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Communication

Azospirillum baldaniorum Sp245 Induces Anatomical Changes in Cuttings of Olive (*Olea europaea* L., cultivar Leccino): Preliminary Results

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Abstract: The purpose of the research was to assess the potential effectiveness of *Azospirillum baldaniorum* Sp245 in stimulating the induction processes related to adventitious root formation. In this short communication, we present the preliminary results obtained for the first time for the Leccino olive cultivar (*Olea europaea* L.). Semi-hardwood cuttings were collected at different sampling times (autumn, winter, and spring) and subjected to root-inductive treatments with *Azospirillum baldaniorum* strain Sp245 (AZO) and indole-3-butyric acid (IBA), the most employed synthetic auxin used in nursery production of olive plants. Moreover, two growing substrates, perlite (an inorganic medium) and Elepot® (a blend of peat, coconut fiber, and perlite) were tested. Histological assays were carried out to identify the main cellular changes leading to adventitious root formation. The highest rates of cuttings showing more advanced stages of rooting inductive signals were observed with the spring sample. The events observed after AZO treatments were comparable to those observed with IBA, which were mainly associated with the Elepot® growing substrate. The positive responses of semi-hardwood cuttings of Leccino cultivar to AZO could provide the possibility of replacing IBA and develop perspectives in a context of organic nursery systems where alternative compounds, able to improve the rooting rate of cuttings, could substitute the non-permitted synthetic rooting agents. AZO induced significant cellular activities and could be considered a valid alternative rooting agent.

Keywords: agamic propagation; olive; PGPR; semi-hardwood cuttings; histology

Citation: Bartolini, S.; Pappalettere, L.; Toffanin, A. *Azospirillum baldaniorum* Sp245 Induces Anatomical Changes in Cuttings of Olive (*Olea europaea* L., cultivar Leccino): Preliminary Results. *Agronomy* **2023**, *13*, 301. https://doi.org/10.3390/agronomy13020301

Academic Editor: Samir C. Debnath

Received: 23 December 2022
Revised: 13 January 2023
Accepted: 17 January 2023
Published: 18 January 2023

1. Introduction

Multiplication by cuttings is an agamic propagation strategy, widely employed to produce genetically uniform plants from stock germplasm [1]. In the olive nursery sector (*Olea europaea L.*), agamic multiplication is usually performed using semi-hardwood cuttings. The rooting success depends on several factors such as the cultivar and its physiological status, growing environmental conditions (i.e., temperature, relative humidity, light), substrates, and root-promoting products [2]. The best root-promoting compound is indole-3-butyric acid (IBA), a synthetic auxin that is usually employed in olive plant production [3]. However, IBA, as a synthetic chemical product, is not permitted in an organic agricultural context in Italy and the European Union (Commission Regulation (EC) 834/2007 and 889/2008). Thus, new propagation protocols involving alternative compounds able to improve the rooting rate in semi-hardwood olive cuttings are currently encouraged.

Plant growth-promoting rhizobacteria (PGPR) can indeed provide benefits to plants, and their employment has spread in recent decades, mainly in organic systems [4]. Popular PGPR are frequently free-living soil bacteria, such as *Azospirillum* spp., that affect the growth and yield of numerous plant species [5]. In particular, *Azospirillum baldaniorum*

strain Sp245, previously classified as *Azospirillum brasilense* strain Sp245 [6], has multiple and synergic mechanisms of action such as nitrogen fixation, phytohormone (abscisic acid, gibberellins, and indole-3-acetic acid), polyamine, and trehalose production; these are compounds that enhance root growth and induce drought stress resistance [7–10]. The predominance of each mechanism can depend on substrate and climate conditions, the presence of minerals in the soil, and the type of plant the bacteria is engaging in a relationship with [9]. Furthermore, *A. baldaniorum* Sp245 has proven to be a valid rooting inducer and enhancer in rootstocks of *Vitis vinifera* L. [11,12]. The most recent and interesting results about *A. baldaniorum* Sp245 in the olive agamic propagation have been obtained on the hard-to-root cultivar Santa Caterina [13]. Indeed, the best rooting response was obtained by *A. baldaniorum* Sp245 in comparison with IBA treatments. These encouraging results inspired new comparative trials focused on the histological events happening during the phases leading to the adventitious root emergence. The appearance of adventitious roots is a three-phase process: (i) induction phase—period preceding any visible histological event (also comprising molecular and biochemical events); (ii) initiation phase—first histological events take place (formation of small cells with large nuclei and dense cytoplasm); (iii) expression phase—development of the typical dome shape structures, intra-stem growth, and emergence of root primordia [14]. It has been stated that these phases occur within 24 days after rooting treatments [15]. Thus, our aim was to develop a protocol for the quantification of histological events to ascertain in advance the efficacy of a new rooting compound, compared to the current methods which last an average of 60–90 days [2]. In this short communication, we present the preliminary results obtained from cuttings of the Leccino cultivar which were subjected to different root-inductive treatments, growth substrates, and sampling times. To our knowledge, a method like this, using *A. baldaniorum* Sp245 as a rooting agent, has not been proposed yet. The Leccino cultivar is one of the most important Italian olive oil genotypes that show tolerance to the bacterium *Xylella fastidiosa* subsp. *pauca* [16], a phytopathogen associated with the "olive quick decline syndrome" (OQDS). Thus, for this cultivar, an improvement in propagation protocols would be desirable for its employment in new olive orchards so as to contain the detrimental impact of OQDS.

2. Materials and Methods

2.1. Plant Material

The olive cultivar tested was Leccino, which has been considered easy to root [2]. Five healthy trees (12-year-olds), growing 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.), were used for the material collection. Semi-hardwood cuttings (10 cm length, retaining 2–3 leaves and 2–3 nodes, about 0.5 cm diameter) were obtained from one-year-old shoots. As significant seasonal variations in the rooting potential of olive stem cuttings were evidenced [7], the trials were carried out in three different seasons: autumn (T1, November), winter (T2, February), and spring (T3, April) throughout 2021–2022. The basal end of cuttings (approx. 1 cm) was immediately dipped in: (i) *Azospirillum baldaniorum* Sp245 (AZO) solution for 15 min. The suspension has been prepared as reported in the study conducted by Mariotti et al. [17]. The inoculum was set up, adjusting the *A. baldaniorum* Sp245 cell number to 10^7 CFU mL^{-1} with sterile water. The number of viable cells in the inoculum as well as their absence in supernatants was verified by standard plate counts on nutrient agar medium; (ii) Indole-3-Butyric Acid (IBA) solution at 3000 ppm for 10 s. Dissolving IBA powder (Sigma, St Louis, MO, USA) was freshly prepared in a 40% v/v aqueous solution of ethanol; (iii) water as a control for 15 min. After treatments, cuttings were placed in two substrates: (i) perlite, an inorganic rooting medium frequently used in olive nurseries, in perforated plastic pots (3 × 3 × 5 cm) at T1, T2, and T3 seasonal trials in 2020–2022; (ii) Elepot® (https://www.elepot.it, accessed on 23 November 2022) [18], in alveolar trays, a hydrophilic mixture composed of peat, coconut fiber, and perlite were used

at the T3 seasonal trial in 2022. Both systems were placed in Plexiglas rooting chambers which were built inside a greenhouse. The conditions under the rooting chamber were the following: the basal average temperature of the rooting medium was maintained at 24 ± 1 °C by the "EH-POWERGROUND" electric heating mats and an "EH-CONTROL" thermostat (distributed by COFILEA S.R.L., Biella (BI), Italy); the relative humidity was kept at 80–90% by the intermittent mist system "humiSteam Xplus", distributed by CAREL Industries (Italy). The average air temperatures were 12.5, 13.8, and 18.5 °C at T1, T2, and T3, respectively. Cuttings were arranged in a completely randomized block design with three replications for each treatment which consisted of 30 cuttings per experimental unit.

2.2. Anatomical Observations

To identify the main anatomical phases that lead to adventitious root formation (i.e., induction, initiation, and expression) cuttings were withdrawn periodically for comparative anatomical observations of the basal portion. Samples (N = 8) were collected just before the starting of experimental trials (day 0, control) and at 2-, 8-, 16-, and 24-day intervals, according to Porfirio et al. [14], after planting cuttings treated with IBA and AZO. At each time, basal stem segments (1 cm long) were fixed in an FAA solution (10% formaldehyde, 5% glacial acetic acid, 45% ethyl alcohol; 1:1:8 v/v) until dehydration in graded ethanol and finally embedded in Histoplast according to standard paraffin procedures [19]. The stem cross sections (10–14 µm), obtained using a microtome (Shandon AS325 Manual Rotary Microtome, Model 0325R), were stained with 0.01% Toluidine Blue. The slides were covered with a drop of neutral resin to make permanent slices. The stem structure of each cutting was observed under a light microscope (Fluophot, Nikon Inc., Minato City, Japan) equipped with a digital camera (Olympus C-2000z, Tokyo, Japan). According to Macedo et al. [15], with modifications, the histological events happening during the first 24 days from the beginning of the trials were classified as follows: (i) before rooting treatments: stage 0 (anatomical structure of the stem without signals of re-acquiring meristematic features); (ii) after rooting treatments—signals about the rooting induction phase represented by the re-acquirement of cell meristematic features: stage 1 (visible dense cytoplasm); stage 2 (visible large nuclei and nucleoli); stage 3 (cell divisions); stage 4 (meristemoid structures); stage 5 (callous formation); stage 6 (morphogenic root primordia). According to Macedo et al. [15] stages lower than 3 were defined as 'non-specific cell activity' (NSCA), whereas stages equal to or greater than 3 were defined as 'organized cell activity' (OCA) corresponding to the initiation phase. The percentage of cuttings showing anatomical features from stage 0 to stage 6 was then calculated.

2.3. Evaluations on Cuttings

During all the seasonal experimental trials, on a group of cuttings, (30 for each treatment and substrate) periodical visual observations were carried out to assess the survival rate (live cuttings with foliar retention). At the end of the trial period, the presence of basal macroscopically visible callous was evaluated. Both parameters were registered and expressed as percentages.

2.4. Statistical Analyses

Statistical analyses were performed using the package GraphPad Prism (version 5.00 for Windows, GraphPad Software, La Jolla, San Diego, CA, USA). Percentage data were subjected to arcsine root square transformation before tests. The standard errors (SE) of the means were calculated for each parameter measured. Data were compared using analysis of variance (ANOVA) and Tukey's multiple range test was assessed to compare the differences among means. Student's t-test was also performed. To evaluate if the factors of the substrate and rooting treatment, and their interaction, had a significant influence on the histological events, a two-way ANOVA was performed. In all analyses, at least the significant confidence level at $p \leq 0.05$ was considered statistically significant.

3. Results

Transverse sections at the basal portion of semi-hardwood stem cuttings in the Leccino cultivar allowed the detection of cell types and the organization of tissues similar to those described for other olive cultivars [20]. No seasonal differences in stem anatomy were noted, in agreement with Denaxa et al. [21]. In Figure 1, the anatomical structure of the stem at the beginning of the trials is reported, when cuttings were just withdrawn (day 0). From the outer layers of the stem cutting, the epidermis (Ep) consists of 5–6 layers of regularly shaped cells. Inward, the cortex (Co) appears as a thick layer of isodiametric parenchymatous cells. Below the cortex, a continuous sclerenchyma ring, constituted by 4–6 layers of isodiametric cells, was detected. As observed on several olive cultivars [22], sclerenchyma tissue consists of masses of sclereids presenting thick walls and small pits. Adjacent to sclerenchyma, the vascular system formed by phloem (Ph) and xylem (Xy) is located with the cambium (Ca) between the two rings (Figure 1). Below the xylem, the pith (Pi), constituted of parenchymatous cells, represents the core of the stem. The sequential histological events happening in the stem after rooting treatments are shown in Figure 2. The first preponderant observed event was the re-acquiring of meristematic characteristics of cells exhibiting dense cytoplasm and large nuclei, in a uniform distribution at the cortex and sub-epidermal tissues (Figure 2A). Cell divisions were mainly in the cortex (Figure 2B) observed, leading to the formation of meristemoid structures constituted by small masses of cells growing by synchronous mitotic divisions (Figure 2C). Afterward, a calloused tissue (Figure 2D) observed only at the cortex level, was constituted by an amorphous mass of loosely arranged thin-walled parenchymatous cells. The last event was the formation of root primordia, which appeared like a polarized clump of cells pushing through the cortex against the epidermis (Figure 2E).

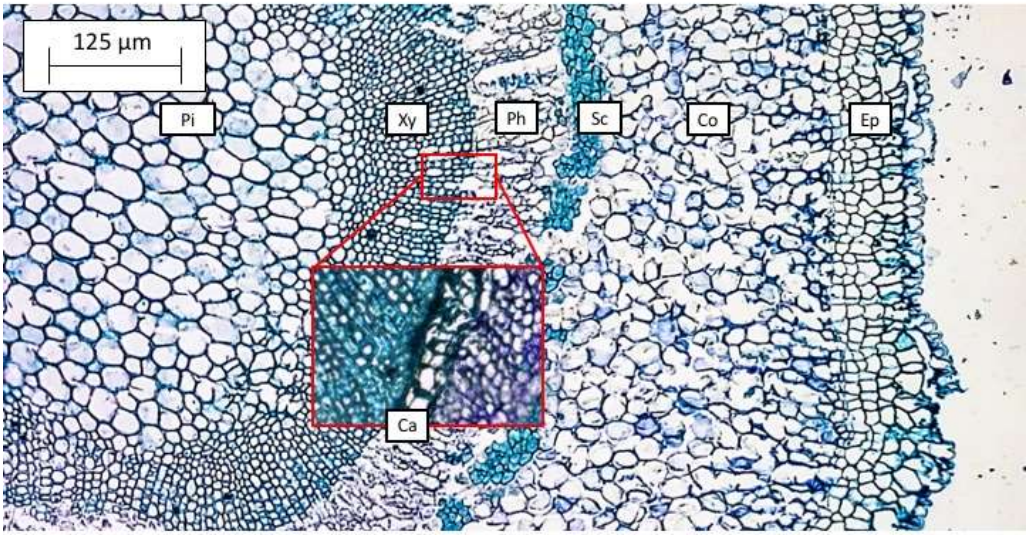

Figure 1. Section of the basal stem region of olive semi-hard cuttings (cv. Leccino). Anatomical structure of the stem prior to rooting treatments (day 0): epidermis (Ep), cortex (Co), sclerenchyma (Sc), phloem (Ph), cambium (Ca), xylem (Xy), pith (Pi).

During the first year of experimental trials, the growing substrate was perlite, commonly adopted in nursery procedures for olive trees. The histological events, related to NSCA and OCA observed after 24 DAT from the beginning of the experimental trial, are summarized in Figure 3, as a function of root-treatments (IBA and AZO) and sampling times (T1, T2, T3).

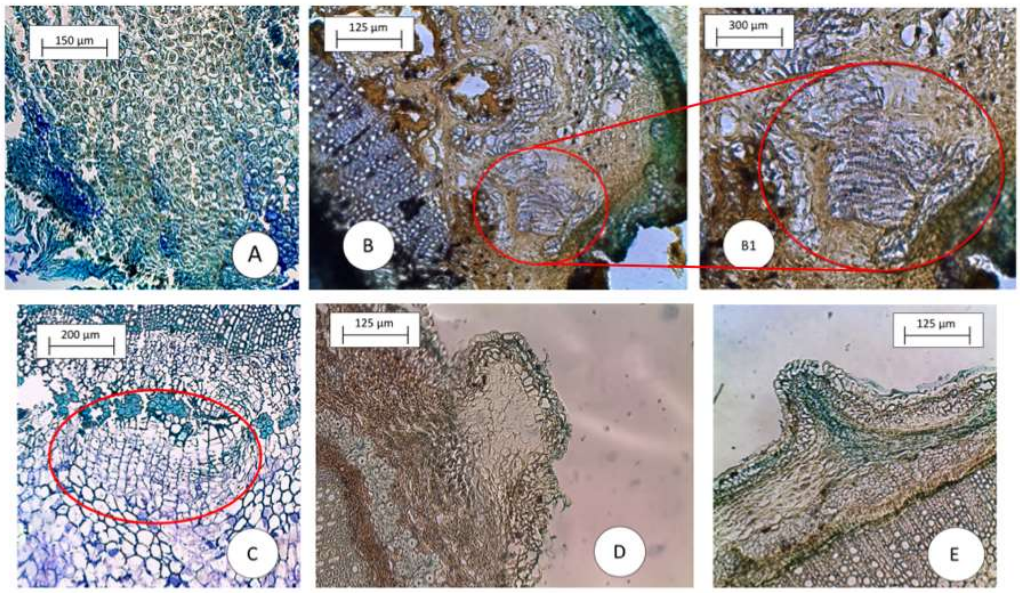

Figure 2. Section of the basal stem region of olive semi-hard cuttings (cv. Leccino). Anatomical structure of the stem after rooting treatments. (**A**) Cells in the cortex re-acquire a meristematic activity, showing dense cytoplasm and large nuclei; (**B**) first cell divisions (**B1** as a detail); (**C**) presence of meristemoid structures (red circle); (**D**) callous; (**E**) root primordia.

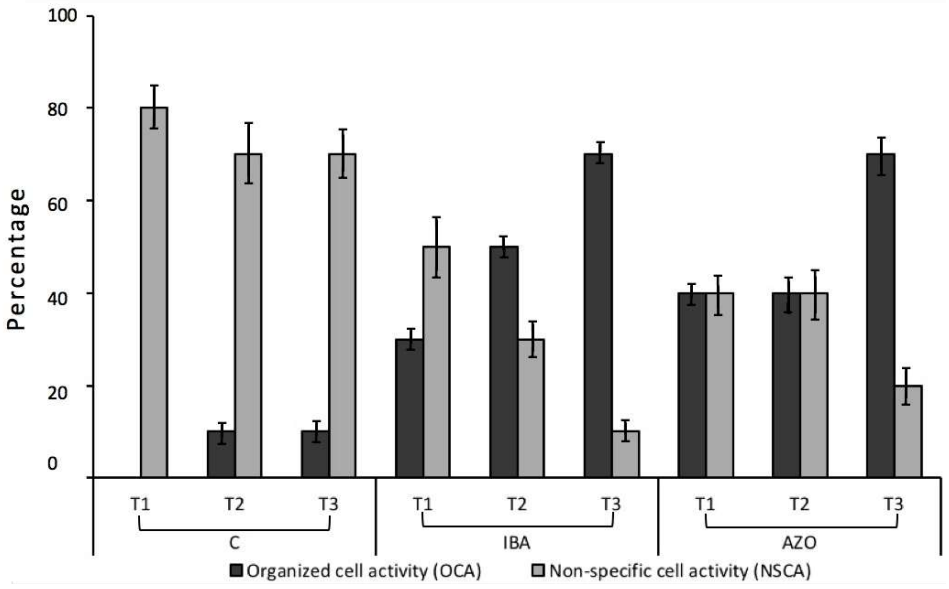

Figure 3. Mean percentages (± SE) of cuttings showing histological events happening during the adventitious rooting process in olive cuttings (cv. Leccino) after 24 d from treatments with Indole-3-Butyric Acid (IBA), *Azospirillum baldaniorum* Sp245 (AZO), and water (control, C) at different sampling times: autumn (T1), winter (T2), spring (T3). The rooting substrate was perlite. Data are expressed as non-specific cell activity (NSCA, stage < 3) and organized cell activity (OCA, stage ≥ 3).

The control cuttings always showed null or very low percentages (12.5%) of OCA which was denoted by stages ≥ 3, from cell divisions to root primordia formation, as indicators of rooting initiation signals. On the other hand, the cytological events linked to cell activity were observed in stem slices from cuttings treated with IBA and AZO. By these treatments, percentages ranged between 37.5–87.5% with seasonal differences, more marked for IBA treatments, where the lowest and highest values were found in autumn (T1) and spring (T3) sampling time. Anyway, at the maximum cell activity detected at T3, percentages of OCA in IBA and AZO cuttings were about 7-fold higher than control.

Considering that the maximum OCA was recognized in the spring season (T3), at this time in the second year of trials, temporal microscopic observations at 2-8-16-24 DAT were performed. In addition to perlite, the Elepot® substrate was also used (Figure 4A,B).

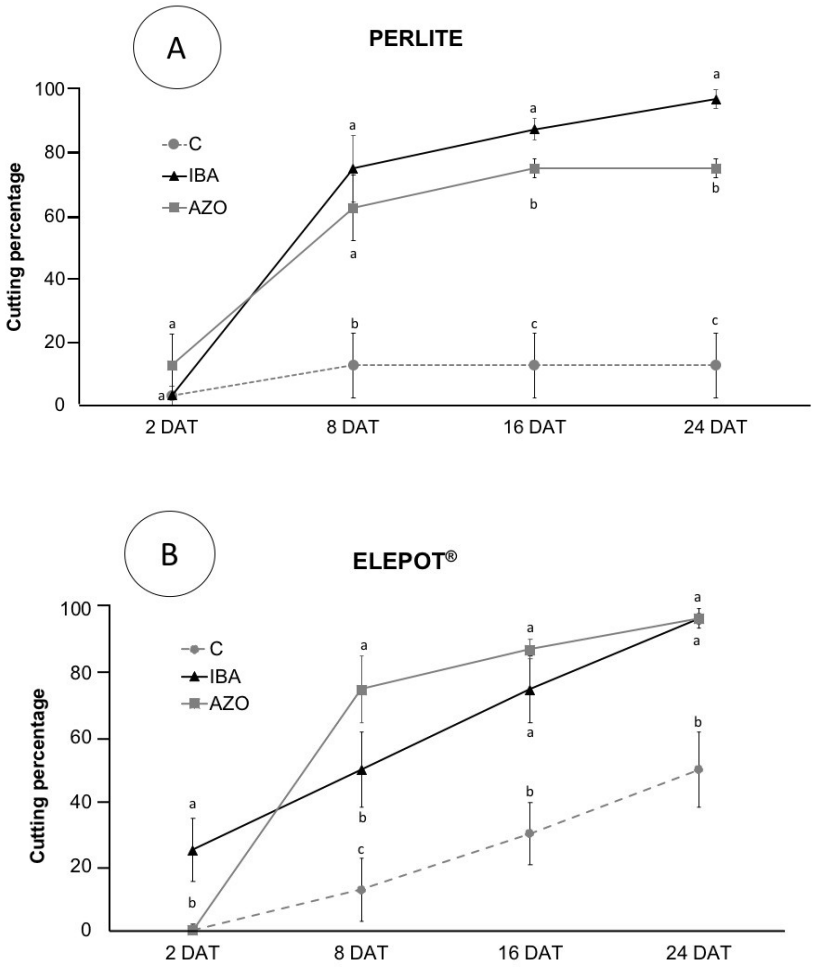

Figure 4. Percentage of olive cuttings (cv. Leccino) showing organized cell activity (OCA, stage ≥ 3) at 2, 8, 16, and 24 days after treatments (DAT) in spring season (T3) with Indole-3-Butyric Acid (IBA), *Azospirillum baldaniorum* Sp245 (AZO) and water (control, C). The rooting substrates were perlite (**A**) and Elepot® (**B**). Different letters correspond to statistically significant differences at each time point ($p \leq 0.05$). Data are means $\pm$ SE.

In both substrates, a similar trend was observed over time. Concerning cuttings on perlite (Figure 4A), any significant changes in the tissue structure were observed at 2 DAT. At 8–16 DAT, significant differences between rooting treatments and control were recorded. Indeed, histological OCA signals were observed in about 62–87% of treated cuttings that reached, at 24 DAT, 75%, and more than 90% in AZO and IBA, respectively. Interestingly, the tracked changes in cuttings treated with AZO were comparable with IBA, similar to the data obtained at T3 during the first year of trials (Figure 3). For untreated samples, cell activity was recorded from 8 DAT onwards, in 12.5% of cuttings without differences among time points.

Regarding cuttings on Elepot® (Figure 4B), no changes in the structure of control and AZO examined sections were observed at 2 DAT. On the other hand, at this time, 25.0% of cuttings treated with IBA showed OCA, which regularly increased, reaching the maximum value (close to 100%) in correspondence with the last cutting collection at 24 DAT. The treatment with AZO determined a strong cell activity, ranging between 75.0 and 87.5% at 8 and 16 DAT, respectively. It achieved the same high values of IBA at 24 DAT. Untreated cuttings (C) showed OCA in a range between 12.5 and 50.0%, starting from 8 DAT.

The two-way ANOVA results were obtained after 2, 8, 16, and 24 DAT did not reveal significant interactions between substrate and rooting treatment (Table 1).

Table 1. Two-way ANOVA results obtained at 2, 8, 16, and 24 days after treatments (DAT) of olive cuttings (cv. Leccino). Main effect: substrate (S) and rooting treatment (T). F- and *p*-values ($p \leq 0.05$) are shown; ns: not significant.

	2 DAT		8 DAT		16 DAT		24 DAT	
Main effect	F	*p*	F	*p*	F	*p*	F	*p*
Substrate	0.75	ns	1.74	ns	0.57	ns	0.80	ns
Treatment	3.41	0.0426	13.87	≤0.0001	19.18	≤0.0001	25.65	≤0.0001
S × T	2.85	ns	1.50	ns	0.71	ns	1.25	ns

Analyzing the histological events happening at 2, 8, 16, and 24 DAT, defined by stages 0 to 6 (Figure 5), it emerged that, regardless of substrate, at each time point cuttings treated with IBA and AZO showed similar stages, always more advanced and significantly different in comparison with control. The mean stage did not overcome stage 2 (visible large nuclei and nucleoli) at 2 DAT. The cuttings treated with both compounds were characterized by stages from 3 (cell divisions) to 5–6 (callous and root primordia) at all other time points.

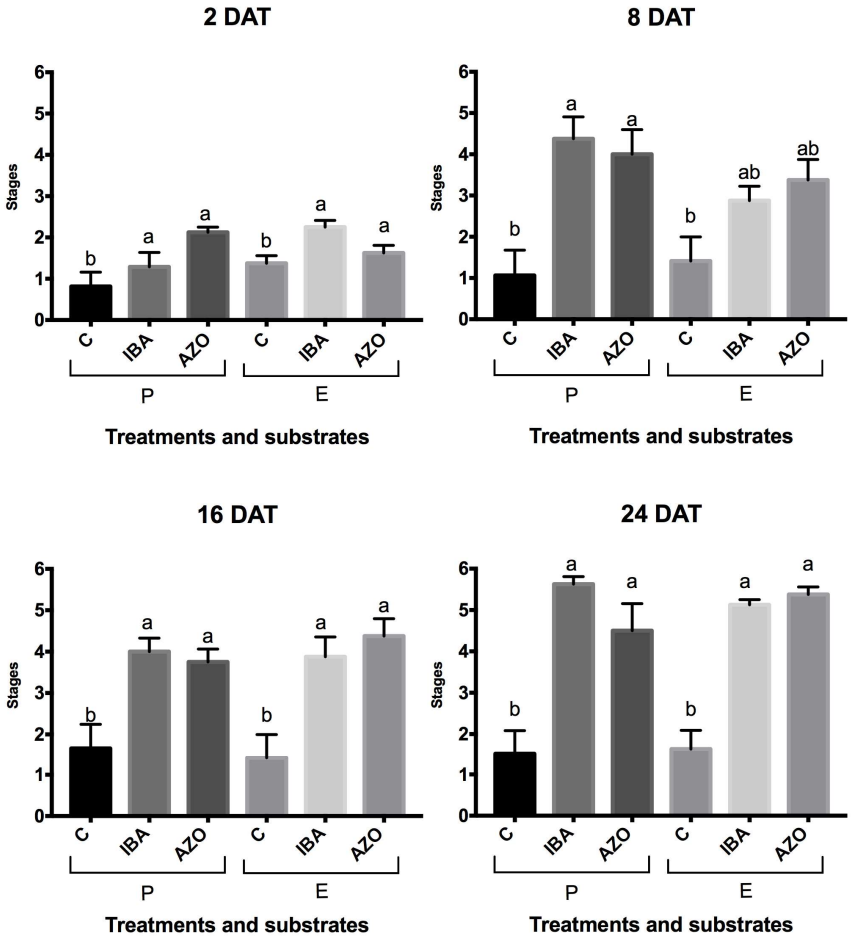

Figure 5. Histological stages recorded in olive cuttings (cv. Leccino) at 2, 8, 16, and 24 days after treatments (DAT) in spring season (T3) with Indole-3-Butyric Acid (IBA), *Azospirillum baldaniorum* Sp245 (AZO), and water (control, C). Substrates: perlite (P) and Elepot® (E). Stage 0 (no signals of re-acquiring meristematic features); stage 1 (visible dense cytoplasm); stage 2 (visible large nuclei and nucleoli); stage 3 (cell divisions); stage 4 (meristemoid structures); stage 5 (callous formation); stage 6 (morphogenic root primordia). Within each substrate, different letters correspond to statistically significant differences ($p \leq 0.05$). Data are means ± SE.

Results from the visual observations of the healthy state of Leccino cuttings, namely with foliar retention and absence of leaf drop, and the presence of basal macroscopically visible callous after 24 DAT in the spring season, are shown in Table 2. Regarding the percentages of leafy retention on perlite, statistical differences were found among control and treated cuttings, ranging from 82.8 (control) to 90.0–95.3 for AZO and IBA, respectively. On the other hand, on the Elepot® substrate, no differences were recorded among treatments (from 89.8 to 91.4). In this substrate, the untreated cuttings showed a lower leaf drop rate in comparison with perlite. Concerning the presence of callous on the basal part of olive cuttings, its formation was mostly stimulated using the Elepot® substrate with both IBA and AZO treatments. Substantial increases were recorded in comparison with perlite, about 65% for IBA and 250% for AZO.

Table 2. Frequencies (%) of leafy and calloused olive cuttings (cv. Leccino) at 24 days after treatments with Indole-3-Butyric Acid (IBA), *Azospirillum baldaniorum* Sp245 (AZO) and water (control, C) using perlite and Elepot® substrates at springtime. For each parameter and within each row, mean values (±SE) with different letters are significantly different (ANOVA, $p \leq 0.05$). At the bottom of each column, asterisks denote significant differences between substrates (Student's *t*-test): $p \leq 0.005$ (**), $p \leq 0.0001$ (***), ns (not significant).

	Leafy Cuttings (%)			**Calloused Cuttings (%)**		
Substrate	IBA	AZO	C	IBA	AZO	C
Perlite	95.3 ± 1.3 a	90.0 ± 1.1 b	82.8 ± 0.9 c	18.2 ± 0.8 a	11.3 ± 0.9 b	1.2 ± 0.1 c
Elepot®	89.8 ± 0.9	91.4 ± 0.9	90.5 ± 1.1	30.0 ± 1.1 b	40.4 ± 1.1 a	2.3 ± 0.6 c
t-test	ns	ns	**	***	***	ns

4. Discussion

Anatomical studies provide information on tissue and organ arraignment and formulate hypotheses on the effects of different treatments in plant development [23]. It is known that particular physiological conditions of plant organs may produce morphoanatomical alterations [24]. In this sense, the anatomical approaches allowed for following the histological changes of the stem organization induced by IBA and AZO treatments. IBA is widely used in olive plant propagation [25,26], whereas AZO, the bacterium *Azospirillum baldaniorum* Sp245, was tested for the first time in the Leccino olive cultivar as a potential and alternative rooting inducer. In particular, the microscopic observations carried out up to 24 DAT with IBA and AZO allowed for identifying the phases leading to the formation of adventitious roots, as a function of rooting treatments, substrate type, and experimental seasons. As evidenced, the regulation of rooting formation is influenced by numerous factors, whose interaction remains poorly understood, and underlying molecular mechanisms governing the process are still poorly investigated [27,28]. The phenological stages and physiological status of the mother plant at the time of cutting collection are crucial factors for achieving successful rooting rates [29]. Marked seasonal variations in cutting rooting ability have been evidenced also in the same olive genotype, ranging from 80% in the spring and summer seasons to 20–30% during the wintertime [7]. Our results are consistent with these findings. In both years, the highest rates of cuttings showing more advanced stages of rooting inductive signals were observed in spring (T3). After applications of AZO and IBA, the cellular events and changes in the internal stem structures were similar to those described exhaustively for easy-to-root olive cultivars by Altamura [20], Salam et Qrunfleh [22], and Macedo et al. [15]. In Leccino, most OCA events (from cell divisions to the appearance of root primordia) occurred in the cortex and sub-epidermal regions of cutting stems where meristemoids were forming, in agreement with Macedo et al. [15]. The same histological events were found in the cambial region of the cutting sections as well [20,22]. From a temporal point of view, cells in the active division and meristemoid formation were mostly observed between 8 and 16 DAT, followed by callous and root primordia appearance. On the other hand, NSCA occurred from 2 to 8 DAT.

The free-living soil bacteria *A. baldaniorum* Sp245 was able to stimulate cells to re-acquire the meristematic features, proving to have an inducer action similar to IBA, the rooting treatment *par excellence* used in the olive nursery sector. Positive results with the employment of *A. baldaniorum* in semi-hardwood olive cuttings were obtained by Dalla Rosa et al. [30]. Recently, interesting findings were obtained from the hard-to-root olive cultivar Santa Caterina where a much more successful rooting treatment in comparison with IBA was recorded [13]. The failure of IBA to stimulate rooting, also recorded in hard-to-root cultivars (i.e., Kalamata), suggests that other physiological and/or biochemical factors might be involved in this process [21]. However, auxins remain one of the most important molecules produced by *Azospirillum* sp. that promote root development, improving plant growth and development [8,31,32]. Therefore, its effectiveness could be explained by other mechanisms such as the production of nitric oxide (an auxin precursor), and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, an enzyme that degrades the precursor of plant hormone ethylene, which acts as a rooting inhibitor [4,33].

Cuttings treated with IBA and AZO responded similarly when grown on perlite; in terms of cutting, percentages showing OCA stages were $\geq$3. However, when cuttings were placed on the Elepot® substrate, the combination with AZO stimulated a significant OCA increase at 8 DAT. This occurrence was also confirmed by the morphological data about the quality parameters of cuttings at the end of the experimental trials (24 DAT). In particular, cuttings that grew on Elepot® and were treated with AZO produced a higher calli percentage than those treated with IBA. As observed from in vitro investigation, the bacterial effect can also depend on the culture media [23]. Bashan and de-Bashan [9] have argued that bacterial phytostimulation is complementary to other mechanisms during plant interaction and could be modified by the chemical and osmotic composition of the culture medium. In addition, the genotype might play a significant role, interacting with the substrate type. This additive hypothesis deserves further in vivo investigation employing a number of different inorganic and organic substrates. Indeed, results obtained with *Azospirillum* sp. in field inoculation trials are numerous but fairly inconsistent [34], suggesting that *Azospirillum* sp. or bacterial auxin biosynthesis may be drastically affected by environmental conditions [35]. In addition, a different rooting behavior has been observed when several substrates were used (i.e., perlite, sand, peat moss) within the same genotype [36,37]. Such a finding was consistent with our results that evidenced differences between the two tested substrates. In particular, untreated Leccino cuttings on Elepot® showed an improvement in cellular activity, suggesting a better growing condition in comparison to perlite. It has been ascertained that type of rooting substrate can greatly affect the success of root formation, as a result of specific properties such as porosity, water retention capacity, and nutrient levels [38]. To our knowledge, there are no published studies that compare perlite and Elepot® growing substrates. We could assume that the success of Elepot® with the Leccino cultivar, in stimulating the callous formation, is due to its ability to balance water retention and oxygen supply. Although the presence of callouses may not relate to the rooted cuttings [16], our preliminary results evidenced a relationship with callous formation (data not shown), in agreement with Peixe et al. [39]. Nevertheless, further investigation is needed to clarify this aspect.

5. Conclusions

One of the main arguments for the use of bacteria or other microorganisms as biostimulants is to verify their efficacy within a short time. The present findings suggest that an anatomical approach may be a valid tool to comprehend the influence of rooting treatments in determining effective histological changes, which led to the induction and expression of adventitious roots. Interestingly, the events observed after AZO treatments were entirely comparable to those observed with IBA, the most employed synthetic auxin in the nursery production of olive plants. The adopted protocol allowed us to identify the crucial histological events related to the emergence of root primordia within a few days of rooting treatments. In this context, evidence may be acquired in advance in comparison to the

current olive propagation methods lasting more than 60 days. The positive responses of semi-hardwood cuttings of the Leccino cultivar to *Azospirillum baldaniorum* Sp245 could provide the possibility of replacing synthetic rooting agents, like IBA, in the context of organic nursery systems.

The results obtained for Leccino seem of particular importance considering that this cultivar is tolerant towards the bacterium *Xylella fastidiosa* subsp. *pauca*. For this reason, an improvement in propagation protocols will be a determinant in ensuring that large numbers of Leccino plants are employed in new olive orchards. However, due to the diversity of rooting behaviors among olive cultivars, further studies involving other genotypes are necessary to confirm the role of *Azospirillum baldaniorum* Sp245 as a rooting promoter.

Author Contributions: Conceptualization, S.B. and A.T.; methodology and formal analysis, S.B., L.P., and A.T.; writing—original draft preparation, S.B., L.P. and A.T.; writing—review and editing, S.B., L.P. and A.T.; funding acquisition, S.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the support of the Apulia Region, BiOSAVEX Project "Olive Biodiversity for Saving Salento from Xylella", PSR Puglia 2014–2020, MIS. 16—Cooperation—sub-measure 16.2—Support for pilot projects and the development of new products, practices, processes and technologies, approval no. 94250034397.

Data Availability Statement: The data generated and analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgments: The authors wish to thank Cristina Ghelardi and Fausta Rocco for their technical assistance, and Anton Trivella for English revision of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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