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Enhancement of adventitious root differentiation and growth of *in vitro* grapevine shoots inoculated with plant growth promoting rhizobacteria

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Summary

The effect of *Burkholderia* spp. strain IF25 on adventitious rooting was evaluated in micropropagated grapevine explants. Data on rooting time, stem length, number of stem nodes, basal callus development, number of roots and root length per rooted microcutting were detected at 6, 8, 10, 13, 19, 26 and 30 days after bacterial inoculation. Results suggest that bacterization of *in vitro* grapevine explants by strain IF25 affected root differentiation, as the earliest rooting occurred in inoculated shoots, whether or not exogenous IBA had been applied, and increased the average number of roots per explant.

K e y w o r d s : *Vitis vinifera*; micropropagation; rooting; biotization; *Burkholderia* spp.

Introduction

Free-living soil bacteria that demonstrated effects on plant growth promotion, rhizogenesis and response to parasite challenge are generally referred to plant growth promoting rhizobacteria (PGPR) (Kloepper *et al.* 1989). Different soil bacterial genera among which *Azotobacter, Azospirillum*, *Acetobacter, Burkholderia* and *Pseudomonas* include PGPR that can be considered as non-phytopathogenic facultative bacterial endophytes which often promote adaptation of plant to stress factors (Glick 1995, Hardoim *et al*. 2008, Rea *et al.* 2013). They are often found in the rhizosphere and can perform their action by the epiphytic or endophytic colonization of root and shoot tissues (Compant *et al*. 2005a, Hardoim *et al*. 2008).

The metabolic response of *in vitro* explants to microbial inoculant leading to developmental and physiological changes in derived plants is known as "biotization" (Nowak 1998). Co-cultivation of *in vitro* disease-free plants of *Vitis vinifera* with PGPR increased plant resistance to biotic and abiotic stress (Ait Barka *et al*. 2000, Ait Barka *et al.* 2006, Fernandez *et al.* 2012). Different studies demonstrated the involvement of PGPR in the production of plant growth regulators (PGRs), with a resulting modification of the hormonal balance in host plant (Goto 1990, Glick 1995). Most of the attention had been focused on the synthesis of phythormone indole-3-acetic acid (IAA) and others auxin-like compounds that are involved in the regulation of plant cell division, root differentiation and growth (Altamura 1996, Miransari 2011). PGPR also regulate the uptake of polyamines such as putrescine (Compant *et al.* 2005a), which can be considered as a marker of *in vitro* adventitious rooting of grapevine explants (Neves *et al.* 2002) and other fruit species (Muganu and Rugini 1994, Rugini *et al.* 1997).

Considering the great potential that the use of beneficial bacteria could have both in improving *in vitro* culture and in field plant adaptation, the aim of the present experiment was to evaluate the effects of *Burkholderia* sp. strain IF25 on adventitious root differentiation and on shoot and root growth of *in vitro* grapevine micro-cuttings.

Material and Methods

In vitro propagation was applied to Malvasia bianca lunga (*Vitis vinifera* L.) nodal buds collected from disease-free plants grown in the vineyard of the University of Tuscia (42°25′21″N-12°04′45″E). Micropropagated shoots were obtained in glass test tubes filled with 8 mL of agarized Murashige and Skoog medium + 1 mg·L^{-1} benzylaminopurine, under 40 µmol·m^{-2}·sec^{-1} white fluorescent light, at 24 °C ± 1 and 16-h photoperiod (Muganu *et al.* 2007). Bacterial inoculum was produced by growing *Burkholderia* spp. strain IF25 at 30 °C in 250 mL Erlenmeyer flasks containing 100 mL King's B liquid medium, according to Compant *et al.* (2005b).The bacterial strain, previously isolated from vineyard soil and assigned to *Burkholderia* spp. (Balestra *et al.* 2005), demonstrated multiple PGPR traits (*i.e.* biostimulation, P solubilization, iron-chelating agents and IAA production) (Di Mattia and Buliskeria 2011, Rea *et al.* 2013). Inoculation of micropropagated explants was performed by dipping the basis of bi-nodal shoots on 50 µL of the bacterial inoculum. Non inoculated shoots were produced as controls by dipping explants in King's B medium. Both inoculated and non inoculated shoots were subsequently transferred in glass culture tubes containing two different agarized rooting media: MS0 (½ Murashige and Skoog basal salts supplemented with 30 g·L^{-1} sucrose, and adjusted to pH 5.8) and MSH (MS0 + 0.2 mg·L^{-1} indole-3-butyric acid (IBA). Twenty-five shoots were used per each treatment and placed in the same environmental condition as described above. During *in vitro* growth data on rooting time and rooting percentage, stem length, number of stem nodes, basal callus development, number of roots and root length per rooted microcutting were detected at 6, 8, 10, 13, 19, 26 and 30 d post inoculation (dpi). Rooted shoot

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was considered to have developed an adventitious root if a whitish cylindrical structure of at least 1.0 mm in length was visible.

Statistical analysis: Data were statistically analyzed by ANOVA and means compared using Tukey's multiple range test ($p \leq 0,05$). A two-factor (± IBA and ± bacterial inoculation) full factorial design was used in the experiment. The dynamics of rooting from the beginning to the end of the experiment was expressed by interpolating the data with a negative exponential function by means of the Systat Nonlin procedure (Systat, Version 10, SPSS. Chicago, IL): RSP = RSPA*(1-exp(-RRSP*(T-TT))) where: RSP = rooted shoots percentage (%); RSPA = asymptotic value of rooted shoot percentage; RRSP = rate of the exponential function (%·d^{-1}); T = Time (d); TT =Threshold value (day) for the beginning of root emission.

Results and Discussion

The effect of shoot inoculation by *Burkholderia* spp. strain IF25 and of exogenous IBA on adventitious rooting percentage during *in vitro* growth is shown in Fig. 1, Tab. 1 and Fig. 2. Results suggest that the inoculation of *in vitro* grapevine explants affected root differentiation, as the earliest rooting occurred in inoculated shoots, whether or not exogenous IBA had been applied. At 8 dpi the rooting percentage of inoculated MS0 and MSH explants raised to 31 and 30 %, respectively, compared to 0 and 11 % rooting percentage of *in vitro* shoots detected at the same time, respectively, in non inoculated MS0 and MSH. The faster start of rooting in inoculated shoots is highlighted by the threshold values estimated by the interpolation of the experimental data (Tab. 1). The lowest threshold value for the beginning of root growth was about five days after transfer to the rooting media. A five-day advance was observed on inoculated shoots with substrate not supplemented with IBA, compared to the non inoculated shoots on the same substrate, which were those with slowest onset of rooting. A lower difference was observed between inoculated and non inoculated shoots grown on MSH media. Subsequently at 10, 13 and 19 dpi the rooting percentage of inoculated and non inoculated MSH shoots increased at higher rate compared to inoculated MS0, showing the positive role of applied concentration of IBA on adventitious rooting (Tab. 1). At the end of the experiment the highest rooting percentage was detected in non inoculated MSH (100 %) and the lowest in non inoculated MS0 (40 %), and bacterized shoots grown on both substrates MS0 and MSH showed similar rooting percentages (87 and 88 % respectively). Similar were the asymptotic values of rooting percentage identified by the exponential function (Tab. 1).

Table 1

Calculated parameters of the function RSP = RSPA*(1-exp(-RRSP*(T-TT))) where: RSP = RSPA (1-exp(-RRSP (T-TT); RSP = rooted shoots percentage (%); RSPA = asymptotic value of rooted shoot percentage; RRSP = rate of the exponential function (% d^{-1}); T = Time (d); TT = threshold value (d) for the beginning of root emission

Thesis	RSPA (%)	RRSP (% d^{-1})	TT (d)	R^2
MS0 control	40.0	0.46	10.0	1.0
MS0 inoculated	88.4	0.17	5.2	0.99
MSH control	97.8	0.69	7.8	0.99
MSH inoculated	86.0	0.57	7.2	0.99

Results detected on stem length, number of stem nodes, number of roots and root length per rooted microcutting and basal callus development in the middle (13 dpi) and at the end of the experiment (30 dpi) are shown in Tabs 2 and 3, respectively. At 13 dpi the highest number of adventitious roots was detected on inoculated MSH samples, and

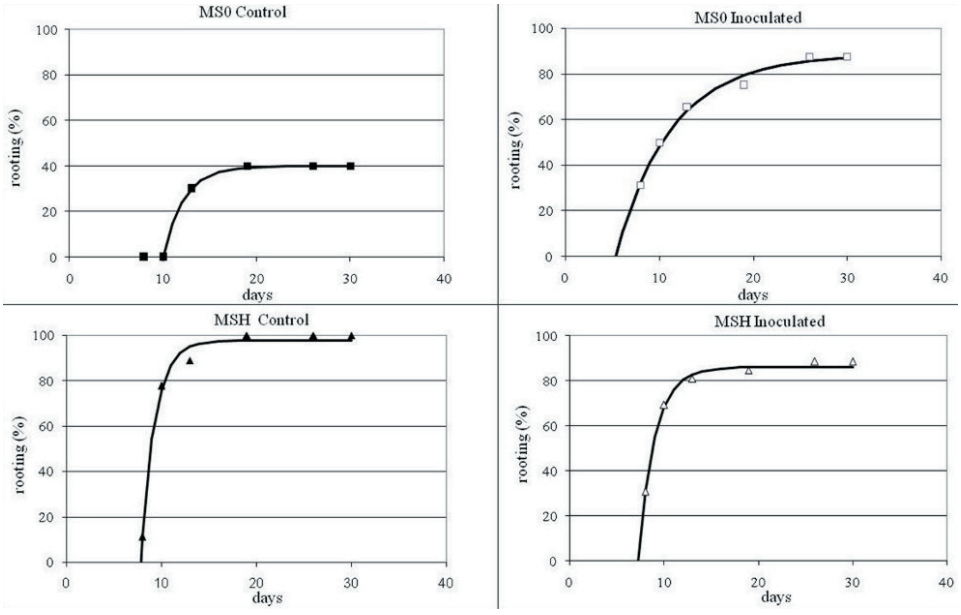

Fig. 1: Rooting percentage as a function of time after transferring inoculated and not inoculated shoots in the different rooting media (MS0: without IBA; MSH with IBA)

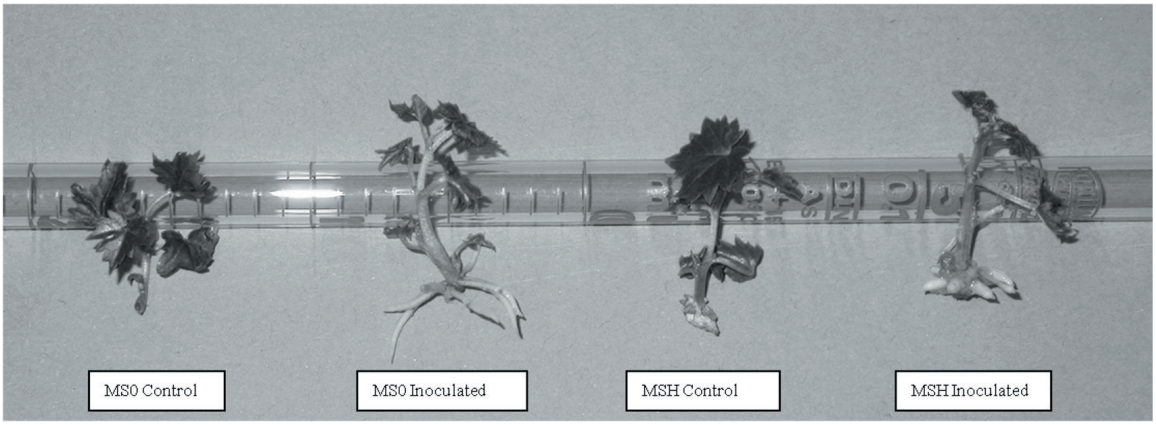

Fig. 2: Development of adventitious roots detected at 13 dpi on *in vitro* grapevine explants grown on inoculated and non inoculated growing media.

Table 2

Average values of shoot and root growth detected in the different rooting media at 13 dpi a)*, **, ***, and ns indicate significance at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, and not significant, respectively, at analysis of variance

Treatment	Stem length (cm)	Stem nodes (n)	Root (n)	Root length (mm)	Size of basal callus
MS0 control	1.8 ab	4.2	0.8 c	2.1 b	nd
MS0 inoculated	1.9 ab	4.6	3.8 bc	7.1 a	nd
MSH control	2.3 a	4.7	8.3 ab	3.0 b	+
MSH inoculated	1.7 b	4.8	11.5 a	3.3 b	+
Factors (signf)[a]					
B. inoculum	ns	ns	*	**	
IBA	ns	ns	***	***	
B. inoculum x IBA	*	ns	*	*	

Table 3

Average values of shoot and root growth detected in the different rooting media at the end of the experiment a)*, ***, and ns indicate significance at $p \leq 0.05$, $p \leq 0.001$, and not significant, respectively, at analysis of variance

Treatment	Stem length (cm)	Stem nodes (n)	Root (n)	Root length (mm)	Size of basal callus
MS0 control	1.8	4.8	3.2 c	24.3	-
MS0 inoculated	2.3	5.2	5.2 bc	20.9	-/+
MSH control	2.8	6.0	10.7 ab	23.3	+
MSH inoculated	2.2	4.7	12.9 a	17.9	++
Factors (signf)[a]					
B. inoculum	ns	ns	*	ns	
IBA	ns	ns	***	ns	
B. inoculum x IBA	ns	ns	ns	ns	

bacterized MS0 shoots did not show significant differences in the number of differentiated roots compared to non inoculated MSH. At the same date significant higher values of root length were detected on inoculated MS0 shoots, compared to other treatments (Tab. 2). At the end of the experiment any significant difference was detected in the values of stem length, the number of stem nodes and root length among different samples. The influence of strain IF25 on root differentiation was confirmed as the number of roots was positively affected by bacterization whether or not exogenous IBA had been applied. No significant interaction between the factors was found and the largest development of basal callus at the end of the experiment was detected in inoculated MSH (Tab. 3).

Different studies, carried out by using *in vivo* and *in vitro* models, showed the effect of PGPR on adventitious rooting supposing the involvement of microorganisms on phytohormone synthesis or induced biochemical changes on plants (Ait Barka *et al.* 2000, Ercisli *et al.* 2003, Thomas 2004, Larraburu *et al.* 2007). Many PGPR

strains have been shown to produce auxin (Arshad and Frankenberger 1998, Barazani and Friedman 1999, Salomon *et al.* 2013), or were linked with the biosynthesis of the phytohormone ethylene (Compant *et al.* 2005a), which regulates auxin transport in adventitious root formation (Negi 2010). The mechanisms that are at the base of strain IF25 involvement on root formation has not been studied in the present experiment, anyway our current results on timing and percentage of root formation of *in vitro* shoots suggest the involvement of strain IF25 in the modulation of auxin biosynthesis and levels during adventitious rooting. This could be asserted considering that bacterial inoculation promoted earlier rooting and higher number of differentiated roots compared with non inoculated MS0. Furthermore strain IF25 induced advanced rooting and high rooting percentage independently of exogenous IBA use, as bacterized MS0 and MSH showed similar rooting time and percentage. These results could be considered in the propagation techniques aimed at the reduction of the use of chemicals. The highest final percentage of rooting was obtained in non inoculated MSH that on the other hand showed slower increase of rooting percentage during the initial rooting period compared to bacterized MSH. These differences could be referred to a possible limiting effect of high concentration of both synthetic and bacterial-derived auxins supply in inoculated MSH at the beginning of the rooting process compared to non inoculated MSH. The high development of the basal callus detected in inoculated MSH can support this hypothesis, considering that callus induction had been related to plant hormonal balance to auxin level (Besse *et al.* 1992, Michalczuk *et al.* 1992).

Conclusions

Nowdays the enhancement of vegetative propagation techniques of woody plants still remain a challenge to overcome both phytosanitary and economic problems. Adventitious rooting is a complex process in which root induction and root differentiation phases are affected by multiple factors including phytohormones, phenolic compounds, tissue nutritional status and genetic characteristics of the vine. The present paper shows the potential of *Burkholderia* spp. strain IF25 in promoting adventitious rooting which is an important goal in both *in vitro* and *in vivo* nursery plant production. In the light of climate change and global warming grapevine bacterization could be usefully applied to enhance the rooting potential of hard-to-root rootstock or cultivars both for grafted or own-rooted vineyards and to increase the vines resistance to abiotic stress by promoting root growth under drought, salinity and nutrient deficiency conditions. Future researches will have to clarify the mechanisms of bacterial influence on plant physiology and on the regulation of root morphology, with derived practical effects on the improvement of water and mineral uptake and on sustainable vineyard management.

Finally bacterization could improve organic propagation techniques that at present are greatly hampered by the necessity of synthetic phytohormones.

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