Using plant growth-promoting microorganisms (PGPMs) to improve plant development under *in vitro* culture conditions

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Abstract

Plant *in vitro* culture techniques are highly useful to obtain significant amounts of true-to-type and disease-free plant materials. One of these techniques is clonal micropropagation which consists on the establishment of shoot tip cultures, shoot multiplication, *in vitro* rooting and acclimatization to *ex vitro* conditions. However, in some cases, the existence of recalcitrant genotypes, with a compromised multiplication and rooting ability, or the difficulties to overcome the overgrowth of endophytic contaminations might seriously limit its efficiency. In this sense, the establishment of beneficial interactions between plants and plant growth-promoting microorganisms (PGPMs) under *in vitro* culture conditions might represent a valuable approach to efficiently solve those restrictions. During the last years, significant evidence reporting the use of beneficial microorganisms to improve the yield of *in vitro* multiplication or rooting as well as their acclimatization to greenhouse or soil conditions have been provided. Most of these positive effects are strongly linked to the ability of these microorganisms to provide *in vitro* plants with nutrients such as nitrogen or phosphorous, to produce plant growth regulators, to control the growth of pathogens or to mitigate stress conditions. The culture of *A. thaliana* under aseptic conditions has provided high-quality knowledge on the root development signalling pathways, involving hormones, triggered in the presence of PGPMs. Overall, the present article offers a brief overview of the use of microorganisms to improve *in vitro* plant performance during the *in vitro* micropropagation stages, as well as the main mechanisms of plant growth promotion associated to these microorganisms.

Main conclusion: The use of beneficial microorganisms improves the performance of *in vitro* cultured plants through the improvement of plant nutrition, the biological control of microbial pathogens or the production of phytohormones that promote plant growth and development.

Keywords: Acclimatization; Biological control; *In vitro* plant-growth promotion; Micropropagation; Plant Growth-Promoting Microorganisms; Phytohormones.

Introduction

Due to their sessile condition, plants in their natural environmental have to cope with the highly fluctuating environmental conditions, including the changes in the nature of soil microorganisms. In this context, plants established close relations with soil-borne microorganisms that can be classified as beneficial or pathogenic (Whipps 2001). In beneficial interactions, none of the two interacting organisms are damaged, whereas pathogenic relationships negatively affect plant physiology, threatening plant growth and health (Schirawski and Perlin 2018). Concerning the beneficial interactions between plants and microbes, most of them take place in a narrow region of the soil called rhizosphere, highly influenced by the root system. It is known that this zone is much richer in microorganisms than the surrounding soil regions as plant roots secrete metabolites that can serve as nutrients to microorganisms (Lugtenberg and Kamilova 2009; Chauhan et al. 2015; Vishwakarma et al. 2020). 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, it is known that other microbes are able to colonise plant tissues and live within the host plants establishing endophytic relationships (Guerrero-Molina et al. 2012; Kusari et al. 2012). Both, rhizospheric and endophytic interactions provide benefits for plants improving the nutrient availability, triggering plant defences or suppressing of the growth of pathogenic microorganisms (Calvo et al. 2014; Vishwakarma et al. 2020; Morales-Cedeño et al. 2021). These beneficial microorganisms are usually known as Plant Growth-Promoting Microorganisms (PGPMs). In this group, Plant Growth-Promoting Rhizobacteria (PGPR) and Plant Growth-Promoting Fungi (PGPF) are generally considered (Lugtenberg and Kamilova 2009; Jahagirdar et al. 2019). Arbuscular Mycorrhizal Fungi (AMF) are also included in this classification 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, to satisfy the World food demand associated to population growth (FAOSTAT, 2018) the use of chemical fertilizers has been increased as a key to improve crop production. This fact has raised a growing concern about the potential impact of the abuse of these compounds with agricultural purposes on human health. Linked to this challenge, a potential solution to help to mitigate the harmful consequences derived from fertilizers might rely on the use of PGPMs as they can act as biofertilizers (Lugtenberg and Kamilova 2009), biological control agents

(Morales-Cedeño et al. 2021), natural phytostimulators (Calvo et al. 2014), or abiotic stress alleviation agents (Alberton et al. 2020).

On the other hand, the growth of plants under *in vitro* culture conditions, represents a very useful technique to produce clones of plants or new genetic variation in a more controlled manner (Hussain et al. 2012). The theorical basis of *in vitro* tissue culture lie on the concept of the totipotency of plant cells whereby a single cell is able to express the whole genome by cell division, differentiating into a whole plant (Thorpe 2007). This ability of plant cells has allowed the development of new methodologies that have made of *in vitro* tissue culture a tool not only merely applied for research purposes, but also as a technique exploited in plant production industry and breeding programs (Thorpe 2007; Akin-Idowu et al. 2009). Nowadays, it is an indispensable approach in agriculture for the production of homogenous disease-free plant material (Hu et al., 2015; Wang et al., 2018), the establishment of new stable genotypes derived from somaclonal variation (Wang & Wang, 2012), the regeneration of plants using *in vitro* embryo cultures (Devi et al. 2017), or the generation of doubled haploid lines as source of completely homozygous parental lines, indispensable for the hybrid seed production industry (Germanà 2010). In plant *in vitro* micropropagation, the first step implies the selection of the plant part, named as explant, from a mother plant cultured *ex vitro* (Hussain et al. 2012). The correct succession of the following steps (establishment of shoot tip cultures, multiplication, in vitro rooting and acclimatization) results in an efficient plant production (Hussain et al. 2012). Nevertheless, this technique when applied to some plant species and genotypes has some limitations that compromise the efficiency in each micropropagation stage. For instance, multiplication and *in vitro* rooting are both limited in recalcitrant and hard-to-root genotypes (Marks and Simpson 2000; Quambusch et al. 2014). In the acclimatization to soil conditions phase, the high losses of plant material are associated with the inability of plants to cope with environmental factors or the presence of soil pathogens (Hazarika 2006; Chandra et al. 2010; Rajamanickam et al. 2018). Over the last years, the number of studies involving the use of beneficial microorganisms to promote plant growth and development have considerably increased. However, most of those studies have been conducted using plants cultured in pots (Ważny et al. 2018; Jain et al. 2020) or soil (Schmidt et al. 2018; Siebers et al. 2018) experiments, and not as much attention has been paid to the use of microorganisms in plant *in vitro* tissue cultures. For that reason, herein, some aspects concerning the use of PGPMs to improve the efficiencies in the different micropropagation phases have been gathered, as well as the most described mechanisms used by these beneficial microorganisms to promote *in vitro* plant growth.

Use of PGPMs to improve *in vitro* micropropagation technique

The *in vitro* application of microorganisms that have the ability to form beneficial relationships with plants can serve to protect *in vitro* cultures while promoting their growth and development. Inoculation of *in vitro* cultures with beneficial microorganisms (including PGPR and AMF), has become the focus of some reviews (Orlikowska et al. 2017b; Soumare et al. 2021) and book chapters (Cassells 2011; Modi and Kumar 2021). It has been demonstrated that bacteria introduced to *in vitro* tissue cultures not only increase the yield of plants produced, but also are a valuable tool in research for plant breeding (Orlikowska et al., 2017b). A clear example of the latter approach is the genetic transformation by the bacterium *Agrobacterium tumefaciens*. This soil-borne bacterium has been used as a universal vector for the introduction of foreign genetic information, thus obtaining transformed plants (Jaiwal et al. 2001; Ceasar and Ignacimuthu 2011). This procedure has led to the development of many *in vitro* transformation protocols of different plant crops including canola (Cardoza and Stewart 2003), finger millet (Ceasar and Ignacimuthu 2011), cowpea (Chaudhury et al. 2007), barley (Trifonova et al. 2001) or apricot (Petri et al. 2008). On the other hand, Digat et al. (1987) reported one of the first studies concerning the use of microorganisms to improve the effectiveness of *in vitro* micropropagation. In this study, *Pseudomonas fluorescens* and *Pseudomonas putida* in artificial substrates attached to *in vitro* plant roots improved 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. In this review, the effect of different PGPMs in each *in vitro* stage, as well as in the *ex vitro* acclimatization phase are listed in Table 1 and Table 2.

Use of PGPMs in *in vitro* multiplication, seed germination and plantlet regeneration

At the multiplication stage, the number of new shoots is exponentially increased by axillary branching, carrying out successive subcultures of the propagules in culture media supplemented with plant growth regulators such as cytokinins (CKs) (Saini and Jaiwal 2002; Hussain et al. 2012). Out of the different CKs, 6-benzyladenine (BA), thidiazuron (TDZ), kinetin, adenine or zeatin are the most commonly used, providing successful results in terms of multiplication in most plant species, including pistachio (Tilkat et al. 2009), *Clitoria ternatea* (Singh and Tiwari 2010), *Capsicum annuum* (Peddaboina et al. 2006) or *Cassia angustifolia* (Siddique and Anis 2007). At this step, many authors have reported the use of beneficial microorganisms as an effective tool to improve its effectiveness. Normally, these microorganisms are

obtained from contaminated cultures, serving as source of microbe inoculants for other *in vitro* plant species. Zawadzka et al. (2014) isolated three bacterial species (*Paenibacillus glucanolyticus*, *Curtobacterium pusillum* and *Methylobacterium extorquens*) from *Hosta* Tratt. 'Paradigm' or *Rubus idaeus* *in vitro* tissues. The three isolates were subsequently proved for their ability to improve the *in vitro* performance of micropropagated shoots of these two cultures as well as *Rosa* L. 'White Gem' and *Gerbera* *jamesonii*. From those experiments, the authors concluded that the inoculation with *C. pusillum* increased the number of axillary shoots in all four genotypes, being the effect on this parameter dependent on the genotype in the case of the other two bacterial strains *M. extorquens* and *P. glucanolyticus*. In other cases, beneficial microorganisms favouring *in vitro* multiplication are natural colonisers of plants. It has been reported that the presence of endophytic microbial strains colonising the explants promoted a successful *in* *vitro* propagation in different *Prunus avium* genotypes (Quambusch et al. 2014). Apart from *in vitro* multiplication, seed germination and plant regeneration have been benefited by using microorganisms. For instance, the incubation of *Allium sativum* cv *Gigante roxo* meristems with *Enterobacter cloacae* and *Burkholderia cepacia* improved the growth and development of the obtained plants in comparison to those plants which were not inoculated (Costa Júnior et al. 2020). Moreover, Regalado et al. (2018) demonstrated that *Bromus auleticus* seeds infected with the endophyte *Epichloë* spp. improved the *in vitro* seed germination from a 57.6% to a 82%, as well as improving callus induction and plantlet regeneration.

Effects of PGPMs in *in vitro* rooting

The formation and development of adventitious roots is undoubtedly, one of the most challenging steps of *in vitro* micropropagation, especially in woody plant species and recalcitrant genotypes, and it is crucial to ensure plant survival to the acclimatization phase (Quambusch et al. 2016; Wiszniewska et al. 2016; Arab et al. 2018). For that reason, it constitutes the process on which many researchers have focused most of their efforts. For an efficient rooting protocol, two processes clearly differentiated should be considered: root induction and root elongation. For root induction, the most widely extended procedure is the supplementation of the Murashige and Skoog (MS) medium (Murashige and Skoog 1962) with auxins, being indole 3-butyric acid (IBA), indole 3-acetic acid (IAA) and α-naphthaleneacetic acid (NAA) the most commonly used (Patel et al. 2014; Shekhawat et al. 2015; Revathi et al. 2018). Also, the culture of explants in a half-strength MS medium (½MS) represents a useful strategy to induce *in vitro* rooting (Amiri and Elahinia 2011). It has been well documented that the low status of some nutrients, such as phosphate (Pi), triggers adaptative responses to facilitate the acquisition of this nutrient from the culture medium (Forde

2002; Misson et al. 2005). In the last years, the pursuit of new alternatives to the use of synthetic growth regulators are urgently needed in the light of the restrictions imposed by the European Commission concerning the use of chemicals, including auxins, in plant production (Pacholczak et al. 2012; Elmongy et al. 2018). The use of natural rooting stimulators instead exogenous auxins may suppose a significant breakthrough in today's society, leading to agricultural policies with a limited impact in public health as well as the environment (Calvo et al. 2014; Alberton et al. 2020). In this regard, the use of microorganisms with plant growth-promoting ability may represent an interesting approach due to their potential ability in producing hormones (Calvo et al. 2014). 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 and Hasnain 2013; Zamioudis et al. 2013). In other studies using *in vitro* fruit tree plants inoculated with *P. oryzihabitans*, it has been suggested that changes in the content of auxins in the culture medium might be related with a higher number of roots (Cantabella et al. 2022). As a result, root morphological changes induced by the PGPMs-produced IAA lead to an enhancement of nutrient uptake from the soil or root exudation (Spaepen and Vanderleyden 2011; Masciarelli et al. 2013). Different mechanisms by which PGPMs produce auxins have been proposed. Some bacteria including *Azotobacter paspali* promote plant growth by direct production of IAA (Lugtenberg and Kamilova 2009), but, in other microbes, this auxin production is strictly dependent on the tryptophan present in root exudates (Lugtenberg and Kamilova 2009; Spaepen and 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 these 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 *B. subtilis* induced a 30% increase of root weight related to those non-inoculated. In addition, GAs are hormones mainly involved in the extension of stem tissue (Vessey 2003), and huge information about the production of these plant growth regulators by PGPMs is available in scientific literature (Hamayun et al., 2009, 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. At this point, the large number of studies available in the scientific literature provide sufficient evidence to support the use of PGPMs to improve the efficiency of *in vitro* induction and development of adventitious roots. The use of microorganisms in *in vitro* culture to promote rooting has resulted in encouraging results in many plant species such as one belonging to *Helleborus* genus (Orlikowska et al., 2017a), *Photinia* x *fraseri* Dress (Larraburu et al. 2007) or *P. avium* (Quambusch et al. 2016). The latter studies combined the supplementation of the culture medium with hormones and the inoculation with microorganisms. Following a similar approach, Cantabella et al. (2021) demonstrated the effect of three rhizosphere microorganisms to improve the *in vitro* rooting of *Prunus* and *Pyrus* rootstocks. In agriculture, the importance of rootstocks relies on their ability to confer tolerance to edaphic conditions; however, the ability to induce rooting of some rootstocks is limited, seriously affecting plant survival during acclimatization (Webster 1995). These rootstocks are known as hard-to-root (Marks and Simpson 2000). In those cases, new strategies are required to achieve a considerable amount of these rootstocks for the selection processes associated to breeding programs. In this sense, Cantabella et al. (2021) demonstrated that the root induction of the hard-to-root genotype *Pyrus* spp. Py12 with two fungi *Cladosporium ramotenellum* PGP02 and *Phoma* spp. PGP03, as well as 10 µM of IBA, favoured an increase of the *in vitro* rooting percentage from a 56.25 to a 100%. The use of PGPMs for *in vitro* rooting may also minimise the costs of the propagation process, supplying nutrients to plants and allowing to remove compounds from the culture medium. In banana (*Musa* spp.) cultures, the application of a combination of bacterial strains during micropropagation allowed the omission of minerals and salts from the growing media (Kavino and Manoranjitham 2018). In this study, a higher number of roots per shoot was observed. Using a hormone-free medium, potato microplants cultured in combination with a strain of *Ochrobactrum cytisi* displayed a higher number of roots than non-inoculated plantlets (Burygin et al. 2019). 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* rooting response in *Pyrus communis* L. cv Dar Gazi microcuttings than those growing on the medium with synthetic auxins.

Effects of PGPMs in *ex vitro* acclimatization

The adaptation to *ex vitro* conditions also constitutes a determinant step for plant survival, being responsible of important losses of plant material (Chandra et al. 2010). The transference of plants from *in vitro* to greenhouse or field conditions, also known as acclimatization or hardening represents the beginning of the autotrophic life of plants (Dobránszki and Teixeira da Silva 2010). *In vitro* plantlets must challenge

the stressful conditions of the environment after their transference to a new substrate (Hussain et al. 2012). It is well-known that abiotic factors including the humidity, temperature and light, as well as biotic factors including the presence of soil pathogens could negatively affect the success of the acclimatization process (Chandra et al. 2010; Maleki Asayesh et al. 2017; Rajamanickam et al. 2018). To avoid the harmful effects of environmental conditions, and thus ensure a normal plant growth and development, hardening must be carried out in a gradual manner (Hussain et al. 2012). In this case, the 'biohardening' by the inoculation with microorganisms might enhance the adaption of plants to greenhouse or soil conditions due to changes in morphological attributes (Chandra et al. 2010). In this regard, beneficial microorganisms may improve *ex vitro* acclimatization as a consequence of the effects induced during *in vitro* conditions. For instance, Cantabella et al. (2020) demonstrated that the inoculation of nectarine embryos with *Pseudomonas oryzihabitans* PGP01 promoted root development of the subsequent *in vitro* seedlings, leading to a greater survival and growth after 4 weeks in acclimatization in greenhouse tunnels build to gradually lower the air humidity. In other cases, explants inoculation with beneficial microorganisms is not always possible at the micropropagation process, and their application at the acclimatization stage should be considered to ensure the adaptation of *in vitro* plantlets to environmental conditions (Orlikowska et al., 2017b). Biohardening of plants with beneficial microorganisms triggers mechanisms of systemic resistance to help plants to cope with stressful conditions (Harish et al. 2008; Rajamanickam et al. 2018). In this sense, the ability of bacteria belonging to the genus *Bacillus* and *Pseudomonas* to promote *ex vitro* hardening has been studied to a greater extent. For instance, the inoculation of micropropagated banana (*Musa* spp.) plantlets under field conditions with *Bacillus* spp. has led to a greater plant growth and resistance to pathogens (Jaizme-Vega et al. 2004; Suada et al. 2015; Rajamanickam et al. 2018). In the same sense, the presence of *Bacillus* as well as *Pseudomonas* spp. in tea micropropagated plants had a positive impact on their *ex vitro* hardening (Pandey et al., 2000; Thomas et al., 2010). In addition, the effects of these bacteria in acclimatization have been also reported in plant species as the case of the medicinal plant *Picrorhiza kurrooa.* In this plant species, Trivedi & Pandey (2007) concluded that bacterial isolates from *Bacillus* and *Pseudomonas* improved plant growth and survival by the control of pathogenic fungi growth. Although little mentioned, it is also noteworthy to remark the role of AMF on the favourable adaptation of *in vitro* plantlets to soil conditions (Vestberg et al. 2002). For instance, the symbiotic relationship between *in vitro* plants and some AMF in the early acclimatization stages improves acclimatization rates, observing an increase in plant

height, leaf area, and biochemical attributes such as the content of colchicine in the medicinal plant species *Gloriosa superba* (Yadav et al. 2013).

In vitro mechanisms of action of PGPMs

The great versatility of *in vitro* tissue culture also makes this technique appropriate to be used as a model for the study of the different pathways underlaying PGPMs enhancement of plant growth and development. Based on the large number of research addressing the mechanisms of *in vitro* plant growth-promotion, the main functions of PGPMs can be grouped in (1) biofertilizer activity, (2) biological control activity and (3) phytostimulating and abiotic stress mitigating activity (Fig. 3).

In vitro biofertilation by PGPMs

The plant growth promotion by supplying plants with nutrients 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 and Kamilova 2009). Nevertheless, this ability has been also attributed to other bacterial genera (Scherling et al. 2009). It has been reported that sugar cane *in vitro* plantlets inoculated with one strain of *Enterobacter* spp. improved their growth and this was related to the ability of this bacterial strain to fix nitrogen (Sajjad Mirza et al. 2001). In a subsequent work, Oliveira et al. (2002) isolated five endophytic bacterial species that contributed to nitrogen fixation in sugar cane micropropagated plantlets. In *Oryza sativa* L., the bacterial strain *Azospirillum amazonense* promoted plant growth by fixing nitrogen instead of using hormonal mechanisms (Rodrigues et al. 2008). Moreover, many authors have reviewed that AMF also play a role in plant nutrition (Vestberg and Cassells 2009; Vejan et al. 2016). It is documented that these microorganisms may also improve plant growth by solubilisation of other important nutrients such as phosphate (Fig. 3), facilitating its uptake by plant roots and promoting plant growth (Della Monica et al. 2015). Likewise, this phosphate-solubilising activity has also been reported for other non-mycorrhizal fungi such as *Penicillium radicum* (Whitelaw et al. 1999), or some bacterial species including *Pseudomonas rhizosphaerae* (Peix et al. 2003).

In vitro biological control activity by PGPMs

PGPMs can also favour plant growth due to their potential role as biocontrol agents (BCAs) (Fig. 3). It is well established that PGPMs, mainly belonging to *Bacillus* and *Pseudomonas*, are able to compete with other pathogenic microorganisms, supressing their growth (Morales-Cedeño et al. 2021). 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 encapsulation of banana shoots with the strain FP10 of *Pseudomonas aeruginosa* increased plant growth while reducing the vascular discoloration caused by the fungus *Fusarium oxysporum*. In a more recent study, Kavino & Manoranjitham (2018) reported that the bacterization of micropropagated banana shoots 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, the induction of defence-related genes 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).

In this regard, it is also noteworthy to mention that the use of PGPMs to control or even supress the growth of endophytic contaminations in *in vitro* cultures can be also considered a less studied way of biological control. Since many years, it is widely assumed that explants micropropagated *in vitro* develop in a culture medium under aseptic conditions, and the presence of most 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 explants, shoots or plantlets 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 often are released to the culture medium or even grow at the basis of the explants. Petrini (1991) and Wilson (1995) described endophytes as microorganisms with the ability of living within plants throughout the whole, or only a part of their life cycle without triggering disease symptoms. 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 some of these contaminations 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 since they can overrun the plant 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 reported (Khan et al., 2018; Lotfi et al., 2020; Shehata et al., 2010). Nevertheless, the introduction of beneficial microorganisms into *in vitro* culture with the aim to remove the presence of endophytic contaminations has not been much considered. In experiments using the temporary immersion system GreenTray® bioreactor (Dolcet-Sanjuan and Mendoza, 2018 and 2020) the ability of *P. oryzihabitans* PGP01 and *C. ramotenellum* PGP02 to control the growth of endophytic contaminations in *Prunus* Rootpac® 20 shoots IBA-root induced was analysed (Cantabella et al. 2022). In the latter study, *P. oryzihabitans* PGP01 was not able to control endophytes population in RP-20 explants at the pH commonly used for *in vitro* culture. In contrast, in the presence of *C. ramotenellum* PGP02 at the same pH, it was observed that endophytes were not detected after 5 days of co-culture. The results suggested a possible role of the culture medium pH in the reduction of these contaminations. A decrease in the culture medium pH to 3 inhibited endophytes growth, controlling these contaminants populations, without reducing RP-20 multiplication and growth (Cantabella et al. 2021b).

In vitro phytostimulation and abiotic stress alleviation by PGPMs

It is quite interesting to remark the role of some of these beneficial microorganisms as natural phytostimulators, modifying the hormonal balance on *in vitro* cultured plants. In this sense, microbial inoculants are able to alter plant growth and development by the production of plant growth regulators such as auxins, gibberellins (GAs) or CKs (Vessey 2003; Drogue et al. 2012). The role of IAA in plant-microbe interactions has been studied in a greater extent than the other plant growth regulators (Vessey 2003; Calvo et al. 2014). In *in vitro* study of plant-microbe interactions, the ability of several bacterial species to produce auxins has been described (Dias et al. 2009; Burygin et al. 2019; Arkhipova et al. 2020). 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 (Vessey 2003; Calvo et al. 2014).

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 (Saravanakumar and Samiyappan 2007; Arkhipova et al. 2020). Under these conditions,

PGPMs that contain the 1-aminocyclopropane-1-carboxylate (ACC) deaminase can considerably reduce the high ethylene (ET) levels present by metabolising its precursor ACC, 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).

Using the model plant *Arabidopsis thaliana* in germ-free conditions for the study of plant-PGPMs 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 (Shekhar et al. 2019; Sánchez-Serrano and Salinas 2021). 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. 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). 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 PGPM. In the Table 3, the effects of some PGPMs in *A. thaliana* plants cultured in sterile conditions, as well as the associated mechanism of action are summarized. It is pertinent to remark that this section of the review does not deal with the effects of PGPMs on the technique of *in vitro* micropropagation. Instead, significant studies providing evidences about the mechanisms of action of these beneficial microorganisms using *A. thaliana* cultured in aseptic conditions have been compiled. Using 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 hair root formation. Similar results were obtained by Trinh et al. (2018) using the strain IHB 13561 of *Pseudomonas nitroreducens*. Contradictorily, Iqbal & Hasnain (2013) studied the effect of one strain of *Aeromonas punctata* and they concluded that this bacterium increases primary root length as well as lateral root density.

In addition, using this methodology, valuable information regarding the mechanisms underlying the root modifications induced by PGPMs has been obtained. For instance, using *A. thaliana* seedlings in a germ-free environment, the role of *P. nitroreducens* in plant nutrition has been demonstrated by an enhancement of nitrate uptake (Trinh et al. 2018). In other cases, the studies involving *A. thaliana* mutants have suggested the involvement of some molecules or signalling processes in the plant growth-promotion observed in response to PGPMs. These results place plant hormones such auxins, ethylene (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, to 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. In addition, other investigations have used *A. thaliana* mutants' seedlings in sterile conditions to study the cross-talk between hormones and antioxidant metabolism in the presence of PGPMs. In the absence of microorganisms, it has been revealed the importance of the redox control mediated by glutathione in the hormonal-mediated control of lateral root development (Passaia et al. 2014). Likewise, a link between the reduced glutathione (GSH) homeostasis and strigolactones (SLs) has been stablished in the regulation of *A. thaliana* root development (Marquez-Garcia et al. 2014). These hormones are closely interacting with auxins creating a loop in which one hormone regulates the levels of the other (Hayward et al. 2009). Developing deeper, SLs are able to control the lateral root development modulating the auxin flux throughout the plant (Koltai 2011; Ruyter-Spira et al. 2011). Altogether, those interactions lead to a complex network mainly based on a close interaction among auxins, SLs and GSH. This cross-talk between plant hormones and redox processes was also

suggested in the presence of *P. oryzihabitans* PGP01 by Cantabella et al. (unpublished data) using SLs (*max2-3*, *max3-9* and *max4-1*) and GSH (*cad2-1*, *pad2-1* and *rax1-1*) defective mutants.

On the other hand, *A. thaliana* seedlings cultured in germ-free MS medium have 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. In this study, the authors demonstrated a higher germination rate (30.7%) of *A. thaliana* seeds inoculated with *P. putida* PS01 in MS medium with 150 mM of NaCl compared to those non-inoculated seeds (9.5% of germination rate). In the same study, it was also observed that *A. thaliana* seedlings inoculated with this bacterium were able to withstand saline concentrations of up to 225 mM NaCl. This greater tolerance to salt stress was correlated with a higher expression of defence genes involved in the jasmonate biosynthesis (Chu et al. 2019). Other studies have also revealed that the inoculation of *A. thaliana in vitro* seedlings with *A. brasilense* strain Sp 245 retarded the water loss rate in 50 days-old-seedlings, mitigating the possible harmful effects caused by drought stress (Cohen et al. 2015). This assumption was reinforced by the increase in ABA contents observed in the presence of this bacterium.

Conclusions

In the light of all the aforementioned, many evidences have been provided in favour of the introduction of PGPMs in aseptic *in vitro* tissue cultures to improve the performance of *in vitro* cultured plants. 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, showing a significant impact on the multiplication and *in vitro* rooting, as well as the adaption of *in vitro* plantlets to the harmful conditions of greenhouse or field (acclimatization). Altogether, this strategy may lead to more competent plant production protocols that are used for commercial purposes. In addition, the *in vitro* tissue culture techniques have been presented as useful procedures to unravel the different mechanisms used by these beneficial microorganisms to promote plant development. In this regard, it has been widely documented that PGPMs in *in vitro* cultures are able to promote plant growth by acting as biofertilizers, biological control agents or natural producers of phytohormones that enhance plant development and help to mitigate the negative effects of abiotic stresses. Although not strictly *in vitro* micropropagation, it seems pertinent to remark that these strategies of plant growth-promotion have been corroborated in the model plant *A. thaliana* cultured in aseptic conditions. Taken together, the presented

review provides a comprehensive overview about the information available in the field of *in vitro* plant-microbe interactions with the aim to solve the main issues presented in *in vitro* micropropagation. Solving these limitations throughout the use of PGPMs will contribute to a more efficient, as well as more sustainable plant production.

Conflict of interest

No conflict of interests has been declared by authors.

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Contributions

D.C. contributed to the elaboration of the draft of the manuscript, and N.T and R. D. S. contribute to the editing of the draft manuscript to obtain the final version. All authors have read the article and agree to its publication.

Data availability statement

Data sharing not applicable to this article as no datasets were generated or analysed in this review article.

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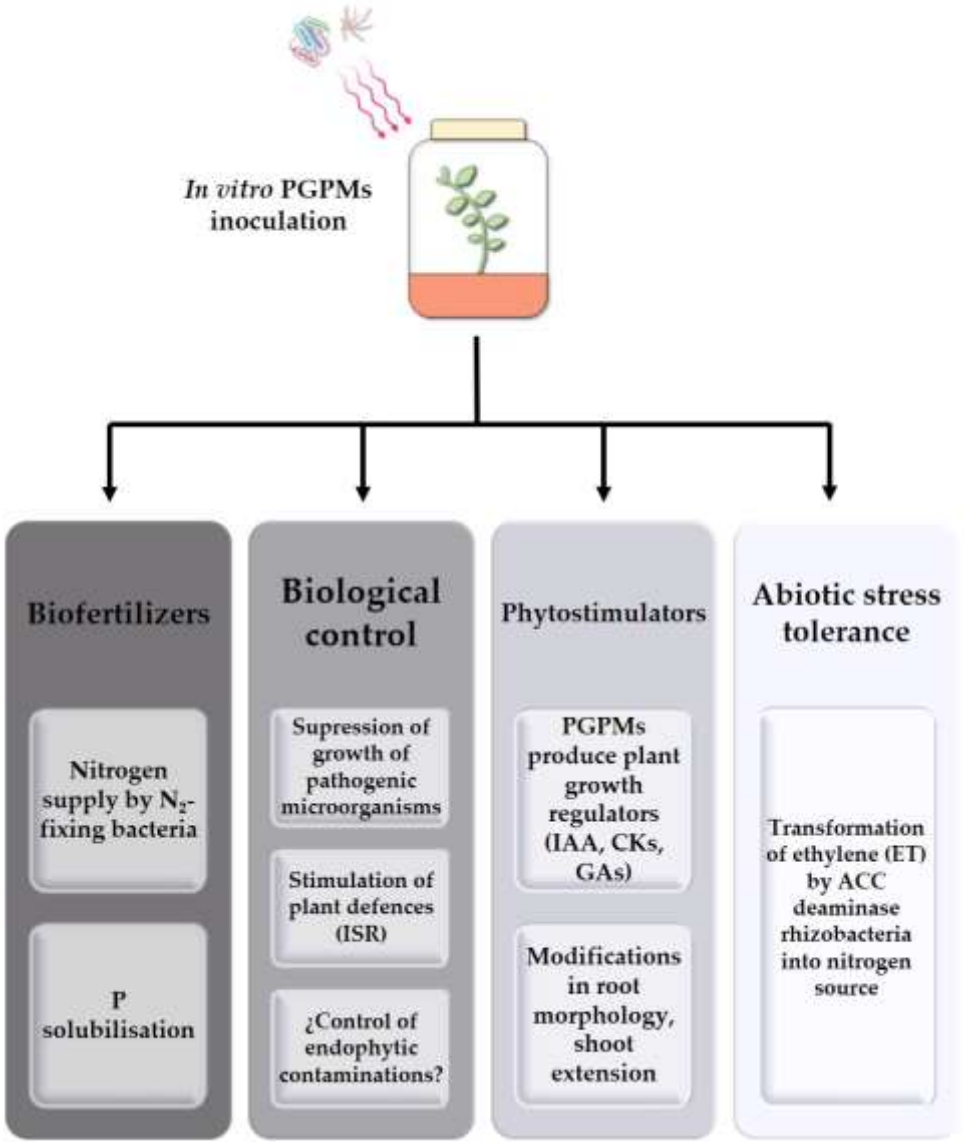

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816 **Figure 1.** Described mechanisms of *in vitro* plant growth promotion induced by PGPMs.

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818 **Table 1.** Overview of different microorganisms improving the efficiency of *in vitro* micropropagation steps and its specific impact.

Microorganism	*In vitro* process	Plant species	Effect	Reference
Paenibacillus glucanolyticus	Multiplication	*Chrysanthemum x grandiflorum* 'Ludo'	Increase of the number and length of axillary shoots	Zawadzka et al. (2014)
Curtobacterium pusillum	Multiplication	*Chrysanthemum x grandiflorum* 'Ludo'; *Gerbera jamesonii* 'Kormoran'; *Hosta* 'Paradigm'; *Rose* 'White Gem'	Stimulation of axillary shoot formation	Zawadzka et al. (2014)
Methylobacterium extorquens	Multiplication	*Gerbera jamesonii* 'Kormoran' *Hosta* 'Paradigm'	Increase of the number and length of shoots	Zawadzka et al. (2014)
Epichloë spp.	*In vitro* seed germination *In vitro* plantlet regeneration	*Bromus auleticus*	Increase of *in vitro* germination percentage Higher percentage of callus induction and plant regeneration	Regalado et al. (2018)
Enterobacter cloacae M19B *Burkhloderia cepacia* CCMA0056	*In vitro* meristems culture	*Allium sativum* cv. "*Gigante Roxo*"	Greater fresh mass and height of the seedlings obtained from inoculated meristems	Costa Júnior et al. (2020)
Microbacterium testaceum *Rhodopseudomonas* spp.	*In vitro* rooting	*Prunus avium* 'Achilleus' *P. avium* 'Fama'	Increase of the rooting percentage and number of roots per shoot	Quambusch et al. (2014) Quambusch et al. (2016)
Burkholderia phytofirmans PsJN	*In vitro* rooting	*Helleborus*	Increase of the *in vitro* rooting percentage and number of roots per shoot	Orlikowska et al. (2017)
Cladosporium ramotenellum PGP02 *Phoma* spp. PGP03	*In vitro* rooting	*Pyrus* spp. selection rootstocks Py12	Increase of the rooting percentage from 56.25 to 100% in combination with 10 µM of IBA	Cantabella et al. (2021)
Azospirilum brasilense strains *Cd* and *Sp*	*In vitro* rooting	*Photinia x fraseri* 'Dress'	Higher root fresh and dry weights and higher root surface area	Larraburu et al. (2007)
Pseudomonas fluorescens Pf1 *Bacillus subtilis* strains 10 and 56	*In vitro* rooting	Banana *(Musa* cv. Red Banana*)*	Increase in the number of roots, root length and root FW and DW	Kavino & Manoranjitham (2018)

Ochrobactrum cytisi IPA7.2	*In vitro* rooting	Potato (*Solanum tuberosum* L.)	Increase of the number of roots in the absence of auxins	Burygin et al. (2019)
Pantoea agglomerans	*In vitro* rooting	*Pyrus communis* L. cv Dar Gazi microcuttings	Higher *in vitro* rooting percentage, number of roots, root length and *ex vitro* survival alone and in combination with IBA	Luziatelli et al. (2020)

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Table 2. Effect of different PGPMs on the acclimatization of *in vitro* culture derived plants to greenhouse or field conditions.

Microorganism	Plant species	Effect	Reference
Pseudomonas oryzihabitans PGP01	Nectarine (*Prunus persica* L. cv. Nectarine) *in vitro* rescued embryos	Higher survival rate and growth after 4 weeks in acclimatization conditions	Cantabella et al. (2020)
P. fluorescens Pf1 and CHA0 *Pseudomonas* spp. EPB22 *Bacillus* spp. EPB5)	Banana plantlets in secondary hardening stage	Reduction of Banana bunchy top disease by an induction of systemic resistance	Harish et al. (2008)
B. subtilis strains PP and CL3	Banana plantlets (*Musa* spp. cv Grand Naine) in primary and secondary hardening stages	Increase of plant height, number of leaves and leaf area Induction of defence enzymes such as peroxidase, polyphenol oxydase, phenylalanine ammonia lyase and pathogenesis-related proteins	Rajamanickam et al. (2018)
B. subtilis *Bacillus* spp. *Pseudomonas corrugata* 1 *Pseudomonas corrugata* 2	Tissue-cultured tea plants	Higher survival rate in greenhouse conditions	Pandey et al. (2000)
Trichoderma harzianum *Azospirillum brasilense* *P. fluorescens*	Hardening tea (*Canellia sinensis*) plantlets	Higher shoot length, number of leaves, number of roots, plant FW, and survival rate in the presence of microorganisms alone or in combination	Thomas et al. (2010)
Rhizophagus intraradices *Funneliformis mossae* *F. geosporum*	Wheat (*Triticum aestivum*)	Alleviation of drought stress	Mathur et al. (2018)
Acaulospora laevis	*Gloriosa superba* L.	Increase of plant height, leaf number and tuber length	Yadav et al. (2013)

840 **Table 3.** Effects of PGPMs on *A. thaliana* plants in germ-free experiments and associated plant growth-promotion mechanisms.

Microorganism	Effect on *A. thaliana* plants	Proposed mechanism of action	Reference
Pseudomonas fluorescens	Inhibition of primary root growth Stimulation of lateral roots and hairy root formation	Changes in root plasticity induced by ethylene, jasmonic acid and auxins IAA signalling induced by cyclodipeptides	Zamioudis et al. (2013); Ortiz-Castro et al. (2020)
Aeromonas punctata PNS-1	Increase in primary root length and lateral root density	Auxins and ACC deaminase production	Iqbal & Hasnain (2013)
Pseudomonas nitroreducens	Inhibition of primary root growth Increase in the number of lateral roots	Increase in nitrate uptake by a higher expression of the nitrate transport gene *NRT2.1*	Trinh et al. (2018)
Phyllobacterium brassicacearum STM196	Lateral root growth promotion	IAA transport and signalling	Contesto et al., (2010)
Pseudomonas putida	Inhibition of primary root growth Stimulation of lateral roots and hairy root formation	IAA signalling induced by cyclodipeptides	Ortiz-Castro et al. (2020)
Bacillus megaterium	Inhibition of primary root growth Increase in lateral root number, growth and root hair length	Root architecture alterations by auxins and ethylene-independent signals	López-Bucio et al. (2007)
Pseudomonas oryzihabitans PGP01	Inhibition of primary root length Promotion of lateral root development (increase in number of lateral roots and lateral root density)	Cross-talk among auxins, strigolactones and glutathione	Cantabella et al., unpublished data
P. putida PS01	Greater seeds germination rate under 150 mM NaCl Higher seedling survival rate under salt up to 225 mM NaCl	Alleviation of salt stress by up-regulation of defence genes	Chu et al. (2019)
A. brasilensis Sp25	Higher main root length Higher content of ABA in rossettes	Mitigation of drought stress by a reduced water loss and increased ABA contents	Cohen et al. (2015)

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