

## Research Article

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# Effectiveness of *Azospirillum brasilense* Sp245 on young plants of *Vitis vinifera* L.

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**Abstract:** Information about the influence of the plant growth promoting rhizobacteria *Azospirillum brasilense* Sp245 on the development of grapevine plants could be promoted to enhance sustainable agricultural practices for globally important fruit crops such as grapes. Thus, this study was initiated to evaluate the potential influence of *A. brasilense* Sp245 on two separate experimental trials, A) potted young grape plants: cv. ‘Colorino’ grafted onto two rootstocks 420A and 157/11 which received a fixed volume of inoculum at different times of vegetative cycle; B) hardwood cuttings from rootstock mother-plants of 420A and 775P inoculated during the phase of hydration, before bench-grafting in a specialized nursery. Overall, our results revealed that *A. brasilense* Sp245 has considerable potential to enhance the root apparatus and vegetative development in grapevines. Potted plants inoculated with *A. brasilense* Sp245 showed an improved development. Repeated supply was revealed to be more effective, inducing a greater accumulation of dry mass. Interesting findings were obtained in nursery experiments resulting from a considerable enhancement of the final quality of roots. The stimulating effect of *A. brasilense* Sp245 on vegetative development of rootstocks and vines may indeed improve sustainability in viticulture, through the promotion of healthy growth and a reduced dependence on fertilizers and other chemicals.

**Keywords:** grapevine, Colorino, rootstock, 420A, 157/11, 775P, PGPR, bacterial inoculums

## 1 Introduction

*Azospirillum* spp. represents one of the best-characterized free-living diazotrophs among plant growth-promoting rhizobacteria (PGPR), able to colonize hundreds of plant species [1]. *Azospirillum* are proteobacteria mainly associated with the rhizosphere of important cereals and other grasses; they stimulate the plant growth through several mechanisms including nitrogen fixation, production of phytohormones and molecules with antimicrobial activity [2]. *Azospirillum brasilense* was additionally shown to induce remarkable changes in root morphology [3]. *A. brasilense* Sp245 was initially isolated from the rhizosphere of wheat (*Triticum aestivum* L.) in Brazil [4] and has been considered a promising strain for cereal inoculation during the 1980s. In recent decades, due to its strong effect as a plant growth promoter and influence on root architecture, *A. brasilense* Sp245 has been tested in several studies aimed at improving growth of woody plants, including the propagation of fruit rootstocks [5,6] and grapevines [7,8]. Grapevine vegetative multiplication is based on rooting of cuttings, and usually performed in nurseries. The rhizogenetic process leads to new adventitious lateral roots, which can regenerate the plant. Since the late 19th century, modern *Vitis vinifera* cultivars have been grafted onto selected rootstocks, which are resistant to insect pathogen, grape phylloxera (*Viteus vitifoliae*). Since then, a wide variety of rootstocks have been selected, obtaining hybrids of American species (*V. berlandieri*, *V. riparia* and *V. rupestris*), to tolerate diverse soil and climate conditions. They are now produced by nurseries worldwide. Some rootstocks, though widely used for their positive characteristics, do not root easily, thus causing significant loss in the production of vines. Additionally, there are numerous other viral, fungal, and insect diseases of grapevine propagation material, which threaten nursery production. Hence, wide application of chemical compounds, such as fertilizers, pesticides and synthetic plant hormones, are being used in conventional nurseries to overcome these events. *Azospirillum* may have a role in this context as producers of plant hormones,

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such as mainly auxins, which are well known to positively influence the rooting process. Therefore, it would likely improve the performance of rootstocks, particularly those which have difficulty rooting. Our understanding of the influence of *A. brasilense* Sp245 on development of grapevine plants is still poor, and should be promoted in order to enhance sustainable agricultural practices on worldwide important fruit species such as grapevines. Thus, this study was conducted to evaluate the potential influence of *Azospirillum brasilense* Sp245 on nursery propagation of rootstocks and during the vegetative development of young grapevine plants.

## 2 Materials and Methods

Two separate experimental trials on (A) young potted grape plants and (B) hardwood cuttings, were performed using bacterial cells of *A. brasilense* Sp245 cultured in liquid Nutrient medium (Oxoid), harvested by centrifugation (10 min at 4300 x g, at 4°C), washed and suspended in equal volumes of 0.9% NaCl solution exactly as described in Russo et al., 2008 [5]. Cell number was assessed by microscopy with Burker counting chambers and verified by plate counts on Nutrient agar medium (Oxoid). A final cell concentration of 10<sup>7</sup> CFU/mL was obtained by dilution in sterile water before use as inoculum.

A) Young grape plants of *Vitis vinifera* L. (cultivar ‘Colorino’) were observed following grafting onto two rootstocks 420A and 157/11 (*V. berlandieri* x *V. riparia*). Before bud swelling (first ten days of April), roots of the grapevine rootstocks were immersed overnight at room temperature in a suspension of *Azospirillum brasilense* Sp245 which was diluted 1:10 in tap water to a final cell concentration of 10<sup>6</sup>. Plants were then transplanted into 25 L pots (33 x 33 cm) containing a sterile 1:1 mixture of peat and inert perlite. Pots were placed outdoor at the Department of Agriculture, Food and Environment (University of Pisa) located in central Italy (Pisa, Tuscany, Lat. 43°43’17” N, Long. 10°51’55” E). To prevent external infection, pots were isolated from the ground with a layer of black plastic sheet. During the vegetative season, 1L of the bacterial suspension diluted 1:10 in tap water to a final cell concentration of 10<sup>6</sup>, was added to the pot mixture surface at different intervals. The inoculated plants were divided into two groups that were treated differently by adding a fixed volume of inoculum. The first group (T1) received only one treatment (end of May), the second group (T2) was treated at regular intervals, three times from the end of May to July. A third group of plants (T3), served as a control, and were not inoculated with *Azospirillum*,

but received the same volume of water. For each grafting combination, 90 plants were used. Of the first batch, 45 plants were used for the first-year observations, and the remaining plants were left over the successive dormancy season for spring observations. These latter plants were pruned at the end of the first vegetative year, leaving 2 buds per spur. A randomized block design (3, with 5 replications) was adopted.

Treatments for adequate development of plants (T1, T2, T3), such as control of weeds, fungicide application and irrigation were also performed. Since bud burst, the shoots were tied and maintained in an upright position, thus ensuring an equal distribution of solar radiation.

From the beginning of bud burst to plant senescence, periodic observations (from 15 to 3-4 days intervals one/twice a week depending on the phenological stage) were performed to track evolution of phenological phases by the BBCH scale [9], shoot number and length measurements, shoot rate growth, and leaf growth rate and number. At the end of active growth phase of the first year, plants were harvested to evaluate the produced biomass which was divided into leaves, shoots and roots for further measurements of fresh and dry weight (held at 65°C until reaching constant weight).

For plants in the second batch, the survival rate was evaluated after bud dormancy overcoming, at the resumption of active growth.

B) Hardwood cuttings (n=100/rootstock) from rootstock mother-plants of 420A (*V. berlandieri* x *V. riparia*) and 775P (*V. berlandieri* x *V. rupestris*) were inoculated during the phase of hydration before bench-grafting in nursery (‘Vivai La Vite’, Cenaia, Pisa-Italy) (Fig. 1). The following parameters were considered: callus diameter at the graft level, at the end of the grafted cutting forcing (15 days); rooting percentage, number of adventitious roots and biomass of the root system, at the end of winter storage. The root biomass was evaluated by fresh and dry weight (held at 65°C until reaching constant weight). Moreover, samples of roots were processed in order to determine the content of: total phenolic compounds in fresh material (0.6 g), according to Ait Barka et al. [10]; content of main mineral macro-elements (N, P, K) and the micro-element Fe in ashes (0.5 g of dried material heated to 600°C for 14 hours), according to Sabir et al. [7]. Chemical analyses were performed at least in triplicate both in control and treated cuttings.

Data, expressed as means, were subjected to statistical analysis by the package GraphPad Prism 5 (GraphPad Software, Inc.). Percentages were converted into angle values by Arcsin transformation. Significant differences between control and inoculated rootstocks in

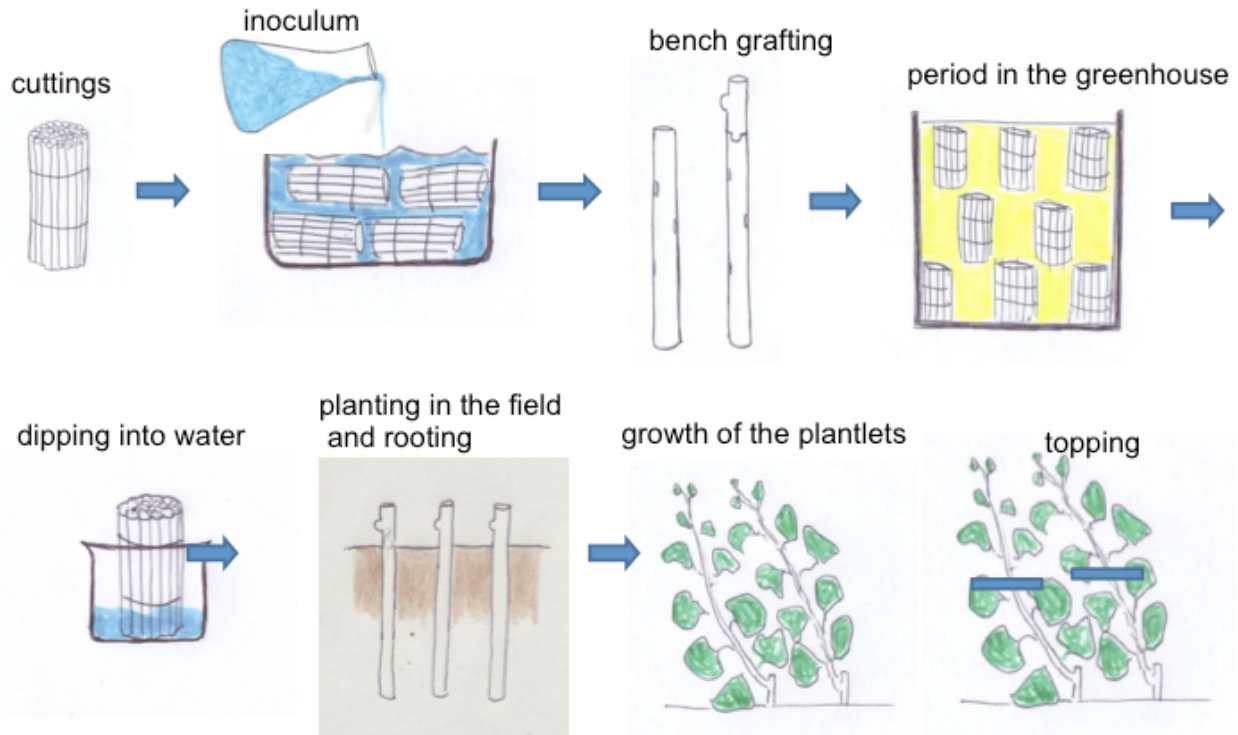


Fig. 1. Phases of grapevine propagation: inoculum with *Azospirillum brasilense* Sp245 cells before grafting (courtesy of Pietro Carrozza).

nursery experiments were tested by Student's *t* test ( $P \leq 0.05$ ). Analysis of variance (ANOVA) was performed and means were compared using the Tukey's test at  $P \leq 0.05$  significance level.

**Ethical approval:** The conducted research is not related to either human or animals use.

### 3 Results

During the vegetative cycle of the young grapevine plants, defined by the interval between onset of budbreak (07 BBCH stage) and leaf falling (91 BBCH stage), observations revealed several notable outcomes.

A positive effect of rhizobacterium on the growth parameters of roots and shoots of 'Colorino' plants was observed. At the end of the vegetative phase (Fig. 2), the plants that were subjected to repeated bacterial suspension inoculation (T2) manifested an improved development relative to control plants. Interestingly, the single treatment group (T1), inoculated at the end of May, produced an effect similar to that obtained by repeated treatments (T2) in the case of 157/11 graft combination.

The effects of treatment with *A. brasilense* Sp245 on vegetative activity of potted grapevines are presented in Table 1. With regard to the leaves, repeated treatments (T2)

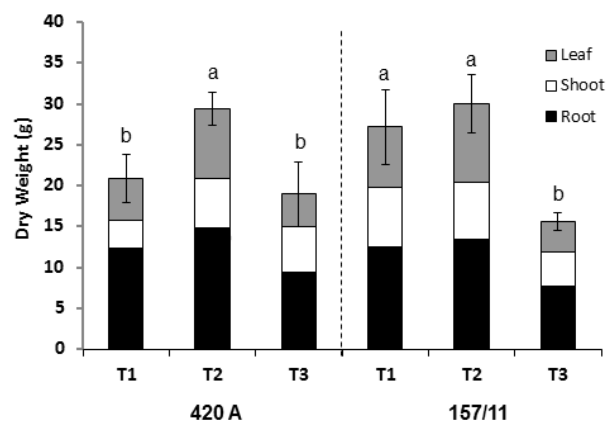


Fig. 2. Cv. 'Colorino' grafted onto 420 A and 157/11 rootstocks. Effect of *Azospirillum brasilense* Sp245 on final biomass (aerial part and root). T1 = one treatment; T2 = repeated treatment; T3 = control. For each rootstock, means ( $\pm$  standard error) with different letters are significantly different ( $P \leq 0.05$ ).

induced a significant increase in numbers relative to the control, in both graft combinations. Differences between T1 and T2 in 157/11 rootstock were not detected.

The number of shoots per plant did not differ, yet the mean length was higher in the combination treatment group with 420A. Specifically, T2 showed a growth increase of about 35%.

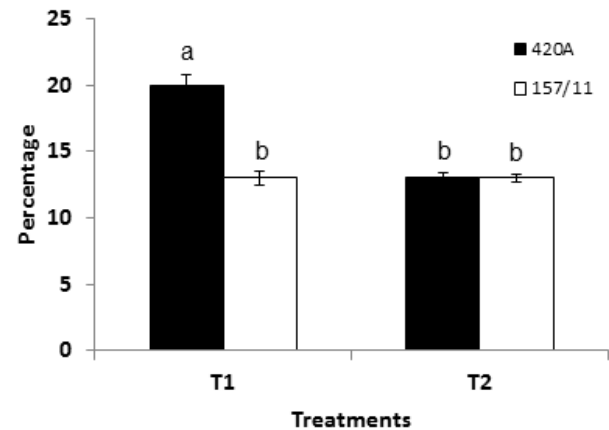
In general, the bacterial treatments induced a significant increase in the growth rate of shoots, with

the exception of T1 in 420A, which did not differ from the control. On the other hand, shoot growth cessation occurred about 70 days after budbreak in both graft combinations (data not shown), without differences between treatments.

As a consequence of the severe water deficit occurring during the summer season, the rate of plant survival at the resumption of growth of the second year (Fig. 3) showed differences between control and treated plants. A positive influence of the *Azospirillum* supply was observed as significant survival percentage increases in comparison to control plants (Table 1), particularly in 420A with one time treatment (T1).

The effects of *A. brasilense* Sp245 obtained in nursery experiments on morphological parameters of rootstocks 420A and 775P are summarized in Table 2. At the end of the grafted cutting forcing, an increase of the callus diameter at the graft junction was recorded in both inoculated rootstocks. However, only in 420A, were significant differences noted relative to the control. Concerning the rooting ability of rootstocks, the control cuttings already showed high percentages (> 90%) that the bacterial treatment did not enhance. On the other hand,

a positive effect with *A. brasilense* Sp245 on other growth parameters such as primary root number and root total biomass, were obtained. Treatment induced significant increases, ranging from about 10 to 36% as compared



**Fig. 3.** Cv. 'Colorino' grafted onto 420A and 157/11 rootstocks. Effect of *Azospirillum brasilense* Sp245 on plant survival. Increase percentage ( $\pm$  standard error) with respect to control after one treatment (T1) and repeated treatment (T2). Different letters indicate significant differences at  $P \leq 0.05$ .

**Table 1.** Cv. 'Colorino' grafted onto 420A and 157/11 rootstocks. Effect of *Azospirillum brasilense* Sp245 on aerial parts of grapevine grafted onto 420A and 157/11. T1 = one treatment; T2 = repeated treatment; T3 = control; p. = plant). For each treatment rootstock ( $n = 15$ ), means ( $\pm$  standard error) with different letters are significantly different ( $P \leq 0.05$ ).

Rootstock/treatment	Leaves/p. (N°)	Shoots/p. (N°)	Shoot length (cm)	Growth rate (cm/day)
<b>420A</b>				
T1	51.7 $\pm$ 2.3 b	7.3 $\pm$ 0.7 ns	29.2 $\pm$ 2.6 b	0.39 $\pm$ 0.01 b
T2	70.5 $\pm$ 1.5 a	7.7 $\pm$ 0.3	39.9 $\pm$ 2.6 a	0.55 $\pm$ 0.03 a
T3	47.0 $\pm$ 6.0 b	9.7 $\pm$ 1.7	29.8 $\pm$ 4.1 b	0.41 $\pm$ 0.01 b
<b>157/11</b>				
T1	76.0 $\pm$ 5.1 a	9.0 $\pm$ 1.1 ns	42.9 $\pm$ 5.4 ns	0.59 $\pm$ 0.04 a
T2	81.3 $\pm$ 2.7 a	8.0 $\pm$ 2.6	39.2 $\pm$ 3.4	0.67 $\pm$ 0.05 a
T3	38.5 $\pm$ 5.5 b	7.7 $\pm$ 2.9	37.9 $\pm$ 5.5	0.53 $\pm$ 0.03 b

**Table 2.** Nursery experiment: effect of *Azospirillum brasilense* Sp 245 on morphological parameters of grafted cuttings of rootstocks 420A and 775P. Within each rootstock, the asterisk indicates significant differences between control and treatment by Student *t*-test ( $P \leq 0.05$ ); ns (not significant). Values are means  $\pm$  standard error ( $n = 50$ ).

	420A		775P	
	Control	Treatment	Control	Treatment
Callus diameter (mm)	17.0 $\pm$ 1.2	19.1* $\pm$ 0.9	17.1 $\pm$ 1.1	17.9 $\pm$ 1.3 ns
Rooting (%)	90.0 $\pm$ 1.5	84.8* $\pm$ 1.8	96.1 $\pm$ 2.5	94.2 $\pm$ 3.1 ns
Primary roots (N°)	6.0 $\pm$ 0.3	6.8 $\pm$ 0.5 ns	11.2 $\pm$ 0.4	17.6* $\pm$ 0.6
Root Biomass (g)	58.0 $\pm$ 3.2	64.3* $\pm$ 2.5	59.3 $\pm$ 4.2	80.7* $\pm$ 6.1

to the uninoculated control cuttings. In particular, the greatest differences were noted in 775P rootstock.

Results concerning the chemical analysis of roots showed that the bacterial inoculation influenced the content of some compounds (Table 3). In both rootstocks, a significant positive effect of *Azospirillum* on total phenols and iron (Fe) concentrations was observed when compared to the control. On the contrary, for the macro-elements nitrogen, phosphorus and potassium (N, P, K), treatments did not improve the contents which remained similar or reduced with respect to controls.

## 4 Discussion

Our results revealed that plants inoculated with *A. brasilense* Sp245 produced more aerial and root mass, also stimulating the growth rate of shoots, similar to earlier reports where positive effects of different bacterial strains on vegetative development were shown [11-13,7]. In particular, in a recent work where *A. brasilense* Sp245 was examined to test its influence on rooting processes of some rootstocks that do not easily root through conventional techniques, the treatment improved propagation and the quality of the root system [8]. However, the effects are not always uniform, suggesting an influence of genetic diversity [14], nutritional and hormonal status of the cuttings [15,16]. Moreover, the interaction of these factors with environmental conditions may be responsible for the response variability to *Azospirillum* treatments [8].

The repeated treatments (T2) showed the greatest improvement in 420A, yet in 157/11 but only a slight influence was observed, possibly indicating a key role of the genotype. Although the considered rootstocks have the same genetic origin (*V. berlandieri* x *V. riparia*), this finding suggests that bacteria would positively stimulate the vegetative development in rootstocks characterized by a more moderate vigour, such as 420A [17]. From a practical

point of view, the favorable effects of treatments could be taken into account once critical agronomical conditions occur, particularly when sustainable practices are adopted, such as organic and biodynamic management. In this context, the results obtained on the capacity of treated plants to survival following a severe summer water deficit seems to be of considerable significance. This finding is in accordance with authors who have claimed that root inoculation with *Azospirillum* sp. is able to improve plant growth under water stress conditions [18-19]. This effect could be related to an increase in root growth which is known to improve root hydraulic conductance [20]. The most important effects of *Azospirillum* on plant growth have been obtained in limiting environments or under sub-optimal conditions. Examples of *Azospirillum*-mitigated environmental stresses include drought, salinity, heavy metal toxicity and extreme pH situation [21].

The nursery technique of asexual propagation of grapevines presents some critical phases of development, mainly related to rooting formation of rootstock cuttings and callus development on the graft wound, which can lead to serious economic loss for farmers. To avoid these difficulties, conventional chemical compounds are usually used. However, in view of more sustainable agricultural practices, alternative compounds are under study including the *A. brasilense* Sp245. Although the influence of this strain on some grapevines has been reported previously [6-8], experimental data need to be extended to verify the potential efficacy on a wide range of genotypes. To this end, we confirmed the growth promotion activity of *A. brasilense* Sp245 in the nursery for two different rootstocks (420A and 775P) known to have low and high rooting aptitude, respectively [17]. The treatment with *A. brasilense* Sp245 was ineffective in improving the rooting in both rootstocks, likely a consequence of high percentages recorded also in control cuttings. This phenomenon could be attributed to the complex rooting process influenced

**Table 3.** Nursery experiment: effect of *Azospirillum brasilense* Sp 245 on chemical compounds of grafted cuttings of rootstocks 420A and 775P. FW (Fresh Weight); DW (Dry Weight). Within each rootstock, the asterisk indicates significant differences between control and treatment by Student *t*-test ( $P \leq 0.05$ ); ns (not significant). Values are means  $\pm$  standard error ( $n = 50$ ).

	420A		775P	
	Control	Treatment	Control	Treatment
Total phenols (g/kg FW)	7.5 $\pm$ 1.1	9.4* $\pm$ 2.3	4.1 $\pm$ 1.2	4.5 ns $\pm$ 0.9
Nitrogen (% DW)	2.9 $\pm$ 0.7	2.2 $\pm$ 0.6 ns	3.5 $\pm$ 0.8	2.9 ns $\pm$ 0.4
Phosphorus (mg/kg DW)	325.6 $\pm$ 30.1	248.2* $\pm$ 24.5	292.5 $\pm$ 21.3	316.3 ns $\pm$ 17.3
Potassium (mg/kg DW)	2250.4 $\pm$ 150.2	1750.5* $\pm$ 110.5	3000.5 $\pm$ 230.0	2500.8* $\pm$ 115.3
Iron (mg/kg DW)	400.6 $\pm$ 35.3	650.1* $\pm$ 41.4	450.3 $\pm$ 27.4	750.4* $\pm$ 53.7

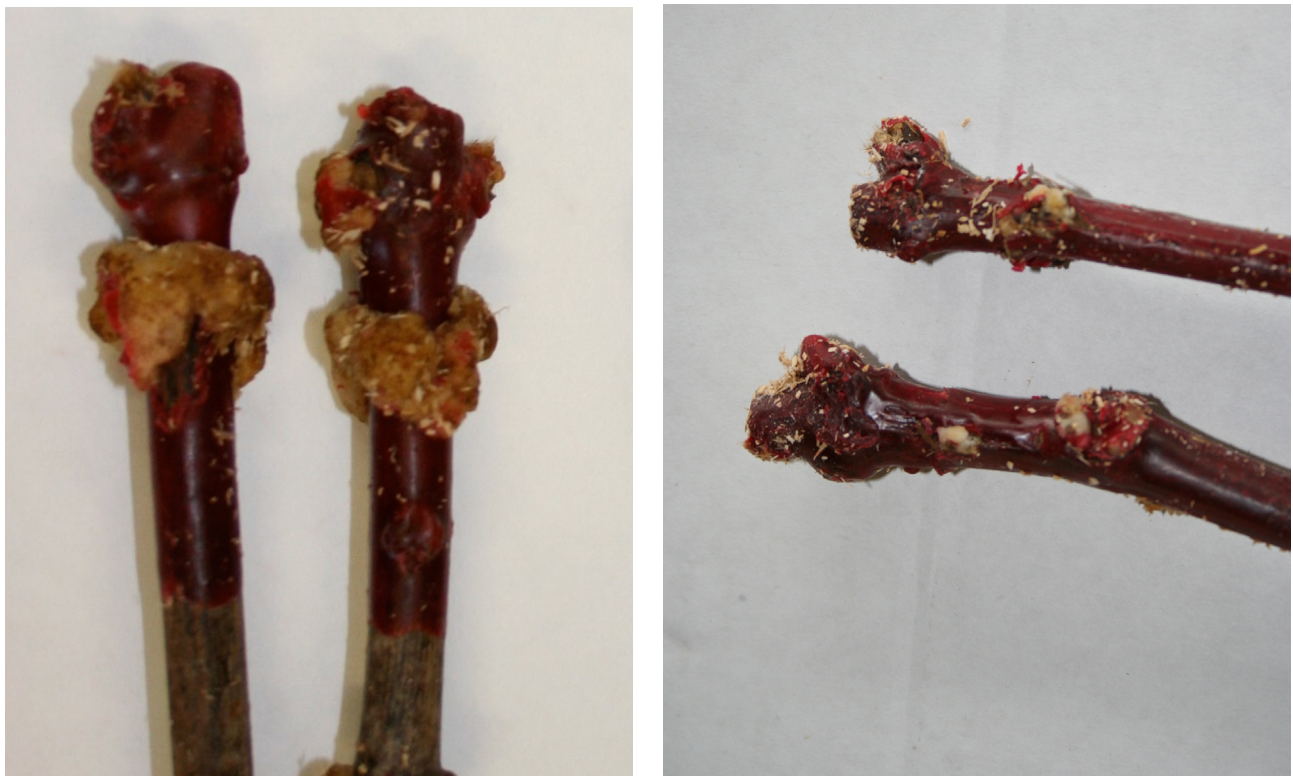


by cofactors and other cutting conditions, such as the quality of material collected from mother plants. It has been established that favorable agro-climatic conditions [22] likely play a key role in determining the nutritional status of the hardwood cuttings, which is an important factor for rooting and plant establishment [23,24,15]. Certainly, the stimulation of the root apparatus in both rootstocks, indicated in primary roots and final root biomass, confirmed previous evidence obtained in other species and grapevine rootstocks [3,7,25]. Moreover, it has been shown that *Azospirillum* inoculation may also exert strong stimulatory activities in plants, by inducing changes in root architecture provoking a more symmetric root system [8]. These relevant results may represent a useful tool for farmers considering that an improvement in propagation technique relating to rooting development, is a first step to trigger a sequence of other physiological phenomena that also involve the plant's aerial structures. Indeed, bacteria play an important role in the synthesis of bioactive molecules, such as phytohormones, as well as an enhancing the content of a variety of nutrients in plant tissues [21].

Another favorable effect of cutting inoculation was observed on the callus formation at the graft junction, as a consequence of improved proliferation of the secondary

meristem at the extreme parts of the cuttings (Fig. 4). The most significant increase in callus diameter was found in 420A, probably as a consequence of its genetic origin (*V. berlandieri* x *V. riparia*). In fact, different extents of callus formation have been defined by Galet [24] who observed that in *V. riparia* and *V. berlandieri*, a hybrid callus develops on both extremities, while in other genotypes (e.g. *V. rupestris*) it is formed predominantly on the upper extremity. In addition, the callus improvement could be related to the additional biosynthesis of beneficial substances induced by the bacteria in the grafted cutting tissues, similar to the promotion of cell proliferation observed in *V. rupestris* Du Lot in presence of the *Bacillus megatherium* [26]. A better graft union stability represents an important aspect to ensure enhanced hardness of the grafted cutting during nursery schedules, up to the commercialization.

One of the best-studied aspects of the interaction between plant and rhizosphere microorganisms is their ability to induce positive effects on plant growth by enhancing the uptake of nutrients [27]. In general, the bacterial strain assayed in the present study did not prove to affect the root uptake of mineral macro-elements (N, P, K). On the contrary, a considerable improvement in iron (Fe) content was recorded in both rootstocks treated with



**Fig. 4.** Effect of *Azospirillum brasilense* Sp245 on callus formation. Examples of callus at the end of the grafted cutting forcing of 420A rootstocks inoculated during the phase of hydration before the bench-grafting (left) and not inoculated (right).

*A. brasilense* Sp245, confirming the ability of rhizobacteria to increase the mobility of Fe by releasing chelating compounds known as siderophores [27]. Iron is a critical element for plant development, since it is involved in many biochemical processes and, often, due to the low solubility of the natural Fe sources in soil, plants suffer from Fe deficiency, especially when grown on calcareous soils.

The augmented content of total phenols in roots of treated cuttings is of notable importance considering the prominent role of these compounds in the enhancement of plant defence [10,28,29]. Several authors have reported the effects of systemic resistance induced through PGPRs in different crops, including grapevines, and it has been associated with the enhancement of plant defence. More recently, evidence of induced defense responses in PGPR-treated tomato plants were associated with an enhanced accumulation of antioxidant peroxidase (POX) and polyphenol oxidase (PPO) enzymes [30].

Overall, our results revealed that *A. brasilense* Sp245 seems to have considerable potential in enhancing the root apparatus and vegetative development in grapevines. The potted plants of cv 'Colorino', grafted on rootstocks 420A and 157/11, showed a better development when inoculated with *A. brasilense* Sp245. The repeated inoculation (T2) during the vegetative cycle proved to be more effective, as evidenced by a greater accumulation of dry mass. In the combination with 157/11 rootstock, 'Colorino' also showed a positive response with single rhizobacterium inoculation too (T1).

Certainly interesting findings were obtained in the experiments conducted in the nursery on a specialized farm. The bacterization of cutting rootstocks showed considerable positive influence on enhancing the final quality of root apparatus.

In conclusion, the stimulatory effect of *A. brasilense* Sp245 on vegetative development of rootstocks and vines, may indeed contribute to enhanced sustainability in viticulture. Moreover, the better survival ability of treated vines is an interesting finding which will warrant further study in other wood species, including fruit and ornamental trees and species for bioengineering works [31], also verifying the influence of *A. brasilense* Sp245 on stress tolerance to biotic and abiotic factors.

**Conflict of interest:** Authors state no conflict of interest

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