The influence of *Azospirillum baldaniorum* Sp245 on cutting propagation and plant growth after transplantation in Leccino cultivar (*Olea europaea* L.)

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Abstract

The employment of plant growth promoting bacteria (PGPB) is a developing practice mainly involved under organic agricultural contexts. In particular, the use of alternative rooting agents may successfully contribute to the development of the olive sector propagation. This issue has let to assess the potential effect of the free-living soil bacteria *Azospirillum baldaniorum* Sp245 on olive cultivar Leccino as i) root inductor for cuttings and ii) biostimulant on growth of nursery olive young plants. During the first nursery phase related to the rooting inductive process, comparative analyses were carried out between *A. baldaniorum* Sp245 and indole-3-butyric acid (IBA), a synthetic auxin usually used as root-promoting compound. Moreover, the bacterial suspension was periodically applied to the growing medium of hardened young plants, and compared with treatments by cell-free supernatant derived from the bacterial culture. Positive results were obtained on the assessment of the early histological changes related to the adventitious root formation. Interestingly, similar responses between cuttings treated with *A. baldaniorum* Sp245 and IBA were observed. Young plants provided with the bacterial suspension showed a general growing improvement of both hypogeal and epigeal apparatus. These features suggest that *A. baldaniorum* Sp245 could be usefully employed as rooting inducer, promoting a possible IBA replacing. Moreover, the improved quality of young olive plants determined by *A. baldaniorum* Sp245 supply, make it a profitable biostimulant in a context of organic nursery systems.

Keywords: agamic propagation, olive, PGPR, cell-free supernatant, nursery, semi hard-wood cuttings, histology

INTRODUCTION

Olive plants propagation is successfully performed using semi-hardwood cuttings, with the aid of root-inducing substances, such as the synthetic auxin IBA (indole-3-butyric acid). However, in organic contexts, IBA substitutes are required to stimulate root formation (Commission Regulation (EC) 834/2007 and 889/2008). Due to this, microbial biostimulants may represent a powerful and innovating tool to improve the efficiency of olive agamic propagation (Bartolini et al., 2022).

To the propagation phase, the transplantation of the rooted cuttings is followed. Depending on the edaphoclimatic conditions, rooted cuttings can be transplanted in pots and moved outside the greenhouse or can be set directly in the orchard; either way, the success of the process depends on the plant's ability to develop a strong and resilient root apparatus (Fabbri et al., 2004). As reported by Masmoudi Charfi et al. (2011), crucial factors such as water content and soil texture, can favor the root development of newly repotted plants. However, an improved and resilient root apparatus has been favored by the employment of microbial inoculants with plant growth promoting (PGP) compounds (Estaùn et al., 2003).

Among plant growth promoting bacteria (PGPB), *Azospirillum baldaniorum* Sp245 (dos Santos Ferreira et al., 2020) turned out to be of particular interest. It is a free-living soil bacterium which has multiple and synergic mechanisms of action such as nitrogen fixation and phytohormones production (indole-3-acetic acid, gibberellins, and abscisic acid), which are proved to enhance root growth (Bashan et al., 2010). *A. baldaniorum* Sp245 has showed to be a valid rooting inducer on hard-to root woody genotypes, such as some rootstocks of *Vitis vinifera* L. and Santa Caterina olive cultivar (Toffanin et al., 2014; Bartolini et al., 2017; Bartolini et al., 2022). Furthermore, *A. baldaniorum* Sp245 had positive effects on the acclimatization of several fruit tree rootstocks obtained by micropropagation (Vettori et al., 2010). Pellegrini et al. (2020) have recently reported that the cell-free supernatants (CFS), derived from the removal of cells from broth bacterial cultures, might be also valid root-inducer products. In particular, *in-vitro* studies showed that CFS from *A. brasilense* spp. contains remarkable amount of auxins (El-Khawas et al., 1999).

This research aimed to assess the potential effect of *A. baldaniorum* Sp245 on olive cultivar Leccino as i) root inductor for cuttings in comparison with IBA and ii) biostimulant on growth of nursery olive young plants, in comparison with CFS.

MATERIALS AND METHODS

Experimental trials on cuttings

The cuttings were collected from olive trees belonging to Leccino cultivar (12-year-old) located at the experimental farm of the Department of Agriculture, Food and Environment of Pisa University (DAFE, University of Pisa) located in Pisa province (Tuscany, Italy, 43°43'32.02" N, 10°27'37.66" E; altitude 3 m a.s.l.). Semi-hard wood cuttings (10 cm length, retaining 3-4 leaves) were obtained from one-year old shoots collected in early spring. The basal end of cuttings (approx. 1 cm) was immediately dipped in i) *A. baldaniorum* Sp245 (AZO) solution for 15 min; the inoculum was set up adjusting the AZO cell number to 107 CFU mL^{-1} with sterile water according to Mariotti et al., 2021; ii) IBA (Indole-3-butyric acid) aqueous solution of ethanol at 3000 ppm for 10 s; iii) water as control. After treatments, cuttings were placed in alveolar trays containing a hydrophilic mixture composed of peat, coconut fiber and perlite as culture substrate (Elepot® - https://www.elepot.it/), and maintained in plexiglass rooting chambers under controlled conditions (rooting medium basal average temperature 24 ± 1°C; relative humidity 80-90%; average air temperatures 18.5°C). Cuttings were arranged in a completely randomized block design with three replications for each treatment which consisted of 30 cuttings per experimental unit.

A fast protocol, described in Bartolini et al., 2023, was applied to identify the main histological events leading to adventitious root formation. Briefly, after AZO and IBA treatments, comparative anatomical observations of the basal portion of cuttings were carried out from the beginning of experimental trials (day 0, control) and at 8, 16, 24 days after the treatments. The histological events related to the rooting induction phases were defined as 'organized cell activity' (OCA). As described by Macedo et al., (2013), they consist of cell divisions, meristemoid structures (small mass of cells growing by synchronous mitotic divisions) and callus formation (amorphous mass of loosely arranged thin-walled parenchymatous cells). Results were expressed as percentage of cuttings showing such anatomical characteristics.

Experimental trials on transplanted plants

This test was carried out on 5-month-old rooted cuttings of Leccino cultivar obtained in previous trials by the standard method, using IBA as rooting agent and perlite as substrate. Hardened young plants, repotted in larger containers (28 L) containing a 1:1 mixture of peat and inert perlite, were kept outdoor from May 2021 to February 2022. They were regularly irrigated, without application of fertilizers. Three groups of plants (5 for each) were constituted to whom different treatments were applied in a fixed volume (45 ml/pot): i) AZO

bacterial suspension, diluted 1:10 in tap water to a final cell concentration of 10^7; ii) Cell-free Supernatant (CFS) derived by *A. baldaniorum* Sp245 culture $1 \cdot 10^8$ CFU mL^{-1} was obtained via filtration as reported by Mariotti et al. (2021) iii) water as control (C). Considering that repeated supplies with *A. baldaniorum* Sp245 gave positive results on young plants of *Vitis vinifera* L. (Bartolini et al., 2017), the solutions were monthly supplied from June to October. At the end of the nine-month long experiment, non-destructive measurements of chlorophyll content by a SPAD (Soil Plant Analysis Development) meter readings (Konica–Minolta, Inc., Osaka, Japan) on leaves of the five plants (replications) of each treatment were performed (Richardson et al., 2002). Then, plants have been divided into roots, shoots and leaves for the following determinations: i) final count of leaves and roots; ii) compute of the increase rate between the beginning of transplanting and the end of the experiment; iii) fresh and dry mass weight. Finally, using the software for image processing (ImageJ), leaf and root images were analyzed to obtain the surface of the leaves and the number and length of primary and secondary roots (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018.).

Data analysis

Statistical analysis was performed by the package GraphPad Prism 5 (GraphPad Software, Inc.) for assessing the treatment effects. Percentage data were arcsine transformed for analysis. Data were analysed by ANOVA and means were compared by using Tukey's test ($p \leq 0.05$).

RESULTS AND DISCUSSION

Interesting results were achieved by microscopical observations of the stem histological organization after cuttings were treated with a potential and a proved rooting inducer, such as the bacterium *A. baldaniorum* Sp245 (AZO) and the phytohormone IBA, respectively. Within the short period of the experimental trial (24 days), both agents gave similar sequential histological events related to the adventitious root formation (Table 1). In particular, the earliest significant appearance of 'organized cell activity' (OCA), mainly represented by intensive cell divisions and appearance of meristemoid structures (Figure 1A, B), was observed 8 DAT with AZO, in 75.0% of the analyzed stem cuttings. At 16 and 24 DAT, cuttings treated with AZO and IBA were characterized by similar rates (more than 90%) in which meristemoid and root primordia were the most observed structures (Figure 1B, C). These histological markers have been identified as key events leading to the adventitious root emergence (Porfirio et al., 2016). In control cuttings (C), OCA events were statistically different and always lower than AZO and IBA.

Table 1. Percentage of olive cuttings (cv. Leccino) showing organized cell activity (OCA) at 8-16-24 days after treatments (DAT) with *Azospirillum baldaniorum* Sp245 (AZO), Indole-3-Butyric Acid (IBA) and water as control (C). Different letters correspond to statistically significant differences at each time point ($p \leq 0.05$). Data are means ± SE.

	8 DAT	16 DAT	24 DAT
C	12,6 ± 4,9 c	12,6 ± 9,7 b	50,1 ± 11,6 b
IBA	50,1 ± 2,9 b	75,0 ± 7,0 a	95,1 ± 1,5 a
AZO	75,0 ± 5,5 a	87,5 ± 2,5 a	96,0 ± 1,7 a

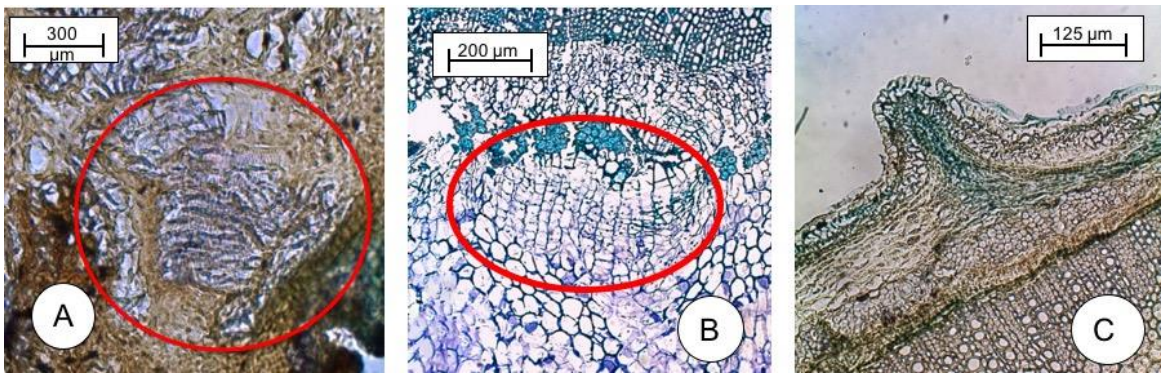

Figure 1. Section of the basal stem region of olive semi-hardwood cuttings (cv. Leccino). Anatomical structure of the stem after rooting treatments with *Azospirillum baldaniorum* Sp245 (AZO) and Indole-3-Butyric Acid (IBA). (A) Cell divisions; (B) Meristemoid structures; (C) Root primordia.

Concerning the performances of transplanted plants, after AZO treatments encouraging results on the growth parameters were obtained (Figure 2). In comparison with CFS and C, the bacterial suspension determined a significant dry weight improvement on both hypogeal and epigeal part of olive young plants. Effects as increased root and shoot biomass have been documented for PGPR-inoculated plants growing in soil (Veresoglou and Menexes, 2010; Walker et al., 2012). The growth promotion by PGPR can result from an indirect stimulation of the plant auxin pathway due to the production of nitric oxide (NO). This compound, involved in the auxins signaling pathway controlling lateral root formation, has been found in several PGPR strains belonging to *Azospirillum* spp. (Creus et al., 2005; Lanteri et al., 2006; Molina-Favero et al., 2008).

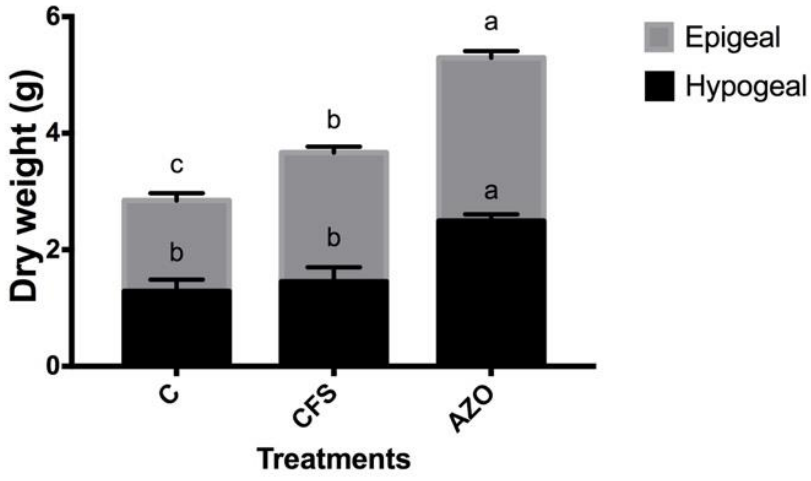

Figure 2. Final biomass of hypogeal and epigeal (leaves and shoots) part of olive young plants, cv. Leccino, after repeated treatments with *Azospirillum baldaniorum* Sp245 (AZO), Cell-free Supernatant (CFS) and water as control (C). Means (± standard error). Different letters are significantly different at $p \leq 0.05$.

In particular, focusing on the rate percentage of growth parameters between transplanting date and end of the experiment (Table 2), significant increases in whole plant fresh biomass

(281,1%) and leaves number (151,4%) were detected with AZO treatments. Moreover, the bacterial suspension determined better final traits of leaves with a greater surface area (430,0 mm^2) and chlorophyll content of tissues (68,9 SPAD unit) compared to CFS and C treatments. In particular, chlorophyll amount (expressed as SPAD units) and nitrogen content in the leaf have been found to be key indicators of plant health (Fotia et al., 2022). Thus, it could be supposed that the supply of AZO cell suspension benefits the nitrogen nutrition, contributing to a better physiological status of young olive plants.

Applications of the cell-free supernatant did not reveal to be efficient, as instead Pellegrini et al. (2020) have found in controlled environment.

Table 2. Olive young plants, cv. Leccino: increase percentage of fresh weight of whole biomass and leaves number, between transplanting date and end of the experiment; final area and chlorophyll content as SPAD unit of leaves. Means (± standard error). Different letters are significantly different at $p \leq 0.05$. ns: not significant.

Treatment	Percentage increase		Leaves	
	Fresh Weight (g)	Leaves (n)	Area (mm^2)	SPAD unit
C	120,7 ± 11,3 c	61,4 ± 7,0 b	293,0 ± 39,9 b	53,4 ± 2,9 b
CFS	180,9 ± 5,7 b	70,1 ± 12,5 b	355,8 ± 25,0 ab	53,1 ± 6,6 b
AZO	281,1 ± 13,5 a	151,4 ± 13,7 a	430,0 ± 48,4 a	68,9 ± 1,1 a

Table 3 shows data on the morphological parameters of root apparatus detected at the end of trials. In general, the applications of AZO after plant transplanting significantly improved the primary and secondary root number (8,1 and 287,3, respectively) which were more than double in comparison with plants treated with CFS and water. On the other hand, concerning the root length, notable differences among treatments were not evidenced. It has been ascertained that the root-hair formation, primary and lateral root growth, is particularly affected by nutrients supply, such as N (López-Bucio et al., 2003), and hormonals exogenous application such as auxins and ethylene (Lynch and Brown, 1997; Himanen et al., 2002). Further investigations will aim to establish whether *Azospirillum baldoniorum* Sp245, similarly to other PGPR, could have improved root development and growth through a better N availability in the growing medium and/or a production of phytohormones or enzymatic activities.

Table 3. Morphological parameters of root apparatus developed in olive young plants, cv. Leccino, after repeated treatments with *Azospirillum baldaniorum* Sp245 (AZO),

Cell-free Supernatant (CFS) and water as control (C). Means (± standard error). Different letters are significantly different at $p \leq 0.05$.

		C	CFS	AZO
Primary roots	Number	2,8 ± 0,9 b	3,6 ± 1,1 b	8,1 ± 1,7 a
	Length (mm)	120,1 ± 12,9 b	156,3 ± 14,8 a	139,5 ± 16,2 a
Secondary roots	Number	119,7 ± 19,6 b	98,7 ± 25,5 b	287,3 ± 17,3 a
	Length (mm)	33,3 ± 5,4 a	45,8 ± 11,4 a	35,4 ± 5,0 a

CONCLUSIONS

The overall results obtained from *Azospirillum baldaniorum* Sp245-treated cuttings and young plants belonging to Leccino olive cultivar suggest its possible employment, both as root inductor and biostimulant of growth.

The bacterial suspension employed on semi-hard cuttings was able to induce histological changes leading to the induction and expression of adventitious roots which were comparable to those observed by IBA treatments.

The bacterial suspension applied on the transplanted rooted cuttings determined a remarkable growth improvement of hypogeal and epigeal part of plants.

Although additional studies are needed to validate these results on other cultivars, our findings seem interesting for a possible use of *A. baldaniorum* Sp245 under a context of organic nursery system, as replacement of not allowed synthetic compounds.

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