Use of beneficial bacteria *Azospirillum brasilense* Sp245 on grapevine rootstocks grafted with cv. Sangiovese

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Abstract

***Azospirillum brasilense* Sp245, a well-known PGPR (plant growth-promoting rhizobacteria), was examined in order to evaluate the effects on nursery propagation. In addition the aim was to test the ability of the inoculated bacterium to improve the rooting parameters of some rootstocks that do not easily root through conventional techniques. Nine rootstocks were tested in a conventional nursery, while two rootstocks were tested in organic nursery: *V. berlandieri* x *V. riparia* 420A, 161-49, 157-11, SO4; *V. berlandieri* x *V. rupestris* 140Ru, 775P, 1103P; *V. riparia* x *V. rupestris* 101-14, 3309C. The quality of the root system was improved in terms of the number of roots, root architecture and the total biomass of vines produced in the organic nursery, while in the conventional nursery the results depended on the rootstock. The results suggest that further study is needed for a better comprehension of the mode of action and to establish how PGPR could be used for the sustainable production of grapevine plants.**

INTRODUCTION

The use of beneficial rhizobacteria can establish plant-microbe interactions that have a positive effect on plant growth and health (Okon and Vanderleyden, 1997; Rosenblueth and Martinez-Romero, 2006). Recently the use of bacteria isolated from the roots and rhizosphere of *Vitis vinifera* was proposed in order to study the mechanisms involved in the plant–PGPR relationship (Salomon et al., 2014). The rooting formation of grafted cuttings is often a critical phase, due to the low rooting ability of some rootstocks, and the low root quality of vines may lead to serious economic losses. *Azospirillum brasilense* produces phytohormones and molecules with an antimicrobial activity (Compant et al., 2005; Somers et al., 2005). In addition *Azospirillum brasilense* strain Sp245 has shown a specific effect on woody plant propagation (Vettori et al., 2010) and root architecture (Molina-Favero et al., 2008). In order to improve the performance of grapevine propagation, *Azospirillum brasilense* Sp245 was tested on *Vitis vinifera* cv. 'Sangiovese' grafted on various rootstock cuttings.

MATERIALS AND METHODS

Bacterial cells of *A. brasilense* Sp245 cultured in a liquid medium (Russo et al., 2008) and suspended in water were inoculated (10^7 CFU/mL) at different stages of the vine propagation in a conventional nursery and in an organic nursery located in Pisa (Italy). The trials were carried out during the scheduled daily work of the host nurseries, who also provided the plant material .

Conventional nursery. The following clonal rootstocks were tested: *V. berlandieri* x *V. riparia* 420A (MIQ88), 161-49 (176F), 157-11 (ISV1), SO4 (31OP); *V.*

berlandieri x *V. rupestris*, 140Ru (101F), 775P (CFC83/20), 1103P (ISV1); and *V. riparia* x *V. rupestris* 101-14 (ISV1), 3309C (143F). The bacterial treatments were carried out at two different times: inoculation of cuttings during the hydration step, before bench-grafting (**A**); inoculation of bench-grafted cuttings during hydration, before field planting after a period of forcing for 15 days at 25°C, high relative humidity, and in the dark (**B**).

Organic nursery. Two clonal rootstocks were tested: *V. berlandieri* x *V. rupestris* 1103P (ISV1) and 775P (CFC83/20). The bacterial treatment was carried out on the grafted cuttings (before forcing) at the beginning of the callus formation period (15 days at 25°C, high humidity rate, and in the dark) just after the bench-grafting (**C**).

The scion used in both nurseries was 'Sangiovese' (clone SS-F9A548), produced under conventional and organic methods, respectively. Bench-grafted cuttings were hydrated for about 5 days and subsequently planted in the field in late spring. The shoots were trimmed mechanically three times during the season to stimulate roots. Rooted vines were harvested in winter.

The following parameters in both nurseries were considered: callus diameter at the graft level in treatment **A** in the conventional nursery, and in the organic nursery grafted cutting forcing was evaluated at the end of the 15 days. The number of nodes per vine was calculated during summer growth in the field (before the scheduled apical shoot cutting), in order to assess the vigor of the growing vines. Percentages of grafted cuttings produced were calculated considering the number of vines with vital shoots.
The number of adventitious roots, percentage of asymmetric roots and total biomass were evaluated after the harvesting of vines and prior to packaging**.** The percentage of asymmetric roots was determined considering those plants that did not have any opposite primary roots as asymmetric.

The experiment design was a randomized block with four replications. A total of 160 grafted cuttings were used for each treatment. The observations on roots and plants were performed on 12 vines per replication. Trials were carried out over three years. Statistical analysis was performed by ANOVA with previous transformation in arcsin of percentage data.

RESULTS AND DISCUSSION

Azospirillum brasilense Sp245 was studied in order to evaluate the effects on nursery propagation, and especially to test the ability of the inoculated bacterium to improve the rooting parameters of some rootstocks that do not easily root by means of conventional techniques in organic nurseries in order to enhance plant propagation.

Conventional nursery

There was a significant increase in the **callus diameter** in the inoculated grafted cuttings of five rootstocks of the nine tested that had received the treatment with *Azospirullum* before bench grafting (treatment **A**). The number of buds recorded in the summer before apical trimming however had slightly increased, although it was significantly enhanced only in 775P (treatment **A**) and in 140Ru, treatment **B** (Table 1).

Callus formation, due to the proliferation of the secondary meristem at the extreme parts of the cuttings follows a polarity depending on the genotype: in *V. vinifera*, a callus is not produced in the apical part; in *V. riparia* and *V. berlandieri* a hybrid callus develops on both extremities, while on *V. rupestris* it is formed more on the upper extremity (Galet, 1993). Different extents of callus formation of several Vitis genotypes have also been reported by Fallot (1964) and Bouard (1963), who during winter

dormancy found that callusing at the base of grapevine cuttings develops slowly or may be completely inhibited, with the exception of *V. rupestris* Du Lot where tissue proliferation was promoted by the presence of *Bacillus megatherium* (Fallot, 1964). Callus formation is also related to environmental conditions such as temperature, humidity and oxygen (Galet, 1993). These can have favorable effects on graft union and protect the rooted cutting during establishment, although callusing takes place before the emission of the adventitious root primordia (Favre and Medard, 1969; Favre, 1973) and is not necessarily directly correlated to rooting.

When a callus produced by control cuttings was pooled by the genetic parentage of hybrids, it was found that *V. riparia* x *rupestris* had a smaller diameter (avg 14.9 cm) than that of *V. berlandieri* x *rupestris* (avg 15.8 cm) and V. *berlandieri* x *riparia* hybrids (avg 16.8 cm). In addition, treatment with *Azospirillum* had a slight beneficial effect (Tab. 1) which was more pronounced in *V. berlandieri x viparia* hybrids (+ 15%).

The percentage of plants produced regarding the bench grafts in the nursery was significantly enhanced by *Azospirillum* treatments on the rootstocks 161-49, 775P (treatment **A**) and 140RU (treatment **B**), indicating variable responses depending on the rootstock and the timing of application (Tables 2 and 3). The *Azospirillum* treatment applied before forcing (**A**) was more effective in increasing the percentage of grafted plants produced in the nursery than the later application (**B**) carried out after the bench graft forcing period.

The percentage of plant produced may depend firstly on a primary effect of root differentiation and graft union during forcing, and secondly on the process taking place in the field. Here soil and aerial environmental conditions affected the synchronization between bud swelling and shoot growth and the root emission that ended with the plant establishment. It is also worth noting that in May and June, when the grafted cuttings were transplanted in the field, there were unusually low temperatures which probably had a negative effect on the time of field rooting. This could result in a random failure of grafted cuttings, especially on rootstocks that may have fewer reservoirs in the wood. Although the diameter of cuttings used was similar, the starch and the nutritional status of the hardwood cuttings (not monitored)which are very important for rooting and plant establishment (Martin and Georgescu, 1968; Galet, 1993; Bartolini et al., 1996).

A more symmetric root system was observed on 157-11 (treatment **A**), while in the other rootstocks and with treatment **B**, the effect of *Azospirillum* on root symmetry was not significant, although the values were sometimes slightly higher or lower than the control (Tabb. 4-5). When the data of root symmetry were pooled according to the rootstock genetic origin, a positive trend effect of *Azospirillum* treatment was observed.

Total biomass per plant was affected differently by the *Azospirillum* treatment **A**, as there was a decrease in weight in the grafted rootstock 161-49**,** but an increase in the SO4 and 420A rootstocks (Tab. 4). In treatment **B**, there was a significant increase in phytomass in 775P (Fig. 1), 101-14 and 1103P (Table 5).

The enhancement of phytomass, has also been observed in other research (Sabir et al., 2012), in our case it did not depend on the average number of primary roots (Tables 2-3) but on the further development of the root system and partially on the shoot growth (data not shown). The variability of responses to *Azospirillum* treatments on different rootstocks, despite a few exceptions, is difficult to explain unless various old hypotheses are valid that deal with physiological bases of rooting (Hess, 1965). Adventitious root initiation requires several conditions : a phenolic cofactor from the bud is translocated through the floem to the base of the cutting to produce, in the presence of sugars and

auxin, the auxin-phenolic complex which is able to stimulate the root differentiation (Haissig, 1992; Galet, 1993). It is possible that the rooting process may be conditioned by the genetic origin of the plant (Haissig and Riemenschneider, 1988) in terms of hormones and cofactors (Bartolini et al. 1986), nutritional status of the cuttings (Bartolini et al., 1996; Kozlsosky, 1992), environmental conditions (Levitt, 1980; Kurkela et al., 1988; Moe and Andersen, 1988; Pearce et al., 1990), state of bud dormancy at the moment of propagation (Basso and Natali, 1975), starch deposit and sugar mobilization (Martin and Georgescu, 1968; Del Canizo, 1978; Bartolini et al., 1996), and by the presence of rooting inhibitor substances on the cuttings (Spiegel, 1954; Bartolini et al., 1991).

Organic nursery

Grafted cuttings of 1103P with 'Sangiovese' treated with *Azospirillum* (treatment **C**) had a favorable effect on root number, phytomass, percentage of plants produced and percentage of symmetric roots, while on rootstock 775P, *Azospirillum* treatment **C** enhanced the percentage of symmetric roots and total biomass per vine (Table 6).

Treatment with *A. brasilense* Sp245 had a variable effect depending on the different rootstocks. In the conventional nursery, there were few significant differential effects between rootstocks, irrespectively of the timing of application. These trials showed that in several cases *Azospirillum* treatments improved the nursery propagation of the grapevine. However this effect can be variable, even though environmental conditions, plant status and genetic diversity may affect the plant response. This makes it difficult to understand the real effect of *Azospirillum* on grapevine. It is known that *Azospirillum* is capable of colonizing the rhizosphere on the root surface and to a lesser extent in the intercellular spaces (Russo et al., 2005). A PGPR (plant growth promoting rhizobacteria) has also been considered because it can produce indole-3-acetic acid (IAA) coded by the gene *ipdC* (Ona et al., 2005). This can have a positive effect on rooting although the influence of cofactors and other cutting conditions depending on the mother plants, may influence the entire rooting process (Zucconi, 1978; Bartolini et al., 1996; Hess, 2000).

In our trials the main focus was to produce better quality grafted vines and not only to increase the rooting percentage. The screening of several rootstocks with a different genetic origin may only partly explain the different responses depending on the specific compositional and features of their tissue, which sometimes can be very positive or very light, or negative (though only in a few cases). The stimulation of plant root growth could be a first step to trigger a sequence of other physiological phenomena that also involve the plant's aerial parts. According to Bashan and de-Bashan (2010) *Azospirillum*-mediated plant growth promotion on grapevine could involve a combination of various mechanisms.

Interestingly, in the organic nursery when the *Azospirillum* was applied on two rootstock hybrids (775P, 1103P) with the same parents, the positive effect on propagation was more evident and not contradictory. This suggests that the cultivation conditions of the mother plants produced cuttings that are more suitable to favorable interaction with *Azospirillum* mediated rooting and establishment.

CONCLUSIONS

In the conventional nursery, the treatment with *Azospirillum brasilense* Sp245 improved the process of nursery propagation, although not always uniformly, thus enhancing the percentage of plants produced from the rootstocks 161-49, 140Ru, 775P and 1103P. In 775P and 1103P, the favorable effects of treatments were more clearly

observed in the organic nursery. The different responses of the rootstocks to the *Azospirillum* treatments may be partially attributed to the genetic diversity and to the possible differences in nutritional and hormonal status of the cuttings used for propagation.

However such promising effects cannot be generalized, suggesting that further study is needed to clarify the mechanism of action and the long-term effects on vines, in order to establish how *Azospirillum brasilense* Sp245 could be used in the sustainable production of grapevine plants. Molecular approaches could thus help to better understand the *Azospirillum*-grapevine interaction.

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Table 1. **Conventional nursery.** Effect of *Azospirillum* (treatment **A**) on graft callus diameter (mm), recorded after forcing and total node number per vine observed in summer.

Rootstock	Treatment **A**						Treatment **B**		
	Callus diam (mm)			Node number/vine			Node number/vine		
	Contr	Treat	Sig.	Contr	Treat	Sig	Cont	Treat	Sign.
157-11	20.2	16.6	n.s.	40.6	44.2	n.s.	40.6	44.0	n.s.
161-49	13.2	16.6	*	45.8	44.2	n.s.	45.8	43.9	n.s.
140RU	14.2	17.2	**	55.0	64.4	n.s.	55.0	73.2	**
420A	18.2	18.6	n.s.	46.6	35.6	n.s.	46.6	45.0	n.s.
775P	15.8	17.6	**	42.4	64.0	*	42.4	51.0	n.s.
101-14	14.4	14.2	n.s.	47.8	55.0	n.s.	47.8	54.2	n.s.
SO4	15.4	18.2	n.s.	44.0	50.4	n.s.	44.0	49.7	n.s.
1103P	17.4	21.2	*	48.0	58.2	n.s.	48.0	45.6	n.s.
3309C	15.3	16.7	n.s.	41.6	53.0	n.s.	41.6	52.1	n.s.
Average	16.01	17.43	**	45.76	49.89	n.s.	45.76	50.97	n.s.
Berl x riparia	*16.8*	*17.5*	*n.s.*	*44.3*	*43.6*	*n.s.*	*44.3*	*45.7*	*n.s.*
Berl x rupestris	*15.8*	*18. 7*	*	*48.5*	*62.2*	*	*48.5*	*56.6*	*n.s.*
Rip. x rupestris	*14.9*	*15.5*	*n.s*	*44.7*	*54.0*	*n.s*	*44.7*	*53.2*	*n.s*

Comparison between treatments along the line * =P<0,05; * =P<0,01; n.s.= not significant

Figures

Fig. 1. Young plants of 'Sangiovese' grafted on 775P control (left) and treated with *Azospirillum brasilense* Sp245 (right).

Table 2. **Conventional nursery.** Effect of *Azospirillum* (treatment **A)** on percentage of plants produced and number of primary roots. Observations made in winter after young vines were pulled out.

Rootstock	% of plants produced			Number of primary roots		
	Control	Treated	Sign.	Control	Treated	Sign.
157-11	37.5	41.8	n.s.	5.3	4.0	n.s.
161-49	40.6	62.0	**	3.5	3.8	"
140RU	46.2	38.6	n.s.	4.0	4.4	"
420A	54.9	42.0	n.s.	6.4	6.0	"
775P	52.3	73.6	**	4.3	3.8	"
101-14	58.9	68.0	n.s.	5.9	6.9	"
SO4	70.1	76.0	"	4.8	4.1	"
1103P	83.1	89.1	"	4.8	4.9	"
3309C	86.9	89.1	"	6.2	6.9	"
Average	58.93	64.45	"	5.02	4.98	"
Berl x Riparia	*50.8*	*55.4*	"	*5.0*	*4.5*	"
Berl x Rupestris	*60.5*	*67.1*	"	*4.4*	*4.4*	"
Rip. x Rupestris	*72.9*	*78.5*	"	*6.1*	*6.9*	"

Comparison between treatments along the line * =P<0.05; * =P<0.01; n.s.= not significant

Table 3. **Conventional nursery**. Effect of *Azospirillum* (treatment **B**) on percentage of plants produced and number of primary roots. Observations made in winter after vines were pulled out.

Rootstock	Percentage of plants produced			Number of primary roots/vine		
	Control	Treated	Sign.	Control	Treated	Sign.
157-11	37.05	41.2	n.s.	5.3	5.2	n.s.
161-49	40.6	46.6	n.s.	3.5	3.7	"
140RU	46.2	66.0	*	4.0	2.9	"
420A	54.9	54.5	n.s.	6.4	6.6	"
775P	52.3	52.0	"	4.3	4.3	"
101-14	58.9	50.7	"	5.9	7.0	"
SO4	76.4	68.8	"	4.8	4.3	"
1103P	83.1	83.6	"	4.8	5.9	"
3309C	86.9	82.5	"	6.2	6.8	"
Average	59.59	60.66	"	5.02	5.19	"
Berl x Riparia	*52.2*	*52.8*	"	*5.0*	*5.0*	"
Berl x Rupestris	*60.5*	*67.2*	"	*4.4*	*4.4*	"
Rip. x Rupestris	*72.9*	*66.6*	"	*6.1*	*6.9*	"

Comparison between treatments along the line * =P<0.05; * =P<0.01; n.s.= not significant

Table 4. **Conventional nursery.** Effect of *Azospirillum* (treatment **A**) on percentage of symmetric roots and plant biomass. Observations made in winter after vines were pulled out.

	Percentage of symmetric roots			Total biomass per vine (g)		
Rootstock	Control	Treated	Sign.	Control	Treated	Sign.
157-11	86.2	100	*	97.8	91.1	n.s.
161-49	87.5	91.7	n.s.	93.6	79.7	*
140RU	92.5	100	“	90.5	89.9	n.s.
420A	100	90.0	“	77.2	89.7	*
775P	95.0	91.7	“	80.6	77.6	n.s.
101-14	94.4	100	“	74.1	70.5	n.s.
SO4	100	100	“	65.2	76	**
1103P	83.9	94.4	“	68.5	72.9	n.s.
3309C	90.0	95.8	“	54.9	52.6	“
Average	92.17	95.96	“	78.04	77.78	“

Comparison between treatments along the line * =P<0.05; * =P<0.01; n.s.= not significant

Table 5. **Conventional nursery.** Effect of *Azospirillum* (treatment **B**) on percentage of symmetric roots and plant biomass. Observations made in winter after vines were pulled out.

	Percentage of symmetric roots			Total biomass per vine (g)		
Rootstock	Control	Treated	Sign.	Control	Treated	Sign.
157-11	100	95.8	n.s.	97.8	96.6	n.s.
161-49	87.5	94.4	*	93.6	95.3	“
140RU	86.2	80.2	n.s.	90.5	91.1	“
420A	100	100	“	77.2	81.1	“
775P	92.5	95.8	“	80.6	90.6	*
101-14	95.0	100	“	74.1	80.8	*
SO4	83.9	88.9	“	65.2	67.6	n.s.
1103P	94.4	100	“	68.5	77.0	*
3309C	90.0	100	*	54.9	54.2	n.s.
Average	92.17	94.57	n.s.	78.04	81.59	“

Comparison between treatments along the line * =P<0.05; * =P<0.01; n.s.= not significant

Table 6. **Organic Nursery**. Effect of *Azospirillum* (treatment **C**) on ‘Sangiovese’ grafted on 1103P and 775P cuttings. Data taken in winter after pulling out young vines from the nursery.

Rootstock	Parameter	Control	Treated	Signif.
1103P	Percentage of plants produced	72.6	80.2	*
	Number of primary roots	7.7	8.9	**
	Percentage of symmetric roots	74.3	95.7	**
	Total biomass per vine (g)	64.6	74.3	*
775P	Percentage of plants produced	90.8	89.5	n.s.
	Number of primary roots	4.9	5.0	n.s.
	Percentage of symmetric roots	85.7	97.4	**
	Total biomass per vine (g)	61.1	69.9	*

Comparison between treatments along the line * =P<0.05; * =P<0.01; n.s.= not significant