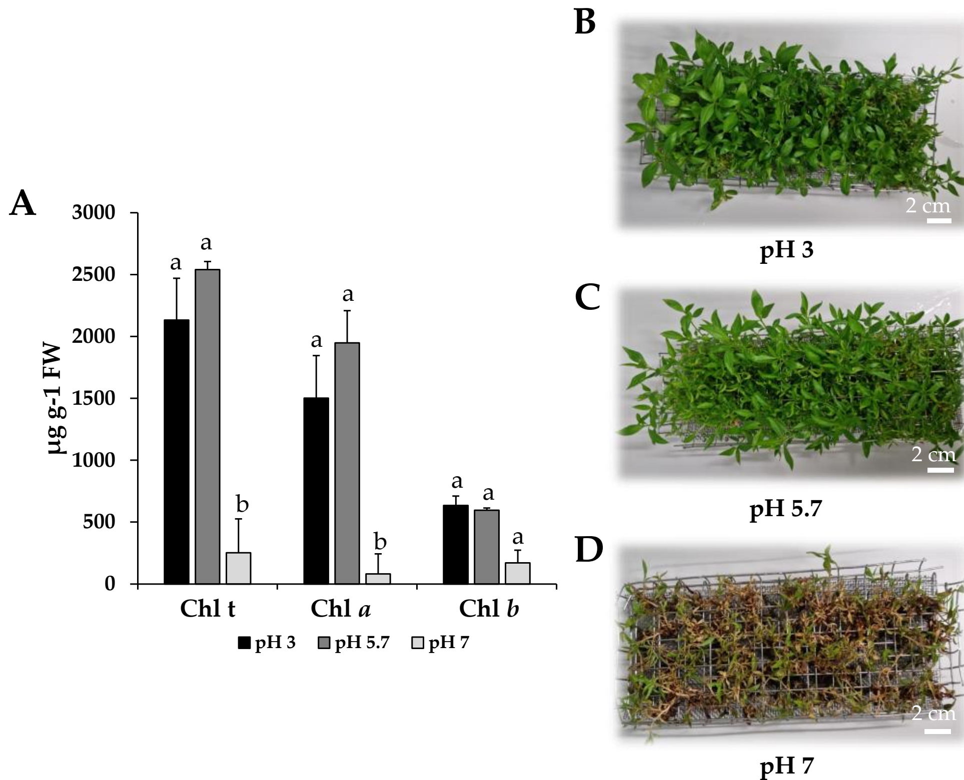

Figure 3.- Chlorophyll content of RP-20 *in vitro* leaves of explants cultured in GT bioreactors at pH 3, pH 5.7 and pH 7 (A) and explants appearance after 8 days of culture (B). Data represents the mean ± standard error (SE) of the measures taken in three bioreactors per treatment. Different letters within each chlorophyll pigment indicate significant differences among treatments according to Tukey HSD test ($P < 0.05$).

After identification, it was revealed that four different microbial species were responsible of these endophytic contaminations of RP-20 cultured in the GreenTray® bioreactor (Table 1). The bacterial species *Bacillus subtilis*, *Roseomonas mucosa* and *Microbacterium oxydans* were identified by MALDI-TOF, and the species *Luteibacter yeojensis* was detected by the sequencing of nucleotides of the 16S rDNA (Table 1). The high scores (2.22, 2.45 and 2.22 for *B. subtilis, R. mucosa* and *M. oxydans*, respectively) as well as the high percentage of similarity (>99%) obtained by both techniques revealed high confidence identifications of microbial species (Table 1).

Table 1. Identification of the different isolated endophytes colonies in *Prunus* RP-20 *in vitro* explants.

MALDI-TOF identification	Score value	Interpretation
Bacillus subtilis	2.22	High confidence identification
Roseomonas mucosa	2.45	High confidence identification
Microbacterium oxydans	2.22	High confidence identification
16S rRNA identification	**% Similarity**	
Luteibacter yeojuensis	> 99%	High confidence identification

4. Discussion

In this study, it has been demonstrated that the pH of the culture medium has an important effect in the growth of endophytes in *in vitro* cultures. This experiment has been possible due to the use of a liquid culture system that avoids solidification problems of the gelling agent (Thorpe et al., 2008). Based on our results, the micropropagation of RP-20 explants in GreenTray® bioreactors could be performed using culture medium with pH 3 since no differences in the number of shoots, shoot length, shoot FW were observed, in comparison to when the process is conducted at pH 5.7, considered optimum for plants growth. These results are consistent with those obtained by Martins et al. (2011) who reported that micropropagation of *Plantago* spp. could be carried out at a lower pH than the commonly used for *in vitro* tissue culture. In contrast, other authors concluded that apple micropropagation could be carried out at a broad range of pH ranging between 5.5 and 7.5 (Shi et al., 2017). However, this was not possible for RP-20 micropropagation since it was drastically affected at pH 7, observing reductions of 86.5 and 83.9% the number of shoots as well as the shoot FW, respectively, compared to the culture at pH 5.7. Under pH 7 conditions, endophytes growth was favoured, leading to higher log CFU mL^{-1} regarding the medium at pH 5.7 at 1, 3, 6 and 8 days of culture. In contrast, it is noteworthy to mention that when the pH of the micropropagation medium was adjusted to 3, endophytes population were somehow controlled, registering lower values of log CFU mL^{-1}. As mentioned, in a previous study conducted in the presence of microorganisms, a relationship between the pH of the culture medium and endophytes growth was established, being the bacterial population considerably reduced at low pH (approximately 2.5 log CFU mL^{-1}) when the inoculation with *C. ramotenellum* PGP02 took place (Cantabella et al., unpublished data). This

microorganism significantly decrease the level of culture pH. In this sense, it is widely known that while bacterial growth is favoured at pH values ranging 6.5-7.0, more acidic pH values below 5.0 seriously compromised bacterial performance (Mossel et al., 1995). In the present study, the uncontrolled growth bacterial endophytes in the RP-20 cultures at pH 7 are most probably the responsible for the negative effects in *in vitro* micropropagation. However, further experiments will be required to corroborate this assumption. All the previous results were supported by those obtained in the content of chlorophyll pigments in *in vitro* RP-20 leaves. After identification, it was revealed that four different microbial species were responsible of these endophytic contaminations. The bacterial species *Bacillus subtilis*, *Roseomonas mucosa* and *Microbacterium oxydans* were identified by MALDI-TOF, and the species *Luteibacter yeojensis* was detected by the sequencing of nucleotides of the 16S rDNA. In this regard, many authors have previously provided evidence concerning the endophytic origin of some of these bacterial species. However, not all of them are described as endophytes in *in vitro* culture. In addition to the endophytic nature of these microorganisms, positive effects in plant growth have been reported. For instance, *R. mucosa* have been found as an *in vitro* endophyte bacterial species in walnut cultures obtained from embryonic tissue (Pham et al., 2017). In a recent work, the endophyte bacterial species *M. oxydans* was isolated from tomato roots (Hernández-Pacheco et al., 2021). In *in vitro* tissue culture, bacterial species belonging to the *Microbacterium* genus have been previously associated with a higher propagation success in cherry (*Prunus avium* L.) genotypes (Quambusch et al., 2014). In addition, many reports are available about the endophytic origin of *B. subtilis* in many plant species (Comby et al., 2017; Fouda et al., 2021). In contrast, very little information is available about the role of *L. yeojuensis* as bacterial endophyte. Nevertheless, other species belonging to this genus have been reported as endophytes in *Quercus* spp (Lasa et al., 2019). In most cases, these endophytes strains have shown beneficial effects in plant growth, increasing plant growth parameters or inhibiting the growth of pathogen microorganisms (Comby et al., 2017; Hernández-Pacheco et al., 2021). For that reason, the isolated microorganisms will be stored for further experiments. These results might represent a paradigm shift in the plant *in vitro* tissue culture that help to mitigate the losses occasioned by the presence of bacterial endophytes. However, further investigations are required in this regard since it is reported that pH requirements for optimal growth are highly depending on the plant species (Leifert et al., 1992). Altogether, it has been demonstrated that endophytes populations in micropropagated explants might be controlled by modulations in the pH of the

culture medium, replacing the addition of antibiotics and contributing to a more sustainable *in vitro* plant production.

5. Declaration of Competing Interest

Authors declare no conflict of interest.

6. Acknowledgements

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7. Data availability statement

Authors can confirm that all relevant data are included in the article.

8. Author contribution statement

DC, NT and RDS contributed to the design of the study. DC, MC, RDS, and CS. conducted all the experiments, as well as the data collection. DC, NT, RT, and RDS performed the data analysis and interpretation. The writing and revision of the manuscript were performed by DC, NT, RT, and RDS. All the authors have read the article and made critical contributions to improve the quality of the manuscript.

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Chapter 6

Optimization of a food industry-waste-based medium for the production of the plant growth promoting microorganism *Pseudomonas oryzihabitans* PGP01 based on agro-food industries by-products

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Abstract

Nowadays, there is a need to find a use for wastes from the food industry to contribute to a more sustainable chain. In this study, three wastes based on potato peels and pulps, tomato seeds and wheat bran were used as basis for the preparation of a cheap medium to produce the bacterium *P. oryzihabitans* PGP01. In flasks experiments, *P. oryzihabitans* PGP01 growth at 25 ºC in a medium based on frozen potato peels and pulp (FPP) with tryptone as a nitrogen source at both 10 g L^{-1} and 20 g L^{-1} resulted in the maximum production ($2.7x10^9$ and $3.6x10^9$ CFU mL^{-1}, respectively) compared to the commercial TSB medium ($2.4x10^9$ CFU mL^{-1}). In the scale-up to 2 L bioreactors, 300 g L^{-1} of FPP supplemented with 10 g L^{-1} tryptone, 10 g L^{-1} molasses, 5 g L^{-1} NaCl and 2.5 g L^{-1} K_2HPO_4 allowed to reach similar biomass production than in the TSB medium. Several conditions were tested to optimize the growth in the bioreactor finding the best results when the agitation and the air flux were set at 400 rpm and 0.75 vvm, achieving a maximum growth of $4.4x10^9$ CFU mL^{-1} after 24 h of culture. Finally, *P. oryzihabitans* PGP01 growing in this optimized medium conserved its biological activity showing the expected effect in root development previously reported for this microorganism. The waste-based-medium developed in the present research could reduce considerably the costs of the production as well as the total amount of wastes generated in the agro-food industry.

Keywords: Plant growth-promoting microorganisms; low-cost production; agro-food by-products; molasses; *in vitro* micropropagated plants.

1. Introduction

From the beginning of the present century, the considerable increase in the amount of greenhouse gasses emitted into the atmosphere because of the application of synthetic fertilizers has led to abandon the existing agricultural practices, moving towards more sustainable ones. In this context, the use of plant growth promoting microorganisms (PGPMs) in agriculture is presented as a promising alternative since they are able to improve plant growth by several mechanisms including mineral solubilisation (Collavino et al., 2010), nutrient uptake (Kuzmicheva et al., 2017), changes in root morphology (Contesto et al., 2010; Iqbal & Hasnain, 2013; Trinh et al., 2018) or abiotic stress tolerance (Barnawal et al., 2017; Kumar & Verma, 2018). Due to their great variability of functions, the use of these microorganisms as biofertilizers can be potentially exploited (Calvo et al., 2014).

Generally, PGPMs are divided in two categories, plant growth-promoting fungi (PGPF) and plant growth-promoting rhizobacteria (PGPR), being the second the greatest studied group so far (Vessey, 2003). Nowadays, a large amount of studies reported the effective use of rhizobacteria as biofertilizers, most of them involve bacteria belonging to *Pseudomonas* or *Bacillus* genus (Esitken et al., 2010; Kumar et al., 2005; Vejan et al., 2016). Within *Pseudomonas* genus, a growing interest in the species *Pseudomonas oryzihabitans* has emerged due to its potential role in plant growth promotion by the enhancement of root development. However, the plant growth-promoting activity of this bacterium species has remained relatively unexplored, and very little information is published in scientific literature. *P. oryzihabitans* is a soil-habiting rhizobacteria with the ability to enhance pea and mustard plant growth, even under adverse conditions, improving root elongation (Belimov et al., 2001). In the last years, an increase in the knowledge of this bacterial species has emerged, being those effects in roots demonstrated in other plant species such as potato plants (Belimov et al., 2015) and in *Prunus and Pyrus* species cultured *in vitro* (Cantabella et al., 2020). Other roles of this bacterial species including the biocontrol activity against root-knot nematodes or the degradation of toluene or organochlorine pesticides have also been reported (Andreoglou et al., 2003; Barragán-Huerta et al., 2007; Vagelas et al., 2007).

The production of the microorganism is the first step to obtain a commercial microbial-based product and one of the main goals is to find a cheap cost medium that ensures the production of large amounts of microorganism cells without compromising their biological activity. In addition, the optimized medium must provide nutrients and energy that supplies microorganism's nutritional demands for cellular metabolism, growth and population stability (Droby et al., 2016). For that

purpose, the use of inexpensive commercial products and by-products has tended to be the most successful approach to obtain high quantities of microbial biomass (Teixidó et al., 2011). This strategy allowed the successful implementation of an optimized biomass production protocol for several biological control agents including bacteria such as *Pantoea agglomerans* CPA-2 (Costa et al., 2001), *Bacillus subtilis* CPA-8 (Gotor-Vila et al., 2017; Yánez-Mendizábal et al., 2012), or yeasts such as *Candida sake* CPA-1 (Abadias et al., 2003) and *Rhodotorula minuta* (Patiño-Vera et al., 2005). In addition, production protocols of some PGPMs such as *Azospirillum brasilense* or *Pseudomonas trivialis* BIHB 745 as biofertilizers have been also developed (Trujillo-Roldán et al., 2013; Vyas et al., 2014).

Several efforts have been made to obtain cheap media to produce microorganisms in a commercial scale and in several cases, they were using by-products from food industries as a carbon or nitrogen sources (Costa et al., 2001; Yánez-Mendizábal et al., 2012). More recently and under the framework of the European Project AGRIMAX (BBI-IA-DEMO-720719), organic wastes from food and agriculture industries were used as by-products to enhance biological processes. It has been estimated that an approximate amount of 88 millions of tonnes of wastes are generated during food manufacturing within the European Union, representing economical losses valued at 143 billion of euros (Stenmarck et al., 2016). In this sense, organic wastes from food and agriculture industries are rich in bioactive compounds that could be used by living organisms to enhance biological processes (Xu & Geelen, 2018). These wastes have a significant relevance as they are obtained from products that are highly consumed within the European market, and they have been identified as raw materials for the extraction of a wide range of products such as biobased coatings and packaging, natural food additives and functional food products, fertilizers and biodegradable pots for agriculture (Fritsch et al., 2017). This project was mainly focused on the sustainable utilization of by-products from potato, tomato, cereals and olive industries that may be useful in other industrial processes including low-cost microorganisms's production. Designing a waste-based medium for microorganism production will help in circular economy not only reducing the industry wastes but also in giving a second life and valorization of these products (Ubando et al., 2020). In previous studies conducted within the AGRIMAX project, several by-products from these food industries showing differences in manufacturing were characterised, and it was found that most of them displayed a high content (approximately 50%) of carbon source and a low proportion of nitrogen source (unpublished data). This fact has made possible the use of these by-products as the basis for the elaboration of a culture medium to grow the yeast *Saccharomyces*

cerevisiae. In contrast, the nutritional requirements are quite different when growing bacteria, requiring compounds that provide a nitrogen supply since it is known that the growth of these microbes is favoured by the presence of nitrogen source (Costa et al., 2002).

The aim of this research was the development of a biomass production protocol for the PGPM *P. oryzihabitans* PGP01 using wastes from the agri-food industry as a more economic and sustainable alternative. This bacterial strain has previously shown to promote root growth and development in *Prunus* and *Pyrus in vitro* plantlets (Cantabella et al., 2020, 2021). The objectives of this study were 1) to optimize the production of *P. oryzihabitans* PGP01 strain growth using a waste-based medium, 2) to scale-up and to optimize the bacterial production of 2 L bioreactor and 3) to use the bacterial population grown in the optimized media to promote explant rooting and improve plant quality in *in vitro* conditions. The present research would set the bases to develop a product based on this microorganism for the fertilisation of fruit tree plants reducing the use of chemicals compounds, promoting a better environmental sustainability.

2. Material and methods

2.1. Microorganism

The bacterial isolate used in the study was *P. oryzihabitans* strain PGP01 belonging to the Postharvest Pathology Group of IRTA (Lleida, Catalonia, Spain). This bacterial strain was isolated from *Prunus* and *Pyrus in vitro* contaminated cultures which displayed a greater growth than those non-contaminated, and a root growth promotion ability was attributed in different *in vitro* plant materials (Cantabella et al., 2020). Bacterial stocks were preserved at 4 ºC after isolation and subcultured on nutrient 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 25 ºC for 48 h when needed. Bacterial cultures were stored for a long-term at -80 ºC using criogenic beads.

2.2. Inoculum preparation

For all the experiments, *P. oryzihabitans* PGP01 inocula were obtained from 48-h-old cultures. Bacterial cells were transferred to phosphate buffer (70 mL KH_2PO_4 0.2 M; 30 K_2HPO_4 0.2 M and 300 mL deionized water). The fresh cell suspension was adjusted to OD 40 ± 5% measured at 420 nm with a spectrophotometer, which corresponded to, approximately $1x10^8$ colony forming unit per ml (CFU mL^{-1}). This concentration was diluted to $1x10^5$ or $1x10^6$ CFU mL^{-1} using sterile distilled water for

shake flasks or bioreactors inoculation, respectively. In both cases, the real applied concentrations were determined by dilution plate technique on NYDA medium.

2.3. Shake flasks experiments

2.3.1. Optimum growth temperature optimization

The effect of incubation temperature was tested to determine the optimal growth temperature of *P. oryzihabitans* PGP01. For that purpose, conical flasks containing *P. oryzihabitans* PGP01 growing in 50 mL of tryptone soy broth medium (TSB: 17 g tryptone; 3 g digested soy flour; 5 g sodium chloride; 2.5 g glucose, 2.5 g K_2HPO_4 in 1000 mL deionized water) were incubated at 20, 22, 25, 28, 30 and 32 ºC under orbital agitation at 150 rpm for 48 h. Samples were taken at 24 and 48 h, and viable cell concentrations were determined by plate dilution technique on NYDA medium. For each temperature analysed, three independent replicates were used.

2.3.2. Agro-food industry by-products assays

In this experiment, *P. oryzihabitans* PGP01 was tested for its ability to grow in media based on different by-products from several industry wastes (frozen potato peels and pulp (FPP), tomato seeds after flotation (TS) and wheat bran (WB). Before media preparation, FPP was grinded to improve homogenization and TS were sterilized to avoid microbial fermentation. Media were prepared in 250 mL conical flasks containing 50 mL of each medium (FPP, TS and WB at 300, 100 and 20 g L^{-1}, respectively) and autoclaved at 121 ºC for 15 min. For each by-product the selected concentration was the highest which could be dissolved for this amount of water without causing seals during growing. The laboratory medium TSB was used as a control. Initial pH of all media was measured before and after autoclaving, and 2.5 g L^{-1} of a sterile solution of K_2HPO_4 0.2 M was added to adjust the initial pH at 7. Upon inoculation with *P. oryzihabitans* PGP01 as described above, flasks were incubated at the optimum temperature and at 150 rpm for 48 h. Cultures were sampled at 24 and 48 h, and viable cell concentration (CFU mL^{-1}) was determined for both sampling periods by plate dilution technique on NYDA medium. Three independent replicates were used for each tested medium.

2.3.3. Optimization of nitrogen sources assays

Media based on frozen potato peels wastes was supplemented with several nitrogen sources such as urea, peptone (PEP), PROSTAR 510A (PS), yeast extract (YE), meat extract (ME), pea protein (PP), maize protein (MP) and tryptone (TRP), and were tested in order to fit the nitrogen requirements for the growth of *P. oryzihabitans*

PGP01 in the waste-based medium. PROSTAR 510A is an isolated soy protein, and represents an adequate nitrogen source since it contains a 90% of protein content (Gotor-Vila et al., 2017). The different nitrogen sources were added at low and high concentration to select the best one (Table 1). As previously, TSB medium was used as control. Samples were collected after 24 and 48 h of growth, and the *P. oryzihabitans* PGP01 population (CFU mL^{-1}) was determined at both sampling periods by plating serial dilutions on NYDA medium.

Table 1. Nitrogen sources tested and concentration.

Nitrogen source	Dose level	g L^{-1}
Urea	High	5
	Low	10
Peptone (PEP)	High	10
	Low	20
PROSTAR (PS)	High	10
	Low	20
Yeast extract (YE)	High	10
	Low	20
Meat extract (ME)	High	10
	Low	20
Pea protein (PP)	High	10
	Low	20
Maize protein (MP)	High	10
	Low	20
Tryptone (TRP)	High	10
	Low	20

2.4. Bioreactor experiments

2.4.1. Optimization of the medium composition

Scaling-up of PGP01 production in FPP-based-media was performed at laboratory scale using 2 L BioFlo/CelliGen 115 modular bioreactors (Eppendorf AG, Barkhausenweg, Hamburg, Germany). In a first experiment, these bioreactors were filled with 1.8 L of the medium based on potato wastes (FPP) and the best nitrogen source at 10 and 20 g L^{-1} to optimise the concentration that provides the best bacterial growth. To improve bacterial production, the FPP-based medium supplemented with a nitrogen source was also complemented with 5 g L^{-1} of sodium chloride (NaCl), 5 g L^{-1} glucose + 5 g L^{-1} NaCl or 10 g L^{-1} of sugar cane molasses (MOL) + 5 g L^{-1} NaCl. All

media were sterilized by autoclaving at 121 ºC for 60 min. Bioreactors were inoculated with fresh *P. oryzihabitans* PGP01 inoculum at $1x10^6$ CFU mL^{-1} as described above. In all cases, the initial pH of the medium was adjusted to 7 with a sterile solution of K_2HPO_4 0.2 M. Moreover, bioreactors containing the laboratory medium TSB were also inoculated and used in the experiment as standard control. The conditions of the process were set at 25 ºC, 400 rpm and 0.5 vvm of air feeding, and pH, temperature and dissolved oxygen (pO_2) were constantly monitored. Antifoam (30% simethicone emulsion USP, Dow Corning®, USA) at 1 mL L^{-1} was added. Samples were taken at 0, 20, 22, 24, 26, 28 and 42 h of growth, and the viable cell concentration (CFU mL^{-1}) was determined at each sampling period by the dilution plate technique to obtain microorganism growth curve.

2.4.2. Optimization of growth conditions

In order to maximize the production yield of *P. oryzihabitans* PGP01 in the potato wastes-based medium (300 g L^{-1} FPP supplemented with 10 g L^{-1} TRP, 5 g L^{-1} NaCl 10 g L^{-1} MOL and 2.5 g L^{-1} K_2HPO_4), different agitations (200, 400 and 600 rpm) and air fluxes (0.25, 0.5 and 0.75 vvm) at 25 ºC were tested. After inoculation of bioreactors with fresh cell *P. oryzihabitans* PGP01 inoculum as described above, samples were taken at 0, 20, 22, 24, 26, 28 and 42 h for 200 and 400 rpm. In the case of 600 rpm, samples were taken at 0, 18, 20, 22, 24, 26 and 42 h due to the faster growth of *P. oryzihabitans* PGP01 in these conditions. Viable cell concentration (CFU mL^{-1}) was determined by dilution plate technique on NYDA medium.

2.4.3. *P. oryzihabitans* PGP01 growth under optimal conditions

Once defined the conditions of the whole production process, the growth curve of *P. oryzihabitans* PGP01 in the optimised medium was deeply studied with the aim of elucidating the performance of the microorganism in the culture medium. In this experiment, 2 L bioreactors containing the optimized medium were inoculated with *P. oryzihabitans* PGP01 at $1x10^6$ CFU mL^{-1}. Growth conditions were set at 25 ºC, 400 rpm and 0.75 vvm of air flux. Samples for the determination of the bacterial population size during the most active growing stage (from 0 to 30 h) were taken every 2 h. In addition, samples were also taken at the stationary phase of bacterial growth (40 and 48 h). Viable *P. oryzihabitans* PGP01 cell concentration was determined at every sampling time by dilution plate technique on NYDA medium and expressed as CFU mL^{-1}.

2.5. Determination of the root growth-promoting activity of *P. oryzihabitans* PGP01 in the optimized medium

The feasibility of the waste-based optimized medium for the growth and maintenance of *P. oryzihabitans* PGP01 effect on root development activity was tested in the *Prunus* rootstock Rootpac® 20 (RP-20) (Agromillora Group, Spain) *in vitro* rooted plantlets. For that purpose, *in vitro* micropropagated RP-20 explants were cultured in Murashige and Skoog medium (Murashige & Skoog, 1962) supplemented with 10 µM of the auxin indole-3-butyric acid (IBA) during 7 days in darkness to promote *in vitro* rooting. After exposition to IBA, RP-20 explants were transferred to the same medium without IBA during another 7 days, under light conditions, to promote root development. Shoots with visible roots were then cultured in root elongation medium with vermiculite to favour the *in vitro* coexistence of rooted plantlets with microorganisms. RP-20 *in vitro* plants were inoculated with 1 mL of fresh cell inoculum of *P. oryzihabitans* PGP01 set at $2x10^8$ CFU mL^{-1} after 24 h of growth in synthetic TSB and optimized by-products based medium. Control plants were inoculated with 1 mL of sterile distilled water. Viable cell concentration was determined in both cases by dilution plate technique on NYDA medium.

2.6. Statistical data analysis

Data representing bacterial population (CFU mL^{-1}) were log transformed (Log CFU mL^{-1}) to improve homogeneity of variances. Data concerning the optimization of the temperature and the by-products as well as the biometrical data from root growth were analysed by one-way ANOVA. However, data concerning nitrogen source optimization as well as the scale-up to bioreactors were analysed by two-way ANOVA. When the model was statistically significant, Tukey HSD test was used for the separation of the means. For all data analysis, JMP Pro software was used (version 14.2.0, SAS Institute Inc., Cary, NC).

3. Results

3.1. Production of *P. oryzihabitans* PGP01 in flasks

3.1.1. Temperature and by-product optimization

Bacterial population data of *P. oryzihabitans* PGP01 when growing at different temperatures (ranged from 20 to 32 ºC), and different by-product based media (potato, tomato and cereal) from industries wastes are shown in Figure 1. After 24 h of growth in the commercial medium TSB, the highest growth of *P. oryzihabitans* PGP01 was obtained at 25 ºC, reaching a value of 9.32 log CFU mL^{-1} (Figure 1A).

Conversely, a poor growth of *P. oryzihabitans* PGP01 was observed at 20 ºC at this period. The highest growth achieved after 48 h of incubation was registered at both 22 and 25 ºC of temperature (9.71 and 9.77 log CFU mL^{-1}, respectively) (Figure 1A). According to the obtained results, 25 ºC was the established temperature to be used in the following experiments.

On the other hand, the growth of *P. oryzihabitans* PGP01 in the commercial medium TSB was 9.32 and 9.77 log CFU mL^{-1} at 24 h and 48 h, respectively. At the same sampling time, lower growth values were obtained when the bacterium was grown in media based on FPP and WB (8.50 and 8.27 log CFU mL^{-1}, respectively), and even lower in TS-based medium (Figure 1B). Even though *P. oryzihabitans* PGP01 growth in the three by-product based media remained significantly lower than the observed for the TSB medium after 48 h (9.77 log CFU mL^{-1}), the medium with FPP provided the greatest values of bacterial production (Figure 1B), being the candidate by-product for medium optimization in the later trials.

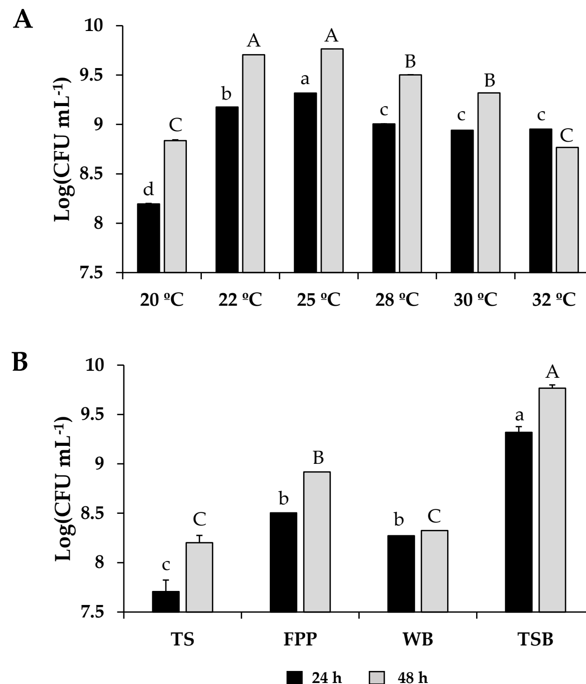


Figure 1. Growth in flasks of *P. oryzihabitans* PGP01 after 24 h (dark bars) and 48 h (grey bars) at different temperatures (A) and using wastes from different industry by-products or wastes (B). Data represents mean ± Standard Error (SE) of three independent replicates, and different letters within each sampling period indicate significant differences according to Tukey HSD test ($P < 0.05$).

3.1.2. Selection of nitrogen source

To improve the growth of *P. oryzihabitans* PGP01, FPP-based medium was supplemented with urea, peptone, PROSTAR 510A, yeast extract, meat extract, pea protein, maize protein or tryptone as nitrogen sources to try to achieve similar amount of bacterial cells that the obtained in the commercial medium TSB. After 24 h, only the combination of FP15 with the highest concentration (20 g L^{-1}) of peptone, PROSTAR 510A, yeast extract or meat extract led to a similar growth of *P. oryzihabitans* PGP01 than when the bacterium was grown in the commercial medium TSB (9.19, 8.29, 9.12 and 9.16 log CFU mL^{-1} for peptone, PROSTAR 510A, yeast and meat extract, respectively), and higher than the FPP-based media by itself (8.15 log CFU mL^{-1}) (Figure 2A). FPP-based media supplemented with other nitrogen sources such as tryptone or pea protein at 20 g L^{-1} (8.72 and 8.66 log CFU mL^{-1}, respectively) as well as meat extract at 10 g L^{-1} (8.59 log CFU mL^{-1}) were able to improve the growth observed when only the FPP by-product was used (Figure 2A). However, at this period, the FPP-based medium supplemented with the lowest concentration (10 g L^{-1}) of yeast extract, pea protein, peptone, PROSTAR 510A or tryptone did not significantly affect *P. oryzihabitans* PGP01 growth compared to the observed in FPP-based media by itself (Figure 2A). Urea and maize protein to the culture medium at both low and high concentration led to the poorest growth of *P. oryzihabitans* PGP01 recorded (<7.5 log CFU mL^{-1}) (Figure 2A).

At 48 h of growth at 25 ºC, the FPP-based media supplemented with peptone at 20 g L^{-1} or tryptone at both 10 and 20 g L^{-1} provided a *P. oryzihabitans* PGP01 growth of 9.42, 9.54 and 9.56 log CFU mL^{-1}, respectively (Figure 2B), being those values similar to the observed for the commercial medium TSB (9.62 log CFU mL^{-1}). The application of the yeast extract at 20 g L^{-1} or pea protein at both concentrations only allowed to obtain better growth values when the FPP (9.31, 9.27 and 9.25 log CFU mL^{-1}, respectively, against 8.98 log CFU mL^{-1}) was used alone for the elaboration of the medium (Figure 2B). Again, urea provided the lowest values of viability since *P. oryzihabitans* PGP01 growth also failed to be detected under these conditions (Figure 2B).

In the light of these results, and considering the lower price of the tryptone compared to the peptone, it was decided to select the first one as the best compound to provide the nitrogen supply on the basis medium in combination with the potato waste FPP. In the next step, the definition of the composition of this basis medium (FPP+TRP) for *P. oryzihabitans* PGP01 growth was carried out in the scaling-up of the production to 2 L bioreactors.

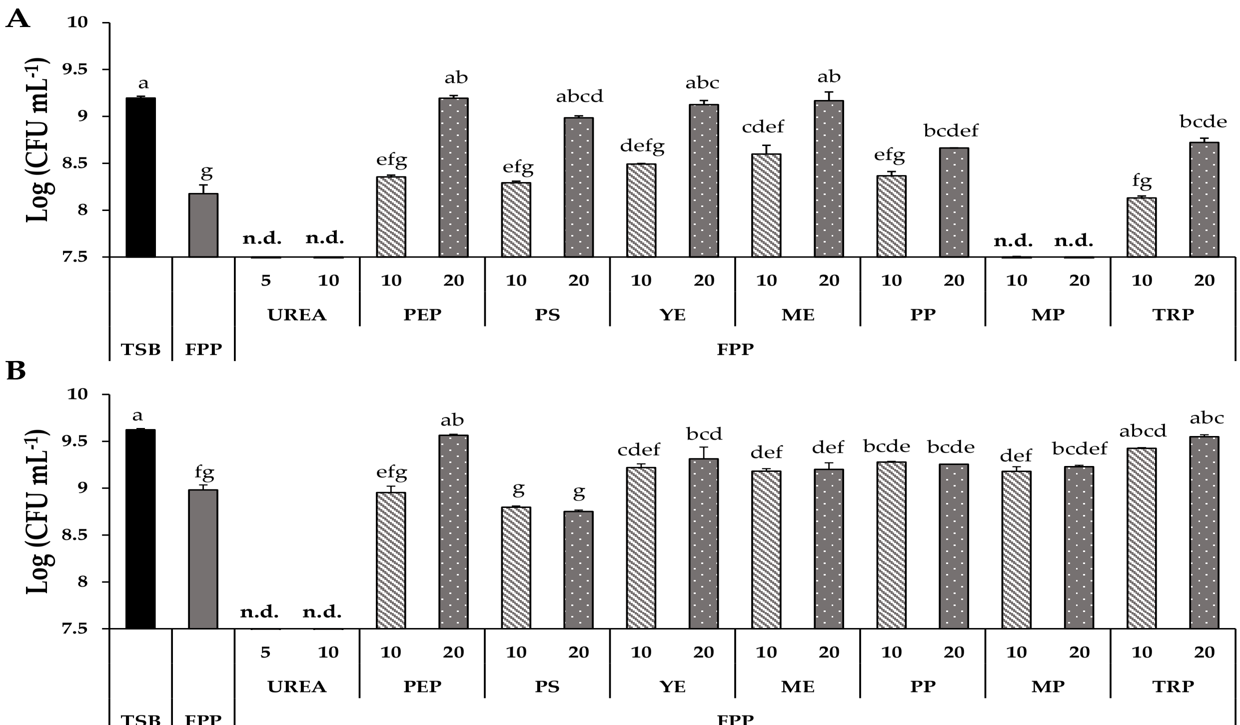


Figure 2. - Growth in flasks of *P. oryzihabitans* PGP01 after 24 (A) and 48 h (B) using potato wastes (FPP) supplemented with several nitrogen sources at low ( ) and high ( ) concentration. *P. oryzihabitans* PGP01 cells were cultured at 25 ºC and 150 rpm. PEP = Peptone, PS = PROSTAR 510A, YE = Yeast extract, ME = Meat extract, PP = Pea protein, MP = Maize protein, TRP = Tryptone. Data represent mean ± SE of three independent replicates, and different letters indicate significant differences between TSB and different media according to Tukey HSD test ($P < 0.05$).

3.2. Production of *P. oryzihabitans* PGP01 in 2 L bioreactors

3.2.1. Optimization of the composition of the culture medium

As a first step, the proportion of the chosen nitrogen source (TRP) needed to be optimised. For that reason, a preliminary experiment in bioreactors with *P. oryzihabitans* PGP01 growing in the presence of the FPP-based medium supplemented with TRP at 10 and 20 g L^{-1} was conducted, and the results are shown in Figure 3. As it was observed in flask experiments, in bioreactors we also did not observe differences in *P. oryzihabitans* PGP01 growth when the potato waste FPP was combined with TRP at 10 or 20 g L^{-1} at the different sampling times (Figure 3). Consequently, TRP 10 g L^{-1} was the definitive concentration used in the potato by-products-based medium (FPP). However, it was observed that *P. oryzihabitans* PGP01 growth on the commercial medium TSB still provided the best production values (Figure 3), and further medium composition still required some improvement.

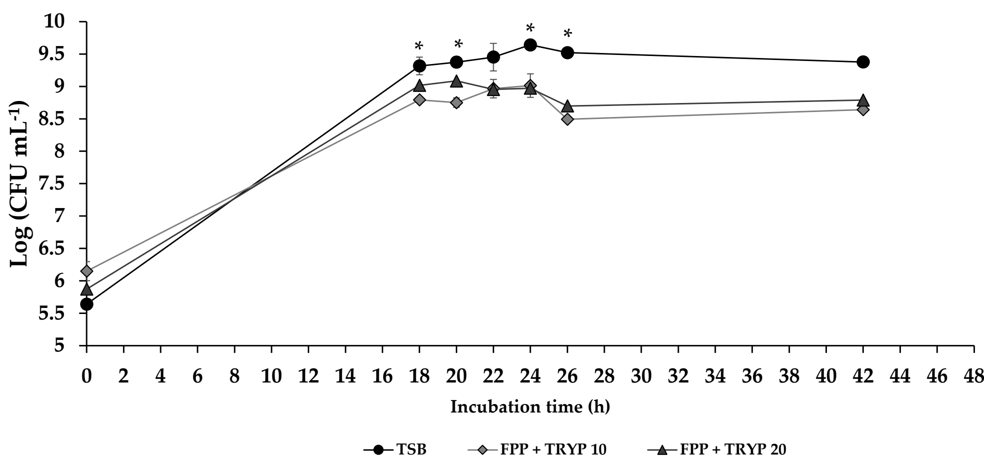


Figure 3. *P. oryzihabitans* PGP01 growth using potato wastes (FPP) supplemented with 10 g L^{-1} (◊) or 20 g L $^{-1}$ of TRP (▲) in a 2 L bioreactor at 25 ºC and 400 rpm. Data represent means ± SE of three independent replicates, and different letters denote significant differences between commercial and by-products optimized media according to Tukey HSD test ($P < 0.05$).

For that reason, in the following steps of optimization, the effect of NaCl as well as glucose and sugar cane molasses as mineral and carbon sources were studied in order to improve *P. oryzihabitans* PGP01 growth in the FPP-based medium FPP+TRP10. *P. oryzihabitans* PGP01 growing in TSB medium peaked its maximum (9.64 log CFU mL^{-1}) after 24 h of culture in 2 L bioreactors (Figure 4). It is important

to highlight that the addition of NaCl at 5 g L^{-1} together with molasses at 10 g L^{-1} allowed to reach a 9.68 log CFU mL^{-1} of *P. oryzihabitans* PGP01 production at the same point, very similar to that obtained when growing the bacterium in TSB medium (Figure 4). When the FPP+TRP10 medium was supplemented only with NaCl 5 g L^{-1}, a production of 9.20 log CFU mL^{-1} was obtained after 24 h of incubation, being this value significantly lower than the observed in the synthetic medium TSB (Figure 4).

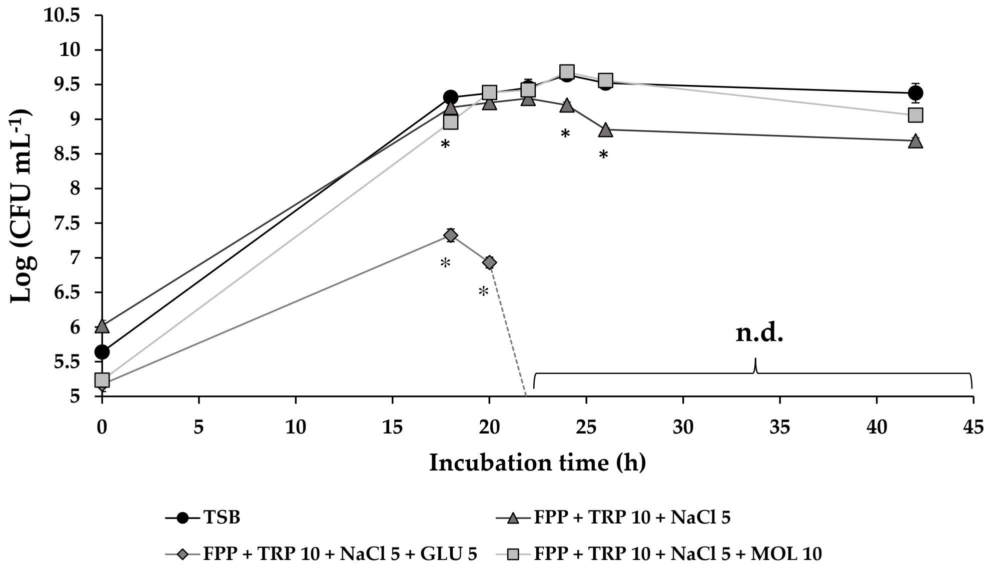


Figure 4. *P. oryzihabitans* PGP01 growth using potato by-products (FPP) supplemented with TRP10 and 5 g L^{-1} of NaCl, 5 g L^{-1} of GLU and 10 g L^{-1} of MOL. Data represents the mean ± SE of three independent replicates. Asterisks (*) symbols within each sampling period denote significant differences between TSB and optimized media according to Tukey HSD test ($P < 0.05$).

On the other hand, the combined addition of 5 g L^{-1} glucose and 5 g L^{-1} NaCl provided the poorest growth since a maximum growth of 7.20 log CFU mL^{-1} was achieved after 18 h of culture. After that, bacterial growth decreased, and it was not detected from 22 h to the end of the production process (Figure 4). Constant pH monitoring revealed a decrease of this factor after the combined addition of 5 g L^{-1} of glucose and NaCl, registering values of 4.5 from 20 h of culture that remained invariable until the end of the fermentation process (data not shown). Thus, the composition of the FPP-based medium which provided similar growth than the observed in the laboratory medium TSB was 300 g L^{-1} FPP, 10 g L^{-1} TRP, 10 g L^{-1} MOL 5 g L^{-1} NaCl and 2.5 g L^{-1} K_2HPO_4.

3.2.2. Effect of agitation and oxygen flux

Once the final composition of a competitive medium based on potato wastes for *P. oryzihabitans* PGP01 growth was established, the agitation and aeration conditions were optimized to obtain the highest bacterial production. In these experiments, the agitation speed had a significant impact on *P. oryzihabitans* PGP01 production at the different sampling times ($P < 0.0001$), while the air flux only showed a significant effect at 20, 22 and 24 h of growth (Table 2). The interaction between the two factors only was significant at 22 and 24 h, coinciding with the maximum growth period (Table 2). Comparing the aeration depending on the agitation speed, it was shown that the lowest values of *P. oryzihabitans* PGP01 production were obtained at 0.25 vvm for both 200 and 400 rpm of agitation at 20 and 22 h of growth (Table 2). In contrast, 0.5 and 0.75 vvm of air flux produced similar *P. oryzihabitans* PGP01 growth values at the same sampling times (Table 2). After 24 h, no differences among the three air fluxes were found on bacterial production at 200 rpm. However, at 400 rpm of agitation, both of the analysed air fluxes (0.5 and 0.75) still produced significantly higher values of *P. oryzihabitans* PGP01 than 0.25 vvm (Table 2). At 600 rpm of agitation, the three air fluxes did not produce significant differences in *P. oryzihabitans* PGP01 production (Table 2). Comparing the agitation depending on the air flux, we found that an agitation of 200 rpm led to the poorest bacterial growth at the three air fluxes and at all times of incubation (Table 2). Overall, agitations of 400 or 600 rpm produced the highest *P. oryzihabitans* PGP01 growth values under 0.25, 0.5 and 0.75 vvm, being these values similar at all of the times of incubation except for 20 and 22 h of incubation (Table 2). Therefore, due to economic reasons, the lower agitation speed (400 rpm) was selected for the production protocol. In addition, this decision was reinforced by the fact that at the most active growth period (22 h), the highest production of *P. oryzihabitans* PGP01 was obtained at 400 rpm and 0.75 vvm of air flux. Regarding air feeding, the highest 0.75 vvm was chosen to provide an adequate oxygen supply since this factor does not show an important economic impact on the production process.

3.3. Growth curve under optimal conditions

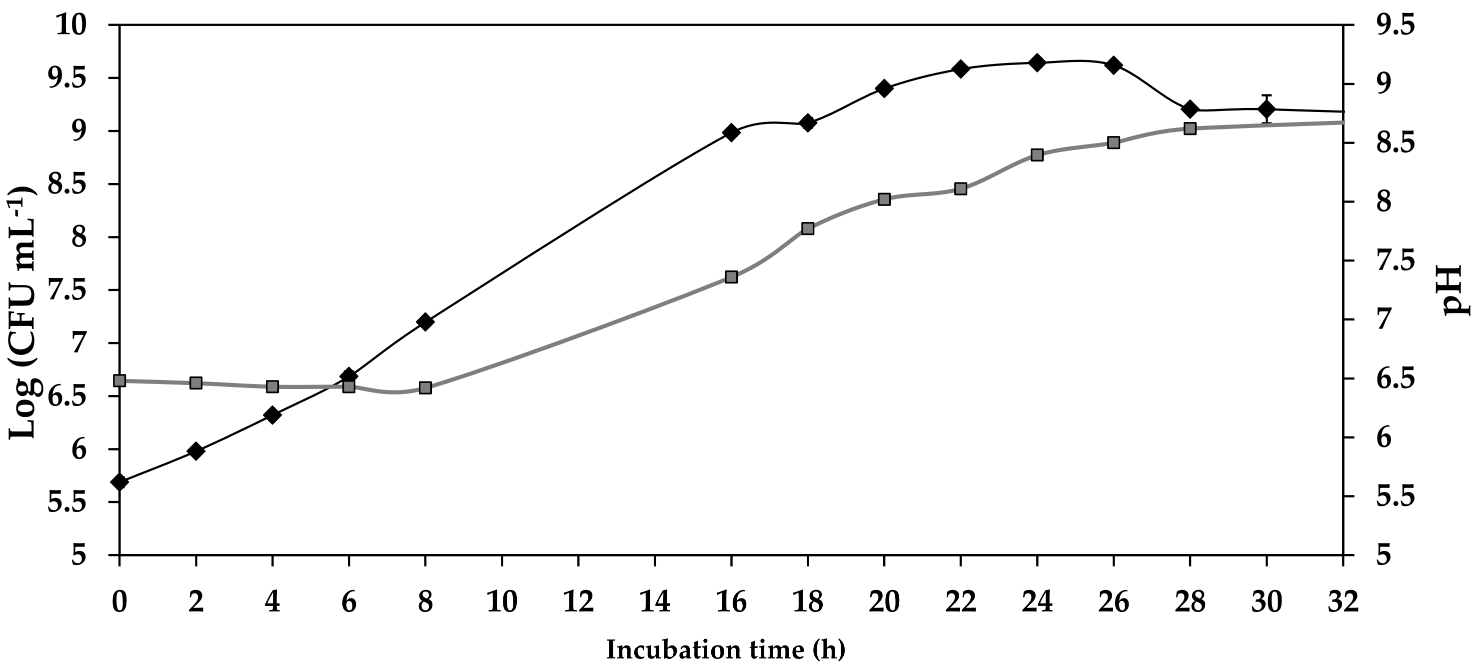
At the initial stages of incubation in the optimized medium, *P. oryzihabitans* PGP01 rapidly reached a 7.19 log CFU mL^{-1} after 8 h of growth (Figure 5). During this period, medium pH remained stable between 6.42-6.48. Then, a 12-h exponential growth phase of *P. oryzihabitans* PGP01 occurred coinciding with a rise on the medium pH until 8.02 after 20 h of growth in the optimized medium (Figure 5). Maximum population of *P. oryzihabitans* PGP01 was reached at 22 h, and it remained stable until

26 h of culture. During this period, a maximum population of 9.64 log CFU mL^{-1}, corresponding to $4.4x10^9$ CFU mL^{-1} of *P. oryzihabitans* PGP01 was registered at 24 h (Figure 5). After 26 h, *P. oryzihabitans* PGP01 population decreased while the medium pH continued increasing during the whole fermentation process reaching a maximum of 8.85 after 48 h of culture (data not shown).

Table 2. Effect of different stirring speeds (200, 400 and 600 rpm) and different air feedings (0.25, 0.5 and 0.75 vvm) on *P. oryzihabitans* PGP01 cells growth in 2 L bioreactors using an agro-food by-product medium based on 300 g L^{-1} of FPP supplemented with 10 g L^{-1} TRP, 10 g L^{-1} MOL, 5 g L^{-1} NaCl and 2.5 g L^{-1} K_2HPO_4. Data represent means ± SE of at least three independent replicates. For each air flux, lowercase letters indicate significant differences among agitation speeds according to Tukey HSD test ($P < 0.05$). For each agitation speed, air fluxes with different uppercase letters are significantly different according to Tukey HSD Test ($P < 0.05$).

	18 h	20 h	22 h	24 h	26 h	28 h
200 rpm						
Air flux (vvm)						
0.25		8.82 ± 0.001 b C	8.89 ± 0.033 b B	8.70 ± 0.067 a B	8.71 ± 0.084 a B	8.51 ± 0.145 a
0.5		8.88 ± 0.056 ab B	8.96 ± 0.029 ab B	8.83 ± 0.035 a B	8.63 ± 0.071 a B	8.63 ± 0.096 a
0.75		9.02 ± 0.025 a B	9.06 ± 0.007 a C	8.94 ± 0.014 a B	8.93 ± 0.066 a B	8.78 ± 0.053 a
400 rpm						
0.25		9.20 ± 0.022 b B	9.35 ± 0.023 b A	9.36 ± 0.013 b A	9.33 ± 0.105 a A	9.26 ± 0.019 a
0.5		9.33 ± 0.052 ab A	9.49 ± 0.080 a A	9.57 ± 0.051 a A	9.40 ± 0.087 a A	9.10 ± 0.088 a
0.75		9.40 ± 0.035 a A	9.59 ± 0.017 ab A	9.52 ± 0.04 ab A	9.32 ± 0.100 a A	9.05 ± 0.070 a
600 rpm						
0.25	9.20 ± 0.012 a	9.36 ± 0.047 a A	9.50 ± 0.055 a A	9.50 ± 0.040 a A	9.46 ± 0.004 a A	
0.5	9.24 ± 0.033 a	9.39 ± 0.023 a A	9.41 ± 0.011 a A	9.52 ± 0.023 a A	9.41 ± 0.060 a A	
0.75	9.23 ± 0.039 a	9.37 ± 0.013 a A	9.44 ± 0.036 a B	9.44 ± 0.014 a A	9.23 ± 0.033 a AB	
***P* - values**						
Agitation (A)		< 0.0001 ***	< 0.0001 ***	< 0.0001 ***	< 0.0001 ***	< 0.0001 ***
Air flux (B)	0.697	0.0006 ***	0.0102 *	0.001***	0.966	0.854
(A) x (B)		0.077	0.007**	0.012**	0.053	0.051

Figure 5. Bacterial growth curve (log CFU mL^{-1}) (■) and pH (◆) evolution of growth curve in a 2 L bioreactor of the bacterium *P. oryzihabitans* PGP01 in the potato wastes optimized medium (300 g L^{-1} FPP, 10 g L^{-1} TRP, 10 g L^{-1} MOL 5 g L^{-1} NaCl and 2.5 g L^{-1} K_2HPO_4) at 25 ºC, 400 rpm and 0.75 vvm of oxygen flux. Each value of the log (CFU mL^{-1}) of the curve represents the mean ± SE of at least three replicates.



3.4. Maintenance of the root growth-promoting activity of *P. oryzihabitans* PGP01 growing on the optimized medium.

The final step of this biomass production protocol was to validate that the *P. oryzihabitans* PGP01 growing in the FPP optimized medium (300 g L^{-1} FPP, 10 g L^{-1} TRP, 10 g L^{-1} MOL 5 g L^{-1} NaCl and 2.5 g L^{-1} K_2HPO_4) maintained its biological activity. In this sense, a significant 64% increase in the number of roots was observed in RP-20 *in vitro* plantlets compared to those non-inoculated plantlets (Figure 6A and B).

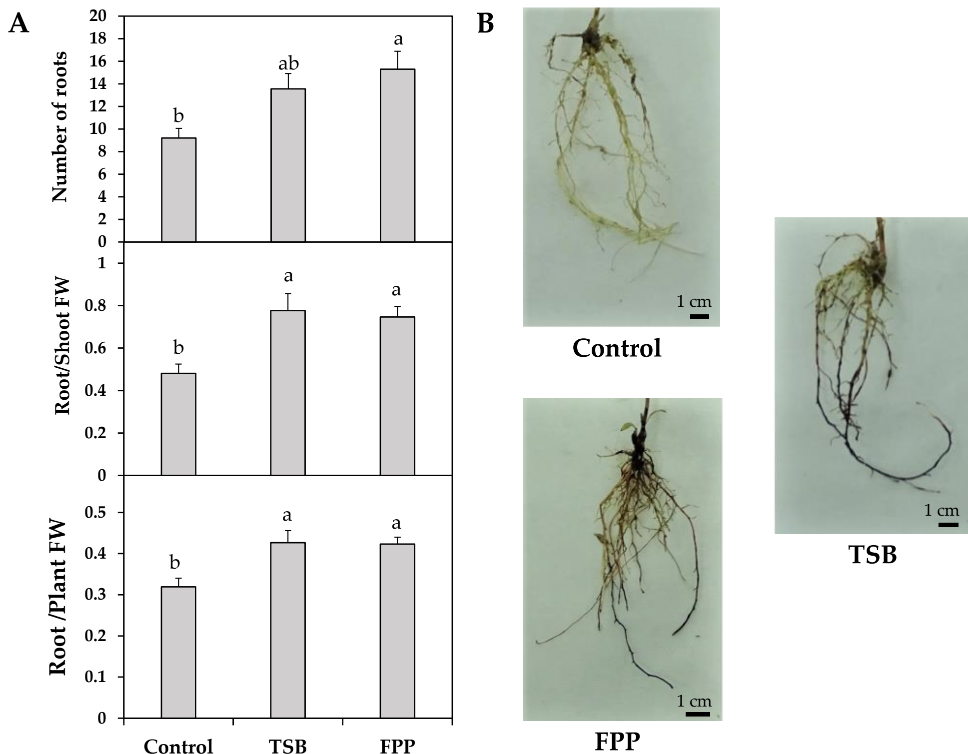


Figure 6. Effect of the bacterium *P. oryzihabitans* PGP01 produced in an optimized potato wastes-based-medium (300 g L^{-1} FPP + 10 g L^{-1} TRP + 10 g L^{-1} MOL + 5 g L^{-1} NaCl + 2.5 g L^{-1} K_2HPO_4) in number of roots, root/shoot FW and root/plant FW ratio (A) as well as in root morphology (B) of *in vitro* rooted Rootpac® 20 plantlets. Data represents the mean ± SE of at least ten independent replicates. Different letters indicate significant differences among control, commercial and optimized medium according to Tukey HSD test ($P < 0.05$).

However, although plants inoculated with *P. oryzihabitans* PGP01 grown in synthetic medium TSB also showed higher number of roots than non-inoculated plants, these differences were not statistically significant. The root-located effect of

the bacterium *P. oryzihabitans* PGP01 growing in the optimized FPP-based medium in RP-20 *in vitro* plants was corroborated since both root/shoot as well as root/plant FW ratio were increased in a 54 and 35% in relation to non-treated plantlets (Figure 6A). A similar trend was recorded in response to the inoculation with *P. oryzihabitans* PGP01 growing on the commercial medium TSB, showing significant 60 and 35% increase in these two parameters (Figure 6A). Moreover, the inoculation of RP-20 *in vitro* plants with *P. oryzihabitans* PGP01 also showed differences in the roots appearance, observing a more robust root system than those non-inoculated plants (Figure 6B).

4. Discussion

Due to the beneficial effects on plant growth and development induced by PGPMs, the development of products based on these microorganisms constitutes a necessary strategy for their large-scale application. In this sense, the optimization of an economic medium that allows to obtain an equal microbe population at a lower price without affecting its biological activity must be firstly considered. In the present study, a protocol for the economic production of the PGPM *P. oryzihabitans* PGP01 was set up using a frozen potato peels and pulp-based medium, providing more evidence about the exploitation of agro-food industries wastes as nutrient sources for microorganism's production. Similarly, by-products generated from dairy sludge or the processing of sweet potato have been also successfully used to produce other bacterial PGPMs including *Rhizobium* spp. (Singh et al., 2013), *Bacillus thuringiensis* (Chang et al., 2008) and *Paenibacillus polymyxa* (Xu et al., 2014) in a more sustainable way. For all those reasons, this research represent an interesting approach to give a second use to wastes obtained from potato processing, which is one of the most widely consumed food products in the European market.

At the very early stages of optimization, the effect of the incubation temperature on *P. oryzihabitans* PGP01 growth was studied. Growth temperature should be one of the first factors to optimize in a biomass production protocol as it is determinant for microbial functionality and development (Andreoglou et al., 2003; Gugi et al., 1991; Kim et al., 2020). In our study, the maximum bacterial population was obtained at both 24 and 48 h when *P. oryzihabitans* PGP01 was grown at 25 ºC, being this the chosen temperature for the biomass production of *P. oryzihabitans* PGP01. The nature of the food industry wastes-based medium was the following step in the developing of the low cost medium. For these experiments, culture medium based on frozen potato, tomato and cereals wastes from AGRIMAX project, were selected as a potential alternative to the use of commercial products due to their wide

diversity of nutrients such as carbohydrates, phenolic compounds, proteins or vitamins that might be used by microorganisms (Balasundram et al., 2006; Ercolano et al., 2015; Fritsch et al., 2017; Wijngaard et al., 2012). According to the obtained results, FPP seemed to be the most promising by-product as an almost 9 log CFU mL^{-1} after 48 h of culture was achieved. Nonetheless, similar production levels than the obtained in the TSB medium were not so far reached. Although the use of TS and WB may have contributed to a cheaper *P. oryzihabitans* PGP01 production protocol due to their lower amount required for the preparation of the media (100 and 20 g L^{-1}) and ease handling in comparison to FPP (WB is a ready-to-use waste), the lower bacterial growth obtained led to the discard these by-products for following experiments. Moreover, the characterization of the three wastes revealed that TS and WB had higher contents of carbon and nitrogen than FPP (AGRIMAX project, unpublished data). However, the carbon and nitrogen forms existing in TS and WB might not be accessible for *P. oryzihabitans* PGP01 metabolism, leading to a poorer bacterial production. It is known that, in some cases, the effective use of wastes by microorganisms depends on the ability to degrade their components (Fritsch et al., 2017). For instance, the yeast *S. cerevisiae* is not able to degrade starch, and complementary strategies such as the addition of starch-degrading enzymes must be followed to favour its utilization (Ostergaard et al., 2000). Accordingly to this study, other authors have reported the effective implementation of potato peels wastes for the reduction of the costs of secondary metabolites and single-cell protein processes by microorganisms (Bacha et al., 2011; Kleekayai & Suntornsuk, 2011). Considering all those reasons, a wastes medium based on FPP was optimized in further experiments for *P. oryzihabitans* PGP01 production.

Another important aspect that must be considered, especially when growing bacteria, is the nitrogen source present in the culture medium (Costa et al., 2002). In the present study, several commercial products were tested at a lower and a higher dose. In this case, the addition of peptone at 20 g L^{-1} or tryptone at both 10 and 20 g L^{-1} to FPP resulted in *P. oryzihabitans* PGP01 populations of 9.56, 9.42 and 9.55 log CFU mL^{-1} after 48 h of growth, respectively. These values were similar to those reached when *P. oryzihabitans* PGP01 was grown in the TSB medium. Peptides such as tryptone and peptone are efficiently used as a substrate in culture media due to their high content in amino acids (Hensley et al., 2016; Taskin et al., 2016). In our research group, both nitrogen sources have been previously tested showing different results depending on the bacterial species. While these two commercial products were not the most effective nitrogen sources for *P. agglomerans* CPA-2 production, peptone resulted in one of the most suitable compounds for the *B. subtilis* CPA-8 mass

production protocol (Costa et al., 2002; Yánez-Mendizábal et al., 2012). In the view of the obtained results, we were able to reduce the concentration of tryptone without seriously affecting *P. oryzihabitans* PGP01 growth. In addition, this tryptone concentration was also lower than the required for the TSB medium composition (17 g L^{-1}). For this reason, tryptone was selected as the best nitrogen source to be added to the FPP-based medium and to produce *P. oryzihabitans* PGP01 in a low cost medium.

In the scale-up to 2 L bioreactors, glucose at 5 g L^{-1} and molasses at 10 g L^{-1} were selected to study the effect of the carbon source on *P. oryzihabitans* PGP01 production, and NaCl at 5 g L^{-1} was added to the optimised medium to maintain osmotic balance. In this regard, Abdel-Mawgoud et al. (2008) and Haddar et al. (2010) reported that the mineral supplementation of the culture medium with trace elements provided a stimulatory effect in cell growth of some *Bacillus* species improving the production process effectiveness. In our study, the addition of 5 g L^{-1} NaCl to the potato waste-based medium (FPP+TRP10) allowed to reach similar values to those obtained in TSB medium after 20 and 22 h of culture (9.23 and 9.42 log CFU mL^{-1}, respectively). However, after 24 h of growth, *P. oryzihabitans* PGP01 population significantly decreased. On the contrary, when the medium was supplemented with both 5 g L^{-1} NaCl and 10 g L^{-1} molasses, it was able to reach a 9.68 log CFU mL^{-1} of *P. oryzihabitans* PGP01 population, being these values similar to the obtained in the synthetic medium after 24 h of growth. Molasses represent a low cost waste from sugar beet, and its high relevance in microorganisms production relies on its high content of mono- and disaccharide such as glucose, fructose and sucrose (approximately 50% w/w) (Costa et al., 2001; Khatun et al., 2020). In previous studies conducted by Yánez-Mendizábal et al. (2012), the combination of molasses with other by-products resulted in the best option to produce the *B. subtilis* CPA-8 strain. A similar response was observed when molasses were used for the mass production of *Bacillus siamensis* using an anaerobic digestate (Pastor-Bueis et al., 2017). The addition of 5 g L^{-1} glucose and 5 g L^{-1} of NaCl to the FPP+TRP10 medium did not produce a positive effect in *P. oryzihabitans* PGP01 production as considerably lower population values of this microbe were recorded after 18 and 20 h of culture, and undetectable values were registered from 22 h to 42 h. The concentration of this substrate added to the optimized medium was the same than the used in the elaboration of the TSB medium (5 g L^{-1}). It is widely known that glucose represents the main carbon sources used for many bacteria genus to ensure cell growth (Bren et al., 2016). Nevertheless, the presence of glucose in the FPP+TRP10 medium produced a decrease on the pH of the culture medium, reaching values of 4.5 after 20 h of incubation. In this sense, it is

widely known that acidic values seriously affect bacterial growth (Mossel et al., 1995). Therefore, the better results obtained in the *P. oryzihabitans* PGP01 production terms using molasses instead might suggest that other compounds existing in this by-product likely favoured bacterial cell growth. In fact, although the 50% of the composition of molasses are sugars, other compounds potentially used for microorganism's growth such as nitrogen compounds or amino acids are also present (Kotzamanidis et al., 2002). Considering the aforementioned, our findings provide a great value to the study, developing an economic protocol for the mass production of *P. oryzihabitans* PGP01 by the re-utilization of wastes from the two highly consumed products potato and sugar beet.

Once the composition of the medium was established, (300 g L^{-1} FPP + 10 g L^{-1} TRP + 5 g L^{-1} NaCl + 10 g L^{-1} MOL+ 2.5 g L^{-1} K_2HPO_4), we focussed on the optimization of the growing conditions in the bioreactors such as the agitation and air flux. Both parameters have been previously shown as critical factors for the successful scaling-up of the production process to bioreactors (Iyer & Singhal, 2010; Mukhtar et al., 2014). In our study, three different agitation speeds (200, 400 and 600 rpm), and three air feeding fluxes (0.25, 0.5 and 0.75 vvm) were analysed in order to maximize the *P. oryzihabitans* PGP01 production in bioreactor. Both parameters and its interaction had a significant impact on *P. oryzihabitans* PGP01 production during the most active period of growth (22 and 24 h). At those points, it was observed that higher bacterial production values were reached when the air flux of the process were set at both 0.5 and 0.75 vvm under 200 and 400 rpm of agitation. In contrast, the higher speed agitation of 600 rpm might provide an adequate oxygen supply, and an effect of aeration was not observed. Regarding agitation speeds, while low agitations (200 rpm) led to a maximum of 9 log CFU mL^{-1} at 22 h under the highest air flux (0.75 vvm) around a 9.5 log CFU mL^{-1} of optimum *P. oryzihabitans* PGP01 growth was reached when the agitation was set up at either 400 or 600 rpm. However, after 22 h of incubation in 2 L bioreactors, the maximum *P. oryzihabitans* PGP01 production was reached at 400 rpm under the highest air flux (0.75 vvm). In this context, a high oxygen supply in bioreactors increase bacterial production since *P. oryzihabitans* is an strictly aerobic bacterium (Gutiérrez-Bustos et al., 2009). Our results differ significantly from those obtained in the production of *A. brasilense* in laboratory-scale pneumatic bioreactors (Carrasco-Espinosa et al., 2015). These authors demonstrated that the highest bacterial biomass was obtained at aerations of 0.1 vvm. Considering the results, 400 rpm and 0.75 vvm were the chosen agitation and air flux conditions for the production of *P. oryzihabitans* PGP01 in 2 L bioreactors. Therefore, in order to reduce the costs of the *P. oryzihabitans* PGP01 process, the selected agitation speed

was 400 rpm. Regarding air flux conditions, no differences between 0.5 and 0.75 vvm were found in terms of *P. oryzihabitans* PGP01. In our study, considering that air feeding is not an expensive factor, the highest air feeding flux 0.75 vvm was fixed for the production protocol of *P. oryzihabitans* PGP01 production.

Observing the growth curve, when the bacterium *P. oryzihabitans* PGP01 was grown in the optimised medium, an exponential growth of this bacterium was recorded from the beginning during the first stage of growth (from 0 to 8 h), increasing the bacterial population from 5.68 to 7.19 log CFU mL^{-1}. This response is quite different to that obtained for the *B. subtilis* CPA-8 and *P. agglomerans* CPA-2 production protocols where a lag phase occurred during the first 4-6 h of bacterial growth (Costa et al., 2002; Yánez-Mendizábal et al., 2012). Those results might suggest that an adaptation to the waste based medium by the bacterium *P. oryzihabitans* PGP01 is not required, obtaining high production values in a shorter time. In this study, the maximum cell growth was obtained at 22 h of culture and a pH of 8.4, reaching a *P. oryzihabitans* PGP01 population of 9.64 log CFU mL^{-1}. In this sense, our study could provide valuable knowledge about the optimum pH for *P. oryzihabitans* PGP01 growth as very little information in this regard is available in literature. Moreover, bacterial population decreased after 26 h of culture in the optimised medium likely due to the depletion of the nutrients in the culture medium or the accumulation of undesirable products that may inhibit bacterial growth.

Although obtaining a high cell biomass using inexpensive compounds constitutes a significant requirement for the development of a microbe-based product, the maintenance of the biological activity of the microorganisms must be ensured for commercial purposes (Costa et al., 2001; Gotor-Vila et al., 2017; Yánez-Mendizábal et al., 2012). For that reason, micropropagated explants of the rootstock Rootpac 20 (RP-20) belonging to *Prunus* genus were inoculated with *P. oryzihabitans* PGP01 grown in optimized medium to corroborate the same effects in roots observed in previous studies with this bacterium grown in laboratory medium (Cantabella et al., 2020). In this case, our study demonstrated the effectiveness of the optimized medium based on by-products on the maintenance of the root development enhancing activity of *P. oryzihabitans* PGP01, observing an increase of the number of roots over non-inoculated plantlets in a greater extent than the laboratory medium. Similarly to previous studies (Cantabella et al., 2021), the root-located activity of this microorganism grown in the optimized medium was corroborated by the 54 and 35% increases on the root/shoot and root/plant FW ratio observed in RP-20 *in vitro* plantlets. These findings might constitute the first step on the development of a more

sustainable biofertilizer with important applications in agriculture. Following a similar strategy, *Paenibacillus polymyxa* produced by the use of wastewater from sweet potato starch production produced an increase of tea yield production as well as polyphenol contents under field conditions (Xu et al., 2014). However, to achieve that goal, it will be necessary to optimize the pilot-scale production and formulation of *P. oryzihabitans* PGP01.

5. Conclusions

In light of the obtained results, we can conclude that an inexpensive medium to maximize the production of the plant growth promoting rhizobacterium *P. oryzihabitans* PGP01 was developed using low cost industry waste's-based medium and commercial products. This medium was proved to be effective to ensure the biological activity of this bacterium, increasing several parameters involving root development. Using a food industry waste as a main component of the medium to grow *P. oryzihabitans* PGP01, the production process was carried out in a more sustainable manner. Although pilot-scale production experiments will be required to finally develop a product based on this bacterium, this study has provided evidence about the revalorisation of food by-products, reducing both economic and environmental impact associated to the generation of wastes in industry.

6. Declaration of Competing Interest

Authors declare no conflict of interest.

7. Acknowledgements

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General discussion

Since the beginning of the 21st century, the number of publications in the field of plant-microbe interactions has significantly increased, going from 244 publications in the 2000 to almost 3000 in the last year (Figure 1). This fact has made this area a much-studied field. Overall, a total of 22787 publications are available in literature concerning the use of microorganisms to stimulate plant growth (FECYT, 2021). However, the total number of studies employing *in vitro* culture techniques does not exceed 322, representing approximately the 1.4% of the total of the studies published in this field.

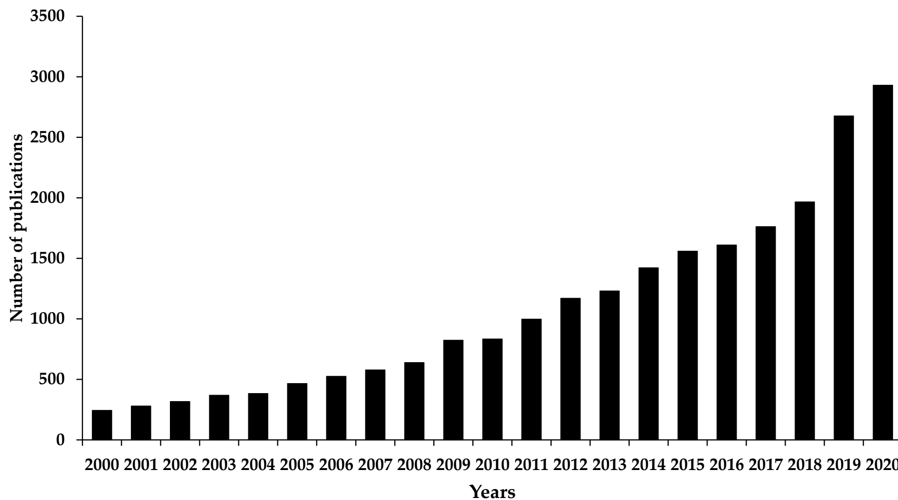


Figure 1. Number of publications in the field of plant growth promoting microorganisms in the 21st century. **Source:** FECYT, 2021.

A few years ago, the introduction of microorganisms into a completely aseptic environment was thought to be unlikely, considering their presence in *in vitro* cultured plants as detrimental (Leifert & Cassells, 2001). However, there has been a change in the mentality of the scientific community due to the fact that *in vitro* cultures are not naturally free of microorganisms. Nowadays, the use of *in vitro* plant tissue cultures tools to study the interaction of plants with beneficial microorganisms is a little more implemented, although an extensive work still needs to be done in this regard.

Plant *in vitro* culture, among its many advantages compared with other plant culture systems, offers the option of developing very versatile approaches that may be used for modelling plant responses to beneficial microorganisms, since large amounts of true-to-type and disease-free plant material can be stored in a relatively small space (Hussain et al., 2012).

In the present thesis, it was studied the effect of three microorganisms whose ability to promote *in vitro* plant growth was supposed from preliminary observations. These microorganisms, were initially found by serendipity, observing that fruit tree *in vitro* cultures belonging to *Prunus* and *Pyrus* genus displayed a better growth than non-contaminated cultures. Furthermore, an attempt was also made to elucidate the mechanisms used by these microorganisms to promote plant growth. The information available in literature regarding mechanisms of plant growth promotion caused by microorganisms is vast, although in the case of these microorganisms, only few studies have been conducted.

Considering the abovementioned, all the results obtained in the present thesis project have been grouped in **six independent chapters** which, taken together, form a very interrelated story that can be based on five well-differentiated pillars: **The isolation of the three microorganisms, the description of the effects of the three microorganisms on *in vitro* plant growth and development**, **the mechanisms underlying this plant growth promotion, the factors modelling this plant-microbe interaction and the production of the microorganism of major interest.**

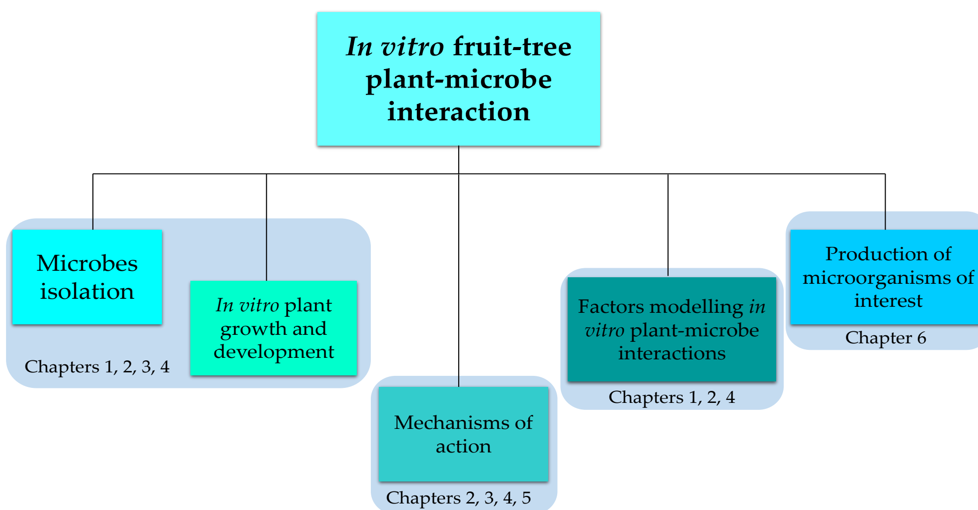


Figure 2. Fundamental aspects covered in this doctoral thesis on the interaction of *in vitro* fruit tree plants and *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03.

1. Isolation of microorganisms and description of their effects in *in vitro* fruit tree plants growth and development

In the presence of contaminants that enhanced the growth of *in vitro* plants, the microorganisms underlying these contaminations were first isolated. In addition, their potential effects in *in vitro* plant growth and development were also analysed (Figure 3).

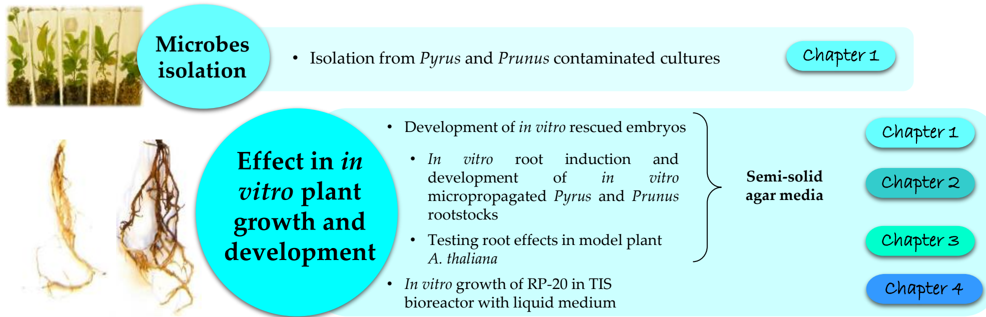
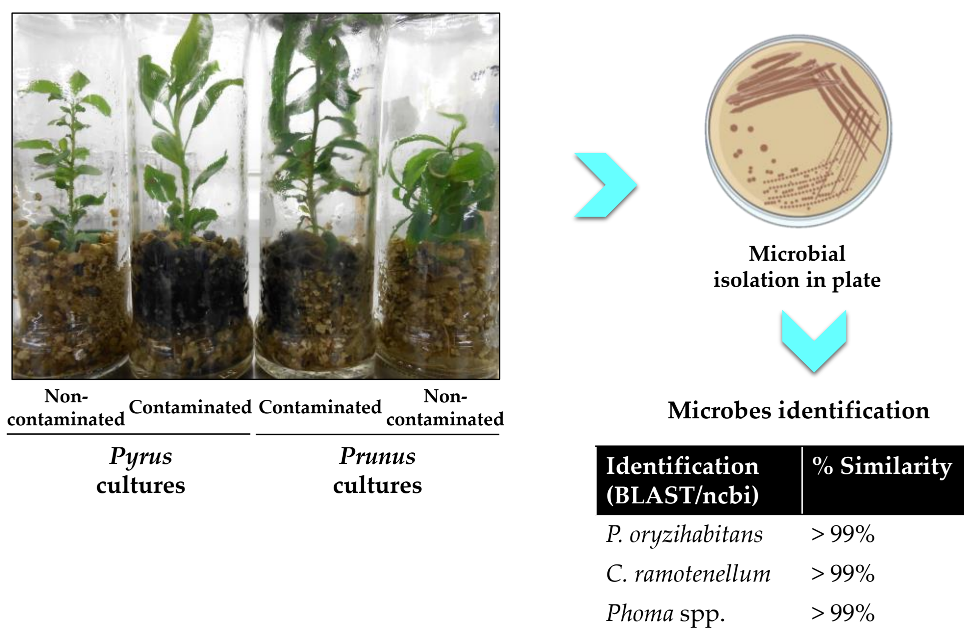


Figure 3. Microbial isolation and study of their effects in *in vitro* plant growth and development.

The first step to take into consideration when selecting promising microorganisms to promote plant growth is the correct isolation and identification of microbial strains (Di Benedetto et al., 2019). As previously said, the microorganisms used for this research were found spontaneously in *in vitro* fruit tree cultures which visually showed better growth than those without contamination (Figure 4). Originally, two bacterial and two fungal apparently different isolates were detected. After microbial classification, bacterial isolates belong to the same bacterial species, being identified as *P. oryzihabitans*. On the other hand, fungal isolates were identified one as *C. ramotenellum* and the other a fungal species belonging to *Phoma* genus. In the latter case, the fungal species could not be detected as *Phoma* genus represents a complicate genus for taxonomists due to unclear boundaries among species (Aveskamp et al., 2010). Once microbial isolates were identified, they were included in the IRTA's PGPMs collection, and named as *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03.

Hereafter, the next step of selection involved the use of the selected microbial strains in plants to determine their biological activity (Barnett et al., 2017). In this sense, in preliminary inoculations using *in vitro* fully developed pear plantlets obtained by embryo rescue, the application of concentrated suspensions of microorganisms already provided promising evidences about the possible role of this

microbial strains in fruit tree plants. From these results, could be extracted that *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 were able to improve parameters determining plant growth such as plant FW and stem and root length. In order to provide a broad spectrum of action of these microorganisms, different types of plant material were used: ***in vitro* rescued embryos (Chapter 1) and *in vitro* micropropagated explants (Chapter 2), *A. thaliana* Col-0 seedlings germinated in MS medium (Chapter 3), and RP-20 explants growing in the GreenTray® bioreactor (Chapter 4).**



Identification (BLAST/ncbi)	% Similarity
P. oryzihabitans	> 99%
C. ramotenellum	> 99%
Phoma spp.	> 99%

Figure 4. Isolation and identification process of *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03. Plate icon extracted from BioRender.

In vitro embryo rescue is a widely established technique for many horticultural crops to obtain early-ripening varieties with a high commercial value (Pérez-Hernández & Grajal-Martín, 2011; Uma et al., 2011). This methodology also tends to reduce the high losses of plant material associated with poor germination due to an abnormal seed development, causing seeds abortion (Devi et al., 2017). The study herein presented constitutes the first report concerning the use of microorganisms that enhance plant development in the process of *in vitro* rescue of peach embryos. Specially, the embryos used to carry out the study of the Chapter 1 proceeded from fruits of three independent nectarine crosses. The application of microorganisms to nectarine embryos, especially the bacterium *P. oryzihabitans*

PGP01, provided promising results as *in vitro* morphological changes that favoured the adaptation to *ex vitro* conditions occurred in the presence of this bacterium. This research might suppose an important breakthrough for fruit tree breeding programmes increasing tolerance of plants to greenhouse transplant for latter agronomical field evaluation. In this experiment, the effect of the application of the three microorganisms was evaluated at three important subsequent steps: **embryos germination, seedlings growth and acclimatization to greenhouse conditions**. For that reason, embryos inoculation with *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 took place at the very early stages of *in vitro* culture, prior to its stratification at 4 ºC and darkness. Microbial concentrations were set to $2x10^8$ CFU mL^{-1} for *P. oryzihabitans* PGP01 and $2x10^7$ sp mL^{-1} for *C. ramotenellum* PGP02 and *Phoma* spp. PGP03. In order to monitor the effect of the co-culture with the microorganisms in embryo germination, the percentage of germinated embryos was calculated at the end of every phase of culture: stratification phase, second stage (14 ºC and 12/12 h) and third stage (24 ºC and 16/8 h). Studies revealing the benefits of *in vitro* application of PGPMs have been reported at different levels including culture establishment through seeds. For instance, Regalado et al. (2018) demonstrated that *Bromus auleticus* seeds inoculated with *Epichloë* strains promote *in vitro* seed germination. In addition, Moeinzadeh et al. (2010) also reported that biopriming of sunflower seeds with *P. fluorescens* promoted germination and plant development in *ex vitro* systems. The results obtained in the first cross revealed that these three microorganisms did not improve germination due to the high effectiveness of the *in vitro* rescue process, reaching almost a 100% of germinated embryos after 24 weeks of *in vitro* culture. These results reinforced the feasibility of the *in vitro* embryo rescue technique in peach breeding programmes. While *P. oryzihabitans* PGP01 did not interfere with seed development, both fungi *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 negatively affected this parameter since at the end of the third stage of culture, around a 50 and 80% of embryos germinated was recorded, respectively, against the almost 100% of germination achieved in non-inoculated and *P. oryzihabitans* PGP01 treated embryos.

Once completed the *in vitro* rescue process, the effects of the inoculation of the first nectarine cross with the bacterium and the two fungi was monitored measuring several growth parameters including plant FW, number of leaves, number of roots, root volume, stem length as well as photosynthetic capacity through the QY. In this regard, subsequent plants from fungi inoculated embryos showed reduced values of FW, root volume, root length, stem length and number of leaves. These results could be expected if we take into account the negative evidence obtained from

the inoculation with *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 in embryo germination. Therefore, in the light of these results, fungi application was discarded for the following two nectarine crosses. On the other hand, in spite of the delay of growth recorded during the stratification phase, *P. oryzihabitans* PGP01 was able to induce some root morphological changes that would later influence in the acclimatization performance. According to these results, it has been previously reported that the application of *Burkholderia phytofirmans* PsJN promoted grapevines *in vitro* plant development under cold temperatures (Fernandez et al., 2012; Theocharis et al., 2012). In this case, although root elongation and number of roots were not stimulated by this bacterium, a thicker and apparently more robust root system compared to non-inoculated embryos occurred in seedlings inoculated in the presence of this bacterium (Figure 5).

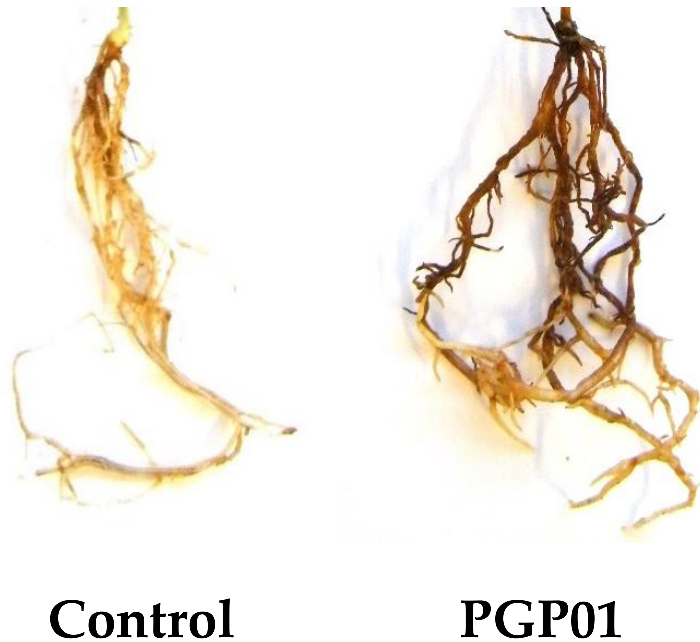


Figure 5. Root morphological changes induced in seedlings obtained from nectarine embryos in the presence of *P. oryzihabitans* PGP01.

These observations were further reinforced by the increase in the root volume measured in those plantlets. In literature, there are no studies that report the use of this bacterial strain in *in vitro* plantlets. However, Belimov et al. (2015) demonstrated that *P. oryzihabitans* increased root biomass up to 57% in pot experiments. In the present thesis, these results represented the first evidence of the effect of *P. oryzihabitans* PGP01 on root architecture of *in vitro* manipulated fruit tree plantlets.

In addition, similar responses were recorded for the second and third nectarine crosses, corroborating that *P. oryzihabitans* PGP01 is able to induce changes in root development.

In the acclimatization process, one of the main factors determining the plant survival is the development of a strong root system. Generally, *in vitro* micropropagated plants present both anatomical and physiological disorders that seriously compromise acclimatization success (Chandra et al., 2010). Plants cultured under *ex vitro* conditions rapidly desiccate by the high losses of water associated with an impaired stomata performance (Pérez-Jiménez et al., 2015). In this sense, the enhancement of the root system development represents a crucial step in water management to ensure plant survival (Nowak & Pruski, 2004). In the Chapter 1, the results obtained showed that the pre-exposition of nectarine embryos to *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 alleviated the high mortality of the resulting plants under greenhouse conditions, improving the survival rate after 4 weeks in greenhouse acclimatization tunnels. However, only *P. oryzihabitans* PGP01 was able to promote plant growth over non-inoculated plants after 4 weeks in acclimatization greenhouse conditions (Figure 6). Biopriming of *in vitro* plants with microorganisms has been shown useful to increase the yield of the acclimatization process (Nowak & Shulaev, 2003; Orlikowska et al., 2017a). Nevertheless, most studies have been conducted applying the microorganisms in hardened plants instead of using *in vitro* micropropagated explants. In these cases, the inoculation with beneficial microorganisms triggers plant defences mechanisms that enhance *ex vitro* acclimatization in a process denominated as "biohardening" (Harish et al., 2008; Kavino et al., 2007; Rajamanickam et al., 2018). By this procedure, a better adaptation to *ex vitro* conditions in response to the application of PGPMs has been reported in several plants such as *Chlorophytum borivilianum* (Mathur et al., 2008), *P. kurrooa* (Das et al., 2017), *Vitis vinifera* (Krishna et al., 2005) as well as *Prunus* and *Malus* rootstocks (Vettori et al., 2010). However, the results obtained in the present thesis would agree with those recently obtained in potato micropropagated plantlets by Kargapolova et al. (2020). These researchers demonstrated that the inoculation with six bacterial strains led to a better plant survival under soil conditions. Moreover, Orlikowska et al. (2017b) also reported that *ex vitro* acclimation of *Helleborus in vitro* plantlets was significantly improved by *B. phytophirmans* PsJN. In the present study, it is important to highlight that the better plant survival observed in plants derived from nectarine embryos inoculated with *P. oryzihabitans* PGP01 was associated with and presumably as a consequence of the morphological changes observed in the roots and not by the application of this bacterium in *in vitro* plants ready to be acclimated.

The results obtained in the next two following crosses did not reveal important effects of *P. oryzihabitans* PGP01 on acclimatization since plants from non-inoculated embryos adapted well to greenhouse conditions. Similar results were obtained in *Lolium multiflorum* inoculated with a strain of *Micronospora* as no changes in acclimatization of *in vitro* plantlets were observed (Della Mónica et al., 2018).

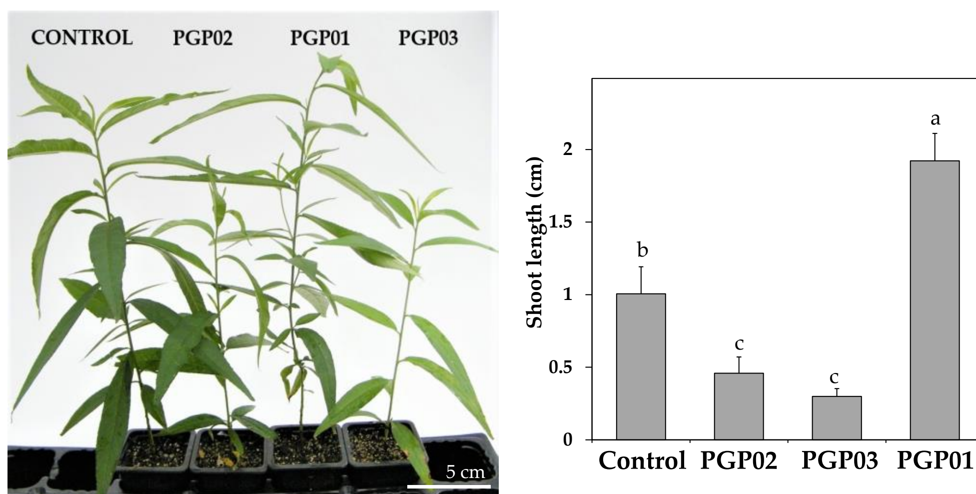


Figure 6. Positive effects in nectarine plants obtained by *in vitro* embryo rescue induced by *P. oryzihabitans* PGP01 after acclimatization to greenhouse conditions.

On the other hand, in *in vitro* micropropagation, the development of adventitious roots usually represents a significant obstacle to overcome, and laboratories have to deal with the high economic costs associated with the high losses of plant material (De Klerk, 2002). This problem is even more acute in woody plant species since genotypes with a considerable agronomic importance are often recalcitrant (Ilczuk & Jacygrad, 2016). Depending on their rooting response, *in vitro* plant genotypes are generally classified as easy-to-root and hard-to-root (Marks & Simpson, 2000). Over the years, the addition of auxins to the culture medium has been established as the most commonly used procedure to induce *in vitro* rooting (Erst et al., 2018; Metivier et al., 2007; Reddy et al., 2001; Velada et al., 2020). Nonetheless, due to the restrictions imposed by the European Commission concerning the use of chemicals in plant production, the interest in seeking rooting-promoting substances or natural root stimulators that reduce the use of auxins to promote *in vitro* rooting is considerably increasing. In this regard, some authors have reported the positive effects of humic acids and other phytoactive compounds in the *in vitro* rooting responses of different plant species (Elmongy et al., 2018; Wiszniewska et al., 2016). However, very little research has been done on the use of PGPMs as natural *in vitro*

root stimulators. The results obtained in the Chapter 2 of this thesis project may provide promising results in this regard since it has been demonstrated that *in vitro* application of PGPMs improved rooting as well as root development of three commercial and advanced selections fruit tree rootstocks belonging to *Prunus* and *Pyrus* genus. Rootstocks have an important impact in agriculture since they offer the possibility of grafting commercial varieties, combining root and shoot characteristics to provide tolerance to edaphic factors related to biotic and abiotic factors (Haroldsen et al., 2012; Prodhomme et al., 2019). In order to demonstrate the effects of the three microorganisms in rooting, three rootstocks showing variable rooting responses were used, being *Prunus* RP-20 and *Pyrus* Py170 easy-to-root rootstocks, and *Pyrus* Py12 a hard-to-root genotype. In addition, two independent methods were performed to study the impact of *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 on *in vitro* root induction and subsequent root development. Due to the negative results derived from the high fungal concentration applied in the Chapter 1, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 dose was reduced at $2x10^6$ sp mL^{-1}.

In a first procedure (Method 1), the effect of the inoculation with the three microorganisms was carried out in combination with IBA treatment to determine if they were able to improve the rooting rates in this routinely used technique. In contrast, whether *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 enhance root induction and development, in the absence of IBA treatment was evaluated in a second procedure (Method 2). Regarding root induction, the results obtained in the present thesis revealed the potential role of *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 as rooting enhancers improving the *in vitro* rooting percentage on the difficult-to-root Py12 rootstock IBA-treated shoots from almost a 60% to 100% (Figure 7). In other woody plant species, Larraburu & Llorente (2015) observed that *Handroanthus impetiginosus* micropropagated plants inoculated with *Azospirillum brasilense* after root induction with IBA led to a 98% of rooting.

In view of the obtained results, several theories were raised to explain these interesting findings, one of them related with possible changes in salts formulations in MS medium caused by the presence of fungi. In this sense, the presence of *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 might lead to an impoverishment of the culture medium that might explain the greater rooting observed in the hard-to-root Py12. It is known that reduced levels of the nutrients in the MS medium promote *in vitro* rooting. For instance, Iglesias et al. (2004) and Patel et al. (2014) reported a greater *in vitro* rooting using a half-strength MS medium (½ MS), and other authors have demonstrated an efficient rooting even in poorer media (¼ MS).

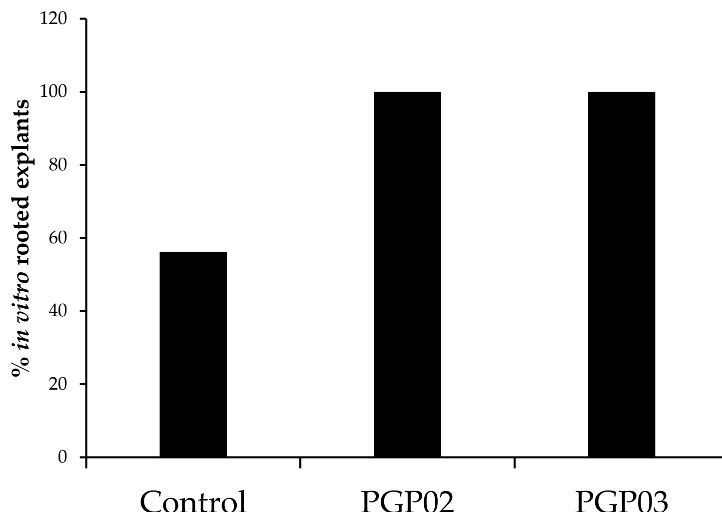


Figure 7. *In vitro* rooting improvement induced by *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 in the hard-to-root Py12 rootstock inoculated after root induction with 10 μM IBA.

The other theory is related to the ability of microorganisms to produce IAA, being this discussed in depth in the section of mechanisms of action. Conversely, microorganisms in the method 2 did not stimulate rooting activity since the 100% of Py12 shoots rooted under control conditions. Differences in *in vitro* root induction in response to the application of microorganisms by two different procedures have been previously reported by Montero-Calasanz et al. (2013) in different olive cultivars. In general, the inoculation with microorganisms in the Method 1 tended to favour the number of roots per explants. However, this increase of *in vitro* number of roots was not accompanied by a better plant growth, since no positive effects in root length and plant, root and shoot FW were observed following this methodology. Only the application of *C. ramotenellum* PGP02 increased the root length in the genotype *Pyrus* Py170.

In the Method 2, where root induction with IBA was not conducted, the application of microorganisms, especially *C. ramotenellum* PGP02 and *P. oryzihabitans* PGP01 promoted a better plantlet development improving shoot and root development in a genotype-dependent manner. In *Pyrus* Py170 rootstock, both microorganisms increased plant, root and shoot FW, and in Py12 rootstocks, those parameters were only improved in the presence of *C. ramotenellum* PGP02. The inoculation with *P. oryzihabitans* PGP01 on this rootstock only improved root FW, being these effects repeated in the *Prunus* rootstock RP-20 regardless of the methodology used. In addition, when the inoculation of microorganisms took place

in fully developed *in vitro* plantlets, *P. oryzihabitans* PGP01 also produced an increase in the number of roots and root FW in *Prunus* RP-20 rootstock without affecting shoot growth. In this regard, a similar response was registered in the resulting seedlings from *Prunus* rescued embryos inoculated with *P. oryzihabitans* PGP01 in the Chapter 1, confirming the specific root-located role of this bacterium in *Prunus* plantlets. Despite observing an increase in the number of roots in the presence of this bacterium in fully developed *Pyrus* Py170 plantlets, the effects in roots were not as evident, being more noticeable in this case, the effect on the shoot FW induced by *C. ramotenellum* PGP02.

As *P. oryzihabitans* PGP01 clearly induced root modifications in *in vitro* plants belonging to *Prunus* genus, *A. thaliana* plants were used in an attempt to elucidate the mechanisms underlying these alterations in the root architecture system. The study of the effect of microorganisms on the root development in this model plant represents an easy approach due to the simplicity of its root system (Kellermeier et al., 2014; Shekhar et al., 2019), consisting mainly on primary root and subsequent lateral roots. In the first part of the experiment developed in the Chapter 3, seedlings of the wild-type genotype Col-0 of *A. thaliana* were evaluated for its root response to the application with *P. oryzihabitans* PGP01. In this case, the application of *P. oryzihabitans* PGP01 promoted root development of *A. thaliana* seedlings, as described in *Prunus* plantlets, **increasing the number of lateral roots** (Figure 8).

Figure 8. Changes in root phenotype in *A. thaliana* Col-0 in response to *P. oryzihabitans* PGP01 in Col-0 root phenotype.

Consequently, lateral root density doubled in comparison to non-treated Col-0 seedlings. Increases on this parameter have been used for many authors as an indicator of the positive effect of microorganisms in *A. thaliana* root architecture.

However, the pathways leading to a greater lateral root density have been shown to be dependent on the microbial species. In this sense, an increase on this parameter in response to the inoculation with *P. brassicacearum* was observed as a consequence of a 50% increase in the number of lateral roots (Contesto et al., 2010). Similar results were obtained by López-Bucio et al. (2007), which reported that *B. megaterium* improved Arabidopsis Col-0 lateral root density by the increase in the number of lateral roots. However, these authors described an inhibition in primary root length. In contrast, the strain PNS-1 of *A. punctata* led to a lateral root density by an increase in primary root length (Iqbal & Hasnain, 2013).

The effects in roots of *P. oryzihabitans* PGP01 were also reported using a liquid culture in the GreenTray® TIS bioreactor. In Chapter 4, RP-20 *in vitro* plants inoculated during 15 days with $1x10^6$ CFU mL^{-1} of *P. oryzihabitans* PGP01 showed a 53% increase in the number of roots than those non-inoculated. Furthermore, these plants also showed a slight increase in the content of total chlorophyll that those non-inoculated, mainly due to an increase in the content of Chl *a* since changes in Chl *b* were not detected. These results, together with the results observed in nectarine embryos, *in vitro* micropropagated plantlets and *A. thaliana* seedlings, provide large evidences about the positive effect of this bacterium in plant root development.

To conclude, the three microorganisms isolated from contaminated *Pyrus* and *Prunus* cultures showed positive effects improving important processes such as *in vitro* peach embryo rescue or rooting development. From the results obtained in the present thesis, it can be extracted that, out of the three microorganisms, *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 provided the most promising results, being even more outstanding the clear effect in the promotion of root development after the application of *P. oryzihabitans* PGP01 in *Prunus* plantlets. This effect was corroborated in *A. thaliana* seedling, observing an increase in the lateral root density by a promotion on lateral root development. Considering all the mentioned above, the main question to be answered was: **Which mechanisms used these microorganisms to promote *in vitro* plant growth?** For that, in the next section, we will try to discuss extensively the possible routes underlying this plant growth-promotion, considering the results obtained during this thesis project.

2. Mechanisms of action of the PGPMs *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 in *Prunus* and *Pyrus in vitro* plants

The study of the mechanisms used by PGPMs to promote plant growth represents an essential issue to be considered if it is intended to exploit their use as biostimulants (Yakhin et al., 2017). Nowadays, this area is quite well-studied; however, although a wealth of information is reported in the scientific literature in this regard, only a few studies used *in vitro* plant material. In addition, most of the studies using *in vitro* culture tools are focused on the alleviation of abiotic stresses or plant diseases. For those reasons, the following step given in the present thesis was the study some of the mechanisms underlying the plant growth promotion observed by these microorganisms (Figure 9).

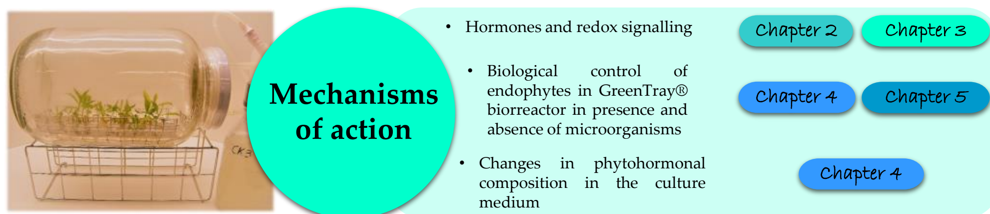


Figure 9. Mechanisms of action of *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 considered in this thesis project.

Among the different mechanisms described, those involving the **microbial production of plant growth regulators** have been the most addressed so far. Phytohormones produced by PGPMs induced changes in plant development patterns, resulting in plants with bigger or more branched roots (Calvo et al., 2014). This response was initially described in the first Chapter of this thesis project, in which the *in vitro* inoculation with *P. oryzihabitans* PGP01 induced root modifications in *in vitro* rescued nectarine embryos. Moreover, in the Chapter 2, root-growth-promoting effects were again reported for this bacterium in *Prunus* rootstocks, but also for both fungi *C. ramotenellum* PGP02 and *Phoma* spp. PGP03, observing an increase in the *in vitro* rooting percentage on the hard-to-root *Pyrus* Py12 rootstock treated with IBA. Out of the different plant growth regulators, the crucial role of auxins in plant root development and morphology has been extensively reported (Sukumar et al., 2013). In the specific field of plant-microbe interactions, many studies involving the use of microorganisms with the ability to produce auxins, mainly IAA, have established a link between this hormone and root growth-promotion (Jiménez-Vázquez et al., 2020; Vicente-Hernández et al., 2019). In the view of these results, theories hypothesizing the role of auxins in these interactions in order to provide an

explanation for the observed results arose. In this regard, the main question to deal with in this part of the thesis was to elucidate if ***P. oryzihabitans*** **PGP01, *C. ramotenellum*** **PGP02 and *Phoma*** **spp. PGP03 were capable of producing auxins**. This assumption was rapidly demonstrated in the Chapter 2, concluding that the three microorganisms were able to *in vitro* produce IAA. It is described that exogenous auxins treatments in Arabidopsis mutants showing defective root system increased the formation of lateral roots which are essential for water and nutrient uptake (Kazan, 2013). Consistent with this fact, combined effects of auxins in the culture medium and the auxins produced by microorganisms might explain the higher number of roots observed in the Method 1. Although it has been reported that auxins are able to promote root development, when in excess, these hormones can inhibit root elongation (Swarup et al., 2007). This affirmation might be confirmed by the results obtained in the inoculation of microorganisms by Method 2, in which a previous treatment with IBA was not performed. Following this method, inoculation with *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 induced root growth, observing higher values of root FW. Apart from the improvement of *in vitro* rooting in the hard-to-root rootstock Py12, no other positive effects in plant growth were observed in the presence of *Phoma* spp. PGP03, being discarded for the following experiments.

Within this thesis project, the role of auxins in *in vitro* root development has been more extensively studied in the case of *P. oryzihabitans* PGP01 since a clear effect in roots of *Prunus* plants was observed. The results obtained in the Chapter 4 using a liquid culture shed more light to those evidences, demonstrating that *P. oryzihabitans* PGP01 promoted root development by altering the auxin levels in the culture medium. It is known that hormones produced by beneficial microorganisms alter plant hormones homeostasis, triggering signalling processes that lead to root morphological changes. In Arabidopsis, it has been described that bacterial species of *Pseudomonas* genus showing the ability to produce auxin-like molecules stimulate plant auxin transport and signalling events (Ortiz-Castro et al., 2011, 2020; Zamioudis et al., 2013). Hormones synthesis, transport and signalling processes are extremely complex, and cross-talk between different hormones are established (Khan et al., 2020). For instance, it has been stablished a link between auxins and strigolactones (SLs) in a dynamic loop in which one hormone regulates the levels of the other (Hayward et al., 2009). The results obtained in the present thesis might be consistent with this affirmation. In the Chapter 3, the results obtained in the defective mutants *max2-3*, *max3-9* and *max4-1* seedlings demonstrated that the root responses observed in *A. thaliana* seedlings in the presence of the IAA-producing microorganism

P. oryzihabitans PGP01 are mediated by SLs. In lateral root development, it has been suggested that the transport of auxins to the roots may be regulated by SLs (Koltai, 2011). In addition, SLs have shown a dual role in lateral root development depending on the auxin status of the plant. When the auxin levels are low, SLs inhibit lateral root formation by reducing auxin import to the roots. However, in the presence of higher auxin levels, a stimulatory effect of SLs in lateral root development occurred due to changes in the auxin optimal level required for this process (Ruyter-Spira et al., 2011). In the presence of *P. oryzihabitans* PGP01, a similar scenario may be occurring since the IAA produced by this bacterium might alter the balance between auxins and SLs, leading to a greater lateral root development (Figure 10).

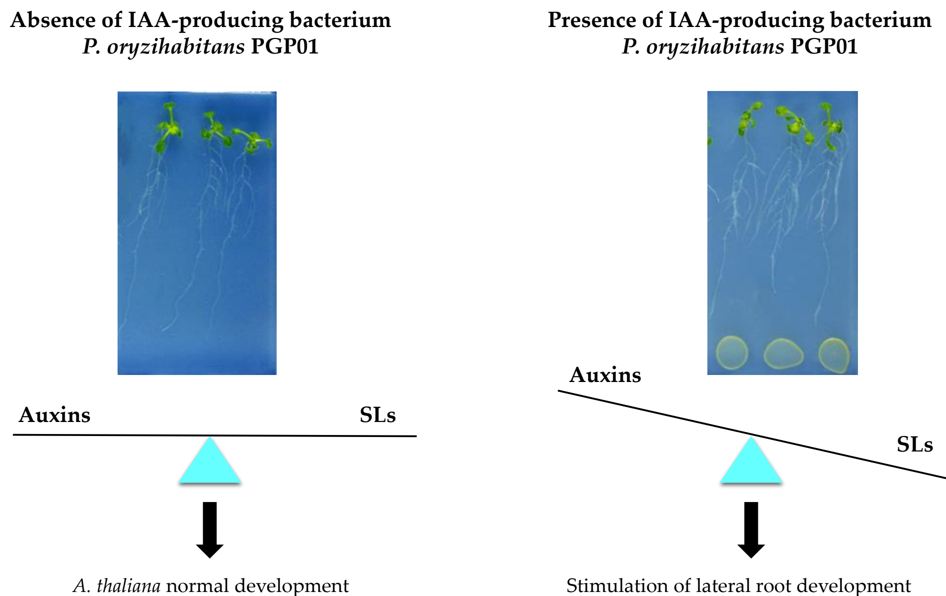


Figure 10. Possible connected role between SLs and auxins in the presence of the bacterium *P. oryzihabitans* PGP01.

However, this interaction network seems to become more complex as a relationship between plant hormones and the antioxidant metabolism also exists. For that reason, we decided to perform the same experiments but using Arabidopsis mutants lacking the two antioxidant molecules ascorbate (*vtc2-1* and *vtc2-2*) and glutathione (*cad2-1*, *pad2-1* and *rax1-1*). All these mutants showed an altered root phenotype, revealing the role of both molecules in root development (Márquez-García et al., 2014; Olmos et al., 2006). The link between antioxidant metabolism and root development remains poorly understood, being this fact more evident in the case of root responses to PGPMs. The results obtained in the present thesis using

Arabidopsis mutants lacking both ascorbate (*vtc2-1* and *vtc2-2*) and glutathione (*cad2-1*, *pad2-1* and *rax1-1*) tried to shed new light to unravelling the role of redox processes in the presence of PGPMs, and their potential connection with hormonal signalling. From this study, it can be extracted that glutathione deficient mutants (*cad2-1*, *pad2-1* and *rax1-1*) showed lower number of lateral roots than the Col-0 Wild-Type genotype in the presence of *P. oryzihabitans* PGP01, suggesting that this molecule is likely required for the greater lateral root development observed in the presence of this bacterium. In previous studies, it has been demonstrated the importance of redox control in *A. thaliana* root development mediated by glutathione (Passaia et al., 2014). However, this molecule would not act independently since it is known that a complex link among glutathione, SLs and auxins exists in the regulation of root architecture (Márquez-García et al., 2014). Taken together, these results might suggest that the root responses observed in *A. thaliana* in the presence of *P. oryzihabitans* PGP01 could be regulated by the close interaction of SLs and glutathione with auxin transport and signalling. However, further investigations in this regard are needed in order to explicitly understand those connections. This study represents an initial approach to unravel the complex relation among root development, hormonal status and antioxidant homeostasis. Other hormones such as GAs, mainly involved in stem elongation, have been also described in beneficial plant microbe-interactions (Calvo et al., 2014). In this research, the predominant shoot growth observed in fully *in vitro* developed *Pyrus* Py170 plantlets in response to the application of *C. ramotenellum* PGP02 suggested the possible role of this hormone in the mechanism of plant growth promotion in these plants. In *ex vitro* conditions, many authors have reported the GAs-producing ability of fungi belonging to *Cladosporium* and *Phoma* genus in the growth and alleviation of abiotic stress in plants. For instance, Hamayun et al. (2009) and Waqas et al. (2012) related the increase in plant growth observed in rice and soybean plants in the presence of *C. sphaerospermum* and *Phoma glomerata* LWL2 with the production of high amounts of bioactive GAs such as GA_1, GA_3, GA_4 and GA_7.

On the other hand, controlling the **growth of other pathogenic microorganisms** also represents a widely described mechanism associated with plant growth promotion induced by microorganisms (Morales-Cedeño et al., 2021). This effect has been broadly reported by many authors using plants cultured under field conditions (Etesami & Alikhani, 2017; Peng et al., 2017; Saravanakumar et al., 2019), being also extended to the field of *in vitro* cultured plants. For instance, Kavino & Manoranjitham (2018) demonstrated that *in vitro* bacterization with some *Bacillus* and *Pseudomonas* strains reduced the incidence of *Fusarium* wilt in banana plants under field and greenhouse conditions. Most of the studies showing *in vitro* biological

control focus their efforts on the reduction of the incidence of a particular plant disease and in the most of cases caused by fungi. In micropropagation, endophytes, mainly bacteria, may act as "vitropathogens", causing negative consequences such as lack of multiplication or losses of plant material (Cassells, 2012; Orlikowska et al., 2017a). In this project, an innovative approach has been followed to prove the ability of the two microorganisms *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 to supress or reduce the growth of bacterial endophytes existing in *Prunus* plantlets micropropagated *in vitro*. These experiments were conducted using the GreenTray® bioreactor as this micropropagation system was recently patented for a more cost-effective propagation of *in vitro* fruit tree plants. In addition, its practical design and ease handling also allowed to follow both inoculated and endophytic microbial population dynamics in the culture medium. The results collected from these studies revealed that none of the two microorganisms *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 showed evidences of a biological control activity under the tested experimental conditions. However, these results provided valuable information that may be useful in the developing of further methodologies to control endophytes growth. For instance, 5 days after inoculation, *C. ramotenellum* PGP02 drastically reduced the bacterial endophytes populations in GreenTray® bioreactors, being unable to be detected by the plate dilution technique. In the light of these results, the first question that arose was if *C. ramotenellum* PGP02 **might show a potential role in the control of bacterial endophytic populations**. This question was rapidly solved when it was observed that RP-20 *in vitro* plants cultured in GreenTray® bioreactors in the presence of this fungus showed negative effects on plants such as decreased values of vegetative growth parameters as well as chlorophyll content. The explanation for these results might be given by the drastic drop of the pH of the culture medium, registering pH values about 2.5 in the presence of this fungi, nutrient depletion in the culture media, and rise of CO_2 levels in bioreactor atmosphere. The pH represents an important factor modulating microbial growth, and it is known that microorganisms displayed the ability to modify the pH of the environment (Ratzke & Gore, 2017). According to this affirmation, *C. ramotenellum* PGP02 altered the culture conditions creating a favourable environment for its growth that, in turn, may also favour the maintenance of the low values of pH, creating a loop that led to the overrunning of the cultures. The high CO_2 and low O_2 values, likely related to fungal metabolism, would help to corroborate these results. It has been described that reported that levels of CO2 above 1% may induce toxicity symptoms in plants (Schwarz, 1999). In addition, these findings are in agreement with the stated by Rousk et al. (2009) in soil, who reported an increase in

fungal growth under low pH conditions while decreasing bacterial growth. Taking into consideration these results, the following questions were raised: If pH level was the determining factor to control endophytes population, **would it be necessary to apply microorganisms to control endophytes?** And even more, **it would be possible to carry out the *in vitro* micropropagation at such low pH without affecting explants behaviour?** Those two questions were attempted to be addressed in Chapter 5 in a subsequent experiment using the GreenTray® bioreactor to evaluate whether a lower pH can control endophytes growth without affecting micropropagation of explants. This experiment was possible due to the use of a liquid culture system without agar which allows to modify the pH of the culture medium avoiding problems of solidification by the gelling agent (Leifert et al., 1992). The results obtained in this study confirmed both hypothesis, demonstrating that endophytes population of RP-20 *in vitro* explants might be controlled at a pH level of 3 without affecting *in vitro* micropropagation. Conversely, when this process was conducted at pH 7, endophytes population was favoured, and micropropagation was seriously compromised, suggesting the negative consequences of the uncontrolled growth of endophytes in *in vitro* cultures. From the knowledge generated in this thesis project, it may be hypothesized an inverse relation between the endophytes population and the micropropagation rate in *in vitro* cultures depending on the pH of the culture medium (Figure 11). However, further investigations are needed in this regard to corroborate this relation in other plant species.

3. *In vitro* factors affecting the interaction between plant and microbes

Although this issue was not directly considered within this PhD project, it is pertinent to mention that the different culture conditions imposed in the different chapters have produced changes in the *in vitro* plant-microbe interactions. In natural environments, interacting plant and microbes often collaborate as partners, and it is expected that imposed environmental conditions affect both organisms (Cheng et al., 2019). This fact is also extended to plant-microbes interaction under *in vitro* conditions. In this context, it may seem logical to think that to observe a positive effect of the application of PGPMs in plant growth, culture conditions must ensure the growth of both components of this interaction. During this PhD, the effect of several factors on the *in vitro* interaction between plants and microorganisms have been considered, establishing two groups clearly interrelated: **Factors depending on the culture conditions, and factors depending on the culture medium composition**.

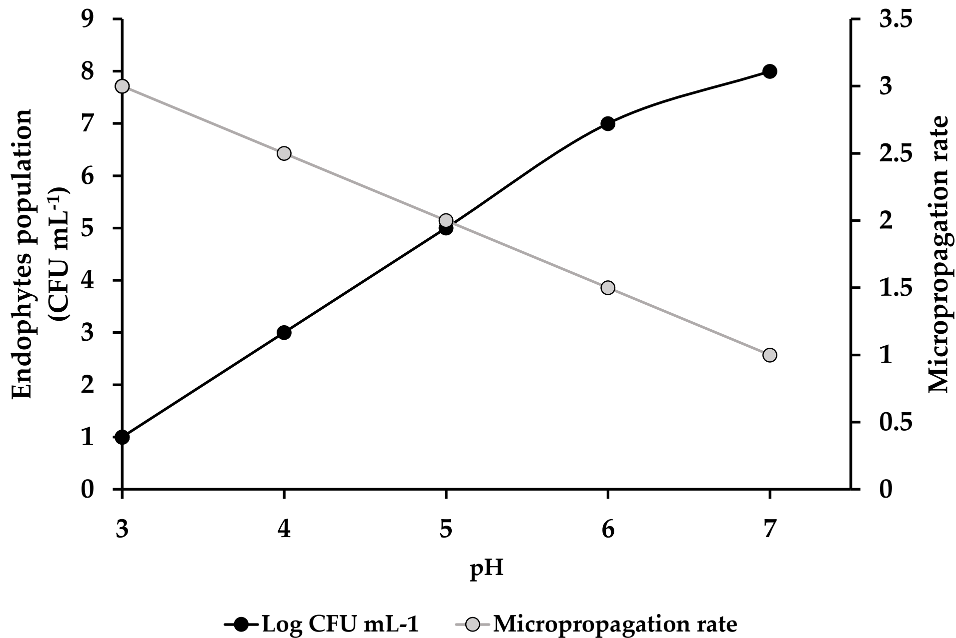


Figure 11. Relation between endophytes population of explants and *in vitro* micropropagation rate depending on the pH of the culture medium.

Concerning those related to the culture conditions, an important effect of **temperature** has been observed in the interaction between microorganisms and *Prunus* rescued embryos (Chapter 1). In those studies, embryos inoculated with microorganisms were submitted to several *in vitro* stages using different temperatures of storage, being the stratification at 4 ºC the most determining step for microbial growth. This represents an essential step when conducting peach *in vitro* embryos rescue to break embryos dormancy and allow embryo germination (Anderson & Byrne, 2002; Daorden et al., 2004). However, the low temperatures at this stage did not favour *P. oryzihabitans* PGP01 growth, leading to a reduction about 2.36 log CFU mL^{-1} after 3 months of culture. Cold temperatures compromise bacterial growth affecting main processes such as nutrients uptake (Nedwell, 1999). *P. oryzihabitans* PGP01 growth was recovered in the following steps of culture and it was also accompanied by the increase on growth, confirming an effect of temperature on bacterial performance in the culture medium. In contrast, these cold temperatures did not disturb *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 growth. These ability of both fungi to grow under these conditions might explain the negative effects on the observed embryo germination.

Regarding the **composition of the culture medium,** it is important to highlight that all the experiments were conducted using an *in vitro* heterotrophic approach, and the traditional mineral recipes (MS and WPM) were supplemented with the incorporation of a 3% of sucrose. In fact, some studies have demonstrated an adequate microbial growth on media commonly used to grow plants such as MS or WPM media (Cagigal & Sánchez, 2017; Leifert & Waites, 1992; Oliveira et al., 2003). It is known that sugars can be used as substrates by microorganisms to ensure their growth and development, especially for fungi (Brannon, 1923). Therefore, the presence of sucrose in the culture medium might represent a factor to consider when co-culturing fungi and bacteria in media for *in vitro* plant growth. On the other hand, the **presence of vermiculite** in the culture medium represents another factor that, together with the presence of sucrose, could have also affected the interaction between *in vitro* plants and microorganisms. Along the years, vermiculite has been used in *in vitro* culture as an efficient supporting material to favour some *in vitro* processes such as micropropagation or root development due to its high porosity (Afreen-Zobayed et al., 2000; Oakes et al., 2016). However, in a recent work, it has been reported that some bacteria belonging to *Enterobacter* or *Pseudomonas* are able to release some elements from the vermiculite that could favour plant development (Pourjasem et al., 2020). Much research is still needed in this area to elucidate if our microorganisms are able to use vermiculite, and how the released compounds may interfere with or promote plant development.

Finally, it was also described the effect of the **pH of the culture medium** as a factor which strongly affected the *in vitro* co-culture since growth of both, microorganisms and plants, is altered by this factor (Leifert et al., 1992). In the present research, the effect of the pH on the interaction was observed in the Chapter 4, and more specifically, in the experiment in which only *P. oryzihabitans* PGP01 was inoculated at pH 5.7 and pH 7. In this experiment, *P. oryzihabitans* PGP01 was inoculated in GreenTray® bioreactors with MS medium adjusted at two different pHs (5.7 and 7) to study its interaction with *Prunus* RP-20 *in vitro* plantlets. These studies were possible as liquid medium was used, preventing the differences in solidification of the gelling agent due to variations of pH (Thorpe et al., 2008). In this experiment, a lower *P. oryzihabitans* PGP01 growth was obtained when the initial pH of the culture medium was set at the normal pH value for plant *in vitro* culture (pH 5.7). In contrast, at pH 7, a typical growth curve was obtained. This was the expected response since it is widely described that bacterial growth is favoured by pH values ranging 6.5-7.0 (Mossel et al., 1995). A greater bacterial growth did not correlate with a better *in vitro* plant performance since no important effects in growth parameters including plant

FW and number of roots were observed after 15 days of co-culture. The effects in roots were observed after 15 days of co-culture at pH 5.7, which represent the optimal pH value for plant growth (Leifert et al., 1992).

4. Sustainable mass production of *P. oryzihabitans* PGP01

Throughout this thesis project, the results collected concerning the use of the three microorganisms revealed that *P. oryzihabitans* PGP01 provided the most promising results inducing important effects in fruit tree root growth and development in several *in vitro* systems and plant material. The last efforts were therefore aimed at trying to develop a product based on this microorganism to scale up its application to field conditions (Figure 12).

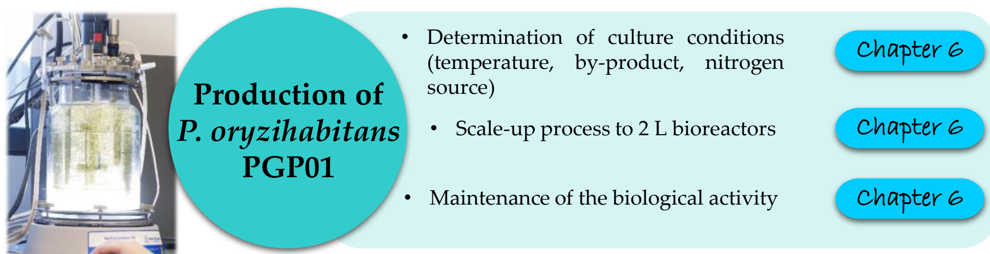


Figure 12. Steps of the production process of *P. oryzihabitans* PGP01 by the use of agro-food industry wastes.

To achieve that goal, the first step is to develop a culture medium that ensures a large amount of microbial production without disturbing its biological activity (Droby et al., 2016). And the second step is to optimize microorganism growth conditions in the developed culture medium. In this study, a competitive medium for the mass production of the PGPM *P. oryzihabitans* PGP01 was obtained and production process was optimized, reaching similar values of bacterial growth than using a commercial medium. A growth medium based on wastes from agro-food industry was performed for *P. oryzihabitans* PGP01 production, making the process cheaper as well as more sustainable. Some studies have demonstrated that the use of by-products generated during the manufacturing of food products can be used as raw materials for the elaboration of media to grow microorganisms. For instance, the use of manure combined with amino acids from animal carcasses led to obtain the maximum biomass of the functional strains of a bio-organic fertilizer (Liu et al., 2016). Other study conducted by Pastor-Bueis et al. (2017) demonstrated the feasibility of wastes from the anaerobic digestion of products from minimally processed vegetables and fruits industries for the eco-friendly production of *Bacillus siamensis*. The

developed product improved efficiency of the use of nitrogen in sweet pepper plants under field conditions.

In this study, after selecting 25 ºC as the optimal growth temperature, three by-products from potato (FPP), tomato (TS) and cereal (WB) industries were tested for their feasibility to ensure bacterial production. These three wastes were obtained from the European Union funded AGRIMAX project, whose main objective was to re-use some agro-food by-products or wastes generated during food processes as raw materials to reduce the costs of industrial processes, including the production of microorganisms. Together with many other potato, tomato and cereal industries by-products differing in their origin, the properties of these wastes were firstly determined, observing a significant carbon content that may be used as a source to grow microorganisms. In the case of this study, FPP waste provided the best results in terms of *P. oryzihabitans* PGP01 growth compared to TS and WB by-products. Potato wastes have resulted in good substrates to produce microorganisms due to its high content in starch and lignocellulosic constituents (Fritsch et al., 2017). Nevertheless, most of the published studies in literature reporting the use of these by-products are addressed to minimise the cost of the production process of secondary metabolites or single-cell proteins (Izmirlioglu & Demirci, 2016; Kleekayai & Suntornsuk, 2011). In this regard, Bacha et al. (2011) studied the use of potato peels for the biomass production of the widely used yeast *Saccharomyces cerevisiae* as protein source in food formulations. The results obtained in the present thesis project provide further evidence of the suitability of potato wastes for microbial production, demonstrating that they are also efficient for the growth of PGPMs. However, growth values of *P. oryzihabitans* PGP01 achieved in the presence of only FPP wastes were still lower than the obtained in the commercial medium TSB, requiring additional supplementation. It is known that after carbon source, an adequate nitrogen supply is also necessary to ensure microbial growth, being especially critical when growing bacteria. For that purpose, different compounds were tested as nitrogen sources for *P. oryzihabitans* PGP01 growth, and among all of them, peptone (PEP) and tryptone (TRP) were the nitrogen sources that best complemented *P. oryzihabitans* PGP01 growth obtained with the by-product FPP in flask experiments. The implementation of those peptides in the culture medium have been reported as useful compounds for many microorganisms (Hensley et al., 2016; Posada-Uribe et al., 2015). The results obtained concerning the use of TRP were not surprising since it is one of the main components of the commercial medium TSB, used as a standard medium to provide the maximum *P. oryzihabitans* PGP01 growth. Finally, tryptone was selected as the nitrogen source for the elaboration of a by-product based medium due to its lower

price compared to other tested compounds. However, the production of *P. oryzihabitans* PGP01 growing in FPP and TRP in flasks was still lower than the obtained when the bacterium grew in the commercial TSB medium, being necessary further optimization.

The composition of the medium was finally optimized and scaled-up in 2 L bioreactors. It has been reported that the use of traditional compounds such as peptone or triptone still lead to high inoculants production costs (Lobo et al., 2019). However, some authors have also used the combination of traditional culture medium components with low-cost substrates for the development of culture media for a more economic PGPMs growth. In this context, Bashan et al. (2011) demonstrated that the substitution of glucose by glycerol or Na-gluconate in the triptone-yeast extract-glucose medium led to a suitable medium for the high-yield production of different strains of *Azospirillum*. In addition, other studies performed by Singh et al. (2013) reported the effectiveness of dairy sludge combined with yeast extract or mannitol for the growth of *Rhizobium trifolii*. In the present study, the combined use of TRP at 10 or 20 g L^{-1} with the potato wastes FPP led to similar yield production, allowing to reduce the concentration of TRP in comparison to that normally added to the TSB medium (17.5 g L^{-1}) and, therefore, reducing the costs of the production process. These results might suggest an effective role of the low content of nitrogen existing in FPP in the growth of *P. oryzihabitans* PGP01. Furthermore, the addition of 5 g L^{-1} of NaCl and 10 g L^{-1} of sugar cane molasses (MOL) allowed to reach finally a bacterial concentration of 4.78×10^9 CFU mL^{-1}, being these values similar to those observed in the TSB commercial medium (4.37×10^9 CFU mL^{-1}). Molasses represents a raw material obtained from the last step of crystallization of sugar, and its application for industrial purposes to obtain high amounts of microbial cells has been extensively studied by many authors due to its high sugar content (Abadias et al., 2003; Bae & Shoda, 2004; Costa et al., 2001; Yánez-Mendizábal et al., 2012). It is known that approximately a 50% of its content is attributed to sugars such as sucrose, glucose and fructose, widely used by microorganisms to grow (Kotzamanidis et al., 2002). In addition, as it is obtained as a waste of the last stage of crystallization of sugar, molasses can be used as an economic carbon source in microbial production processes (Çakar et al., 2014). Taken together, all the obtained results lead to a very promising alternative for the production of the PGPM *P. oryzihabitans* PGP01, developing an economic culture medium mainly based on agro-food by-products. On the other hand, the addition of salts such as NaCl to the culture medium also displayed positive effects in *P. oryzihabitans* PGP01 production since it is known that for certain bacterial species, mineral supplements

represent an important factor to improve growth or the production of their metabolites (Abdel-Mawgoud et al., 2008).

After defining the final composition of growth medium, next step is optimizing process conditions such as agitation and aeration. In most of the production processes involving fed-batch fermenters, those two factors tend to have a significant relevance in the scaling-up determining the mass transfer efficiency between medium and microbial cells (Stanbury et al., 2017). In the present study, variations in *P. oryzihabitans* PGP01 growth were expected under different agitation and aeration since this bacterium is a strictly aerobic microorganism (Gutiérrez-Bustos et al., 2009). In fact, those two factors significantly affected bacterial production at 22 and 24 h of growth, coinciding with the period of most active growth (microbial growth exponential phase). At these points, a maximum $3.16x10^9$ CFU mL^{-1} of *P. oryzihabitans* PGP01 biomass was obtained at higher agitations (400 and 600 rpm) and higher oxygen fluxes (0.5 and 0.75 vvm, air volume/liquid volume x minute). In addition, while under 600 rpm, changes in growth among aerations were not observed, oxygen supply still affected *P. oryzihabitans* PGP01 biomass under 400 rpm, obtaining the highest production values under 0.75 vvm. In that case, 600 rpm provided so much oxygen to bacterial cultures that an extra supply was not able to improve *P. oryzihabitans* PGP01 production. Therefore, to reduce the costs of the process, the conditions of the process were fixed at 400 rpm and 0.75 vvm. Similar responses were observed by Bajaj & Singhal (2010) when producing *Bacillus licheniformis* NCIM 2324. These researchers also reported that high agitation and aeration rates were required to obtain the highest cell growth. In contrast, Carrasco-Espinosa et al. (2015) concluded that low aerations rates (0.1 vvm) led to the highest *A. brasilense* population (10.89 log CFU mL^{-1}) in pneumatic bioreactors. The impact of agitation and aeration needs to be optimized for each microbial species since it has been shown to be clearly genotype-dependent.

Finally, *P. oryzihabitans* PGP01 growth was finally ensured in an optimized potato wastes (300 g L^{-1} FPP) based medium, supplemented with 10 g L^{-1} of TRP, 5 g L^{-1} of NaCl, 10 g L^{-1} MOL and 2.5 g L^{-1} of K_2HPO_4, and at 400 rpm agitation and 0.75 vvm aeration. Under these conditions, a maximum bacterial cell concentration of 4.4 x 10^9 CFU mL^{-1} was reached after 24 h of culture. Furthermore, this optimized medium was considerably cheaper than the commercial medium TSB. The replacement of the papaic digest of soybean meal and glucose for wastes such as FPP and MOL led to a reduction of almost a 70% in the cost of the elaboration of the culture medium (Table 1). The use of this culture medium based on wastes generated during food processing

contribute to a higher sustainability, reducing the amount of wastes generated by their valorisation for microbial production.

Table 1. Economic cost of the optimized medium based on potato wastes in comparison to the commercial medium TSB.

TSB medium			FPP-based medium		
	Conc. ($g L^{-1}$)	**Price (€ L^{-1})**		**Conc. ($g L^{-1}$)**	**Price (€ L^{-1})**
Tryptone	17	1.62	**Tryptone**	10	0.95
Papaic digest of soybean meal	3	1.87	**Potato pulp and peels (FPP)**	300	0.006
Glucose	2.5	0.19	**Sugar cane molasses (MOL)**	10	0.002
KH_2PO_4	2.5	0.23	**KH_2PO_4**	2.5	0.23
NaCl	5	0.07	**NaCl**	5	0.07
TOTAL		**3.98**	**TOTAL**		**1.26**

Finally, the feasibility of the optimized medium for the maintenance of the *in vitro* root development activity of *P. oryzihabitans* PGP01 was demonstrated in *Prunus* RP-20 plantlets (Figure 13), leading to a more robust root system as observed in the Chapters 1 and 2 of the present thesis. In the view of these results, agro-food wastes have been successfully revalued to develop an economic protocol for the sustainable production of the PGPM *P. oryzihabitans* PGP01. The developed culture medium will be included to the list of optimized medium previously elaborated by the research group for bacteria such as *Bacillus subtilis* CPA-8 (Yánez-Mendizábal et al., 2012) or *Pantoea agglomerans* CPA-2 (Costa et al., 2001), and yeasts such as *Candida sake* (Abadias et al., 2000). However, to obtain a product based on this microorganism that can be used in the future, further investigations in terms of the determination of the best conditions to preserve cell viability in formulations based on this microorganism and its large scale-up production to pilot scale will be required.

As a whole, this thesis project represents a highly interdisciplinary approach with the main objective of providing a wealth of knowledge about the interaction between fruit tree and microorganisms from an agro-food perspective. For that purpose, the plant *in vitro* tissue culture techniques have constituted a very useful tool to hypothesise under a controlled environment what might be expected at field conditions. All the results collected during this PhD provided evidence about the

beneficial use of microorganisms to increase the growth and development of fruit tree plants, which show an important agronomic importance. Altogether, this PhD would lay the basis of a research that could contribute, among other things, to the development of a more sustainable agriculture.

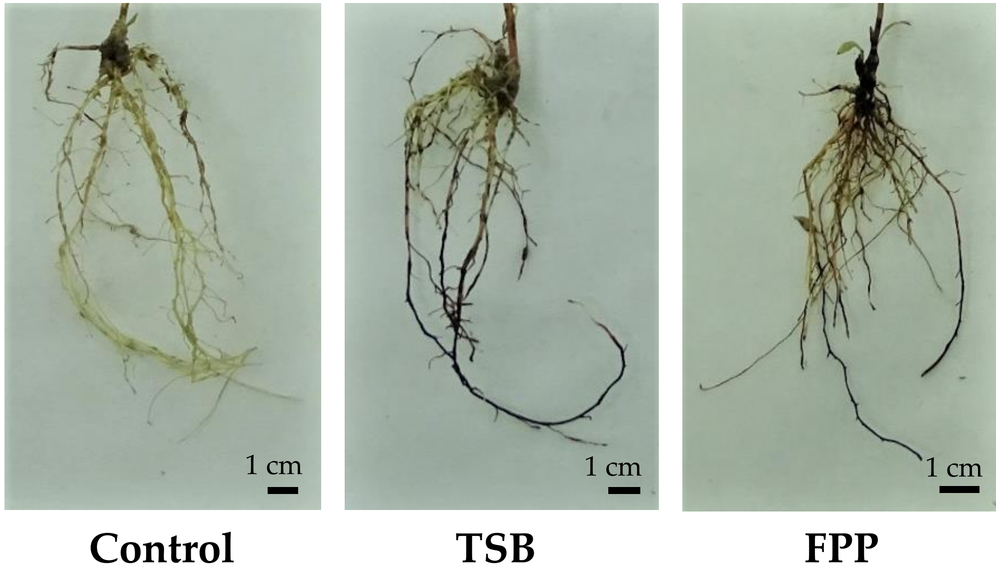


Figure 13. Validation of the root growth promoting effect of *P. oryzihabitans* PGP01 growing in an optimized medium based on potato wastes (FPP).

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Conclusions

Conclusions

The main objective of this thesis project was to study the effect of three microbial isolates in deciduous fruit tree *in vitro* cultured plants. All the results collected in the different chapters of this thesis manuscript (Chapters 1-6) has led to the following conclusions:

1. **Description of the effect of *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 in fruit tree *in vitro* plant growth and development**

1.1. In early ripening nectarine *in vitro* rescued embryos, none of the three microorganisms improved embryos germination since this technique was very effective in the germination of non-inoculated embryos. In addition, seedlings obtained from embryos inoculated with *P. oryzihabitans* PGP01 displayed a more robust root system, favouring their acclimation to greenhouse conditions.

1.2. In *in vitro* micropropagated commercial rootstocks, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 improved *in vitro* rooting percentage of explants of the hard-to-root rootstock *Pyrus* Py12 treated with IBA and directly inoculated with microorganisms.

1.3. The inoculation with the three microorganisms also induced different effects in *in vitro* plant growth and development. Overall, explants inoculated following the Method 1 (root induction with IBA + direct inoculation with microorganisms) showed a higher number of roots. On the contrary, the application of microorganisms by the Method 2 (no root induction with IBA + immersion of the basis of the shoot in microbial suspension) favoured *in vitro* plant growth, leading to increase plant, shoot and root FW, especially in both *Pyrus* rootstocks, Py12 and Py170.

1.4. Regardless the followed methodology, *P. oryzihabitans* PGP01 promoted root development in *in vitro* RP-20 plantlets.

1.5. *P. oryzihabitans* PGP01 effects in root development were confirmed in the model plant *A. thaliana*, observing an increase in the NLR and LRD in the presence of this bacterium.

1.6. The use of the GreenTray® TIS bioreactor with liquid medium represented an effective system for RP-20 *in vitro* micropropagation, increasing the FW and length of the obtained shoots. This system also resulted in an

appropriate system for the study of the interaction between RP-20 explants and PGPMs in a liquid culture, observing an increase in the number of roots of RP-20 in the presence of *P. oryzihabitans* PGP01.

2. **Study of the mechanisms of action underlying the observed *in vitro* plant growth promotion**

2.1. The three microorganisms *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 and *Phoma* spp. PGP03 showed the ability to produce IAA in culture media supplemented with 0.5 and 1 g L^{-1} of tryptophan.

2.2. The root responses observed in *A. thaliana* mutants suggested a connected role of auxins, SLs and GSH in the greater root development in the presence of *P. oryzihabitans* PGP01.

2.3. In GreenTray® TIS bioreactors inoculated with *P. oryzihabitans* PGP01, the greater number of roots in RP-20 *in vitro* plantlets may be related with changes in the IAA contents in the culture medium induced by this microorganism.

2.4. The two microorganisms *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 did not led to an effective biological control of endophytes growth in RP-20 *in vitro* explants cultured in GreenTray® TIS bioreactors. However, it was suggested the effect of a low pH in the control of these contaminations.

2.5. *In vitro* culture of RP-20 explants at pH 3 in GreenTray® TIS bioreactors reduced endophytes populations of RP-20 explants without affecting the *in vitro* micropropagation rates or shoot growth.

3. **Production of *P. oryzihabitans* PGP01 by using agro-food industry wastes**

3.1. In flasks experiments, *P. oryzihabitans* PGP01 growth at 25 ºC and in the presence of potato peels and pulp (FPP) supplemented with Tryptone (TRP) at both tested concentrations (10 and 20 g L^{-1}) resulted in the best combination for the production of this bacterium.

3.2. In the scale-up of the process to 2 L bioreactors, it was determined that the medium providing the highest *P. oryzihabitans* PGP01 growth was the based on FPP and supplemented with 10 g L^{-1} of TRP, 10 g L^{-1} of sugar cane

molasses, 5 g L^{-1} of NaCl and 2.5 g L^{-1} of K_2HPO_4. In this medium, a maximum growth of 4.4×10^9 CFU mL^{-1} was reached after 24 h of incubation.

3.3. The biological activity of *P. oryzihabitans* PGP01 grown in the wastes based medium was maintained, promoting root development of RP-20 *in vitro* cultured plantlets by an increase in the number of roots, root FW/Shoot FW as well as root FW/Plant FW.

3.4. Considering all the above, the use of agro-food industry wastes represents a considerably cheap strategy/process for the growth of the fruit tree root development enhancer microorganism *P. oryzihabitans* PGP01, reducing the costs of the production process as well as maintaining its sustainability.

Conclusiones

El principal objetivo de esta tesis doctoral era demostrar cuál era el efecto de los tres microorganismos aislados en plantas frutales obtenidas mediante el uso de técnicas de cultivo *in vitro*. Toda la información recogida en los diferentes capítulos de la tesis (Capítulos 1-6) ha dado paso a las siguientes conclusiones:

1. **Estudio del efecto de *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 y *Phoma* spp. PGP03 en el crecimiento y desarrollo de plantas frutales cultivadas *in vitro*.**

1.1. En embriones de nectarina rescatados *in vitro*, ninguno de los tres microorganismos mejoró la germinación ya que ésta fue muy alta en el caso de los embriones no inoculados. En las plántulas obtenidas a partir de estos embriones, se demostró que *P. oryzihabitans* PGP01 promovió un mayor desarrollo radicular, que favoreció su aclimatación a condiciones de invernadero.

1.2. En portainjertos comerciales multiplicados *in vitro*, *C. ramotenellum* PGP02 y *Phoma* spp. PGP03 mejoraron el porcentaje de enraizamiento de explantos de *Pyrus* con dificultades para enraizar Py12 tratados con IBA e inoculados directamente con los microorganismos

1.3. La inoculación con los tres microorganismos mediante los Métodos 1 y 2 también produjo diferentes efectos en el crecimiento y desarrollo de plantas cultivadas *in vitro*. En general, los explantos inoculados por el Método 1 (inducción de enraizamiento con IBA + inoculación directa) presentaban un mayor número de raíces. Por el contrario, la aplicación de los microorganismos mediante el Método 2 (sin inducción de enraizamiento con IBA + inmersión de la base del brote en las suspensiones microbianas) favoreció el crecimiento de plantas *in vitro*, observando un aumento del peso fresco de planta, tallo y raíz, especialmente en los dos portainjertos de *Pyrus* Py170 y Py12.

1.4. Independientemente de la metodología aplicada, *P. oryzihabitans* PGP01 incrementó el peso fresco de las raíces de plantas del portainjertos de *Prunus* RP-20.

1.5. Los efectos en la promoción del desarrollo radicular de esta bacteria fueron corroborados en la planta modelo *A. thaliana*, observando un incremento del NLR y LRD en presencia de esta bacteria.

1.6. El uso del biorreactor TIS GreenTray® permitió una eficiente micropropagación *in vitro* de explantos de RP-20, incrementando el peso fresco y la longitud de los brotes obtenidos. Además, este sistema también permitió el estudio de la interacción de estas plantas con los PGPMs, favoreciendo un aumento del número de raíces en presencia de la bacteria *P. oryzihabitans* PGP01.

2. Determinación de los mecanismos de acción usados por los microorganismos para promover el crecimiento de plantas *in vitro*.

2.1. Los tres microorganismos *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 y *Phoma* spp. PGP03 mostraron la capacidad de producir IAA en medios de cultivo suplementados con 0.5 y 1 g L^{-1} de triptófano.

2.2. Las respuestas observadas en las raíces de mutantes de *A. thaliana* sugirieron una posible interconexión entre auxinas, SLs y GSH en el mayor desarrollo de las raíces en respuesta a la aplicación de *P. oryzihabitans* PGP01.

2.3. En biorreactores GreenTray® inoculados con *P. oryzihabitans* PGP01, el mayor número de raíces observado en plantas de RP-20 podría estar relacionado con los cambios observados en el contenido de IAA en el medio de cultivo inducidos por este microorganismo.

2.4. Los dos microorganismos *P. oryzihabitans* PGP01 y *C. ramotenellum* PGP02 no produjeron un control biológico efectivo de las contaminaciones endófitas presentes en explantos de RP-20 cultivados en biorreactores TIS GreenTray®. Sin embargo, se sugirió el efecto del pH bajo para el control de estas contaminaciones.

2.5. El cultivo a pH 3 de explantos de RP-20 en biorreactores TIS GreenTray® favoreció la reducción de la población de endófitos en explantos de RP-20 y por lo tanto su control sin afectar la multiplicación *in vitro* de las plantas.

3. Producción de *P. oryzihabitans* PGP01 mediante el aprovechamiento de subproductos de la industria alimentaria.

3.1. En matraces Erlenmeyer, el crecimiento de *P. oryzihabitans* PGP01 a 25 ºC en presencia de un subproducto basado en pieles y pulpa de patata (FPP) suplementado con Triptona (TRP) a las dos concentraciones testadas (10 y 20 g L^{-1}) resultó ser la mejor combinación para la producción de esta bacteria.

3.2. En el escalado del proceso a biorreactores de 2 L, se determinó que el medio que favorecía un mejor crecimiento de *P. oryzihabitans* PGP01 era el formado por FPP y suplementado con 10 g L^{-1} de TRP, 10 g L^{-1} de melazas de caña de azúcar, 5 g L^{-1} de NaCl y 2.5 g L^{-1} de K_2HPO_4. En este medio, se obtuvo un crecimiento máximo de $4.4x10^9$ CFU mL^{-1} tras 24 h de incubación.

3.3. Este medio optimizado preservó la actividad biológica del microorganismo, promoviendo el desarrollo de raíces de plantas de RP-20 cultivadas *in vitro* mediante un aumento del número de raíces, así como de las relaciones peso raíz/peso tallo y peso raíz/peso total de la planta.

3.4. En base a todo lo anterior, el uso de subproductos de la industria alimentaria representa una estrategia económica para la producción de la bacteria promotora del desarrollo radicular de plantas frutales *P. oryzihabitans* PGP01, reduciendo considerablemente los costes del proceso de producción, manteniendo la sostenibilidad del mismo.

Conclusions

El principal objectiu d'aquesta tesi doctoral va ser l'avaluació de l'efecte dels tres microorganismes aïllats de plantes de fruiters cultivades *in vitro*. Els resultats obtinguts en els diferents capítols de la tesi (Capítols 1- 6) han donat lloc a les següents conclusions:

1. **Descripció de l'efecte de *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 i *Phoma* spp. PGP03 en el creixement i desenvolupament de plantes de fruiters cultivades *in vitro*.**

1.1. En embrions de nectarina rescatats *in vitro*, l'aplicació dels tres microorganismes no va millorar la germinació perquè aquesta va ser molt alta en els embrions no inoculats. No obstant això, les plàntules obtingudes a partir d'embrions inoculats amb *P. oryzihabitans* PGP01 van mostrar un sistema radicular més robust que va afavorir la seva aclimatació a l'hivernacle.

1.2. En portaempelts comercials cultivats *in vitro*, *C. ramotenellum* PGP02 i *Phoma* spp. PGP03 van millorar el percentatge d'arrelament *in vitro* d'explants de *Pyrus* que mostrava dificultats per arrelar Py12 tractats amb IBA i inoculats directament amb els microorganismes.

1.3. La inoculació amb els tres microorganismes mitjançant els Mètodes 1 i 2 va produir diferent efectes en el creixement i desenvolupament *in vitro*. En general, els explants inoculats amb el Mètode 1 (inducció d'arrelament amb IBA + inoculació directa amb els microorganismes) van mostrar un major nombre d'arrels. Pel que fa a la inoculació amb el Mètode 2 (sense inducció d'arrelament amb IBA + immersió de la base del brot en les suspensions microbianes), es va observar un millor creixement vegetal amb increments del pes fresc de la planta, de la tija i de l'arrel, especialment en els dos portaempelts de *Pyrus* Py12 i Py170.

1.4. Independentment de la metodologia aplicada, *P. oryzihabitans* PGP01 va incrementar el pes fresc de les arrels de plantes del portaempelt de *Prunus* RP-20.

1.5. Aquests efectes en el desenvolupament radicular es van confirmar en la planta model *A. thaliana*, observant un augment del NLR i LRD en presència d'aquest bacteri.

1.6. L'ús del bioreactor TIS GreenTray® amb medi líquid va representar un sistema efectiu per a la micropropagació *in vitro* de l'RP-20, incrementant el pes fresc i la longitud dels brots obtinguts. A més a més, aquest sistema també va ser apropiat per l'estudi de la seva interacció amb els PGPMs, afavorint l'augment del nombre d'arrels en presència de *P. oryzihabitans* PGP01.

2. Determinació dels mecanismes d'acció pels quals els microorganismes promouen el creixement de plantes *in vitro*.

2.1. Els tres microorganismes *P. oryzihabitans* PGP01, *C. ramotenellum* PGP02 i *Phoma* spp. PGP03 han mostrat la capacitat de produir IAA en medis de cultiu suplementats amb 0.5 i 1 g L^{-1} de triptòfan.

2.2. Les respostes en les arrels dels mutants d'*A. thaliana* van suggerir el rol combinat de les auxines amb SLs i GSH en el major desenvolupament radicular observat en resposta a l'aplicació de *P. oryzihabitans* PGP01.

2.3. En els bioreactors GreenTray® inoculats amb *P. oryzihabitans* PGP01, l'increment en el nombre d'arrels en plantes de l'RP-20 podria estar relacionat amb els canvis observats en el contingut d'IAA en el medi de cultiu induïts per aquest microorganisme.

2.4. Els microorganismes *P. oryzihabitans* PGP01 i *C. ramotenellum* PGP02 no produïen un control biològic efectiu de les contaminacions endòfites en explants d'RP-20 cultivats als bioreactors TIS GreenTray®. No obstant això, es va suggerir l'efecte del baix pH pel control d'aquests microorganismes.

2.5. El cultiu a pH 3 d'explants de RP-20 en bioreactors TIS GreenTray® en absència de microorganismes, va afavorir la reducció de les poblacions d'endòfits en explants de l'RP-20 sense afectar la seva multiplicació *in vitro*.

3. Producció de *P. oryzihabitans* PGP01 mitjançat l'aprofitament de subproductes de la indústria alimentària.

3.1. En matraus Erlenmeyer, el creixement de *P. oryzihabitans* PGP01 a 25 ºC amb subproductes basats en peles i polpa de patata (FPP) i suplementats amb TRP a les dues concentracions testades (10 i 20 g L^{-1}) va resultar ser la millor combinació per la producció d'aquest bacteri.

3.2. En l'escalat del procés a bioreactors de 2 L, es va determinar que el medi que afavoria un millor creixement de *P. oryzihabitans* PGP01 va ser l'elaborat a partir de FPP amb 10 g L^{-1} de TRP, 10 g L^{-1} de melasses de canya de sucre, 5 g L^{-1} de NaCl i 2.5 g L^{-1} de K_2HPO_4. En aquest medi, es va obtenir un creixement màxim de $4.4x10^9$ CFU mL^{-1} després de 24 h d'incubació.

3.3. Aquest medi basat en subproductes va preservar l'efecte del microorganisme en el desenvolupament radicular, produint un increment en el nombre d'arrels i en les relacions pes d'arrel/pes tija i pes d'arrel/pes fresc de plantes de l'RP-20 cultivades *in vitro*.

3.4. Considerant tot l'anterior, l'ús de subproductes de la industria agroalimentària representa una estratègia molt econòmica per a la producció del bacteri *P. oryzihabitans* PGP01 promotor del creixement d'arrels de plantes fruiteres, reduint considerablement els costos del procés de producció i mantenint la sostenibilitat del mateix.