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ORIGINAL ARTICLE

Azospirillum brasilense inoculation, auxin induction and culture medium composition modify the profile of antioxidant enzymes during in vitro rhizogenesis of pink lapacho

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Abstract Optimal in vitro plant growth can be stimulated by selecting specific nutritional and environmental conditions. However, the culture conditions, dissection, and disinfection of plant material are stressful and may induce disruption of the plant physiological homeostasis. This can be modified by inoculation with rhizobacteria as *Azospirillum brasilense*, by the culture medium type, and by auxin induction. Here, we performed rooting experiments in two auxin-free culture media with 'pink lapacho' (*Handroanthus impetiginosus*) shoots previously induced with 0, 10, 30, or 50 µM indole butyric acid (IBA) for 3 days and inoculated with *A. brasilense* Cd and Az39. Peroxidase (PO), phenylalanine ammonia-lyase (PAL), and polyphenol oxidase (PPO) activities were determined on days 0, 3, 6, 9, 12, and 15. Also, weekly absolute rooting percentage was evaluated. All enzymatic activities were higher in *A. brasilense*-inoculated shoots, linked to early and high rooting percentage. The culture medium type and IBA concentration also affected enzymatic activities. The positive correlation between PO and PAL activities on day 9 and successful final in vitro rooting of *H. impetiginosus* allows using these activities as early markers of rhizogenesis reducing the selection time of easy-to-root plants. The changes in enzymatic levels performed here are discussed on the basis of their role in rooting and in vitro stress and contribute to the knowledge of the physiology of trees and their interaction with rhizobacteria.

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Keywords *Handroanthus impetiginosus* · In vitro rooting · Peroxidase · Phenylalanine ammonia-lyase · Polyphenol oxidase

Abbreviations

IAA	Indol-3-acetic acid
IBA	Indole-3-butiryc acid
PAL	Phenylalanine ammonia-lyase
PO	Peroxidase
PPO	Polyphenol oxidase
PGPR	Plant growth promoting rhizobacteria
ROS	Reactive oxygen species
½WPM	Half-strength woody plant medium
½MSG	Half-strength Murashige and Skoog salts with Gamborg's vitamins

Introduction

Micropropagation is one of the techniques recommended for proliferation of *Handroanthus impetiginosus* (Mart. ex DC.) Mattos (Bignoniaceae), "pink lapacho" a forest tree used as an ornamental plant (Larraburu et al. 2012). In vitro plants grow on nutrient-rich media, under aseptic conditions, with low light intensity and high relative humidity, being continuously exposed to a microenvironment selected to stimulate optimal growth. However, these culture conditions, together with mechanical perturbation, injuries, wounding due to dissection and surface disinfection of plant material, are stressful and may induce a disruption of physiological homeostasis and the release of reactive oxygen species (ROS), which cause oxidative damage (Gaspar et al. 2002). Plants defend from oxidative damage by a broad spectrum of ROS-scavenger systems, including antioxidant enzymes such as superoxide dismutase, peroxidase (PO),

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catalase, phenylalanine ammonia-lyase (PAL), and polyphenol oxidase (PPO) (Cassells and Curry 2001; Rout 2006; Fu et al. 2011).

ROS are traditionally considered as toxic products of aerobic metabolism, but it has been shown that plants also actively produce ROS as important signaling molecules in plant growth and differentiation, modulating processes such as mitosis, cell elongation, adventitious root formation, root hair development, xylem differentiation, programmed cell death, somatic embryogenesis, and response to biotic and abiotic stress (Miller et al. 2008; Jariteh et al. 2015).

Plant peroxidases, which catalyze the oxidation of numerous electron-donor molecules by hydrogen peroxide, play a fundamental role in adventitious root formation (Hatzilazarou et al. 2006). This process consists of successive physiologically interdependent phases, which can be summarized as induction, initiation, and expression. In the induction phase, due to the action of auxin and compounds released by the injured explant, molecular and biochemical events that induce rooting are triggered. The initiation phase is characterized by active cell division and by the organization of root primordia, which emerge from shoots and elongate during the expression phase (Kevers et al. 2009; Li et al. 2009; Pijut et al. 2011). Gaspar et al. (1992, 1997, 2002) have demonstrated that the root induction phase is characterized by an early decrease in PO activity, with higher values in the initiation phase and a gradual decrease in PO activity in the expression phase. Also, plant tissues exposed to stress conditions such as culture initiation (handling, tissue cut, and transplant) increase PO production (Cassells and Curry 2001), which stimulates the oxidative catabolism of auxins. Primordia are formed when endogenous auxin concentration decreases; consequently, the production of antioxidant enzymes modulates rhizogenesis (Kevers et al. 2009). In addition, the characteristics of the PO peak such as precocity of appearance, height, and velocity of decrease have been correlated with subsequent rooting performance (Gaspar et al. 1992, 2002). Thus, plant peroxidases have been proposed as biochemical markers of the successive rooting phases (Gaspar et al. 1992; Metaxas et al. 2004; Hatzilazarou et al. 2006). Similarly, the PAL and PPO activities are altered by different morphogenetic and stress process (Cassells and Curry 2001; Rout 2006; Fu et al. 2011).

Azospirillum brasilense is a plant growth-promoting rhizobacterium (PGPR) that improves plant growth and increases resistance to biotic and abiotic stress factors in some plants. This improvement is due to phytohormone production and induction of plant systemic resistance, among others process (Perrig et al. 2007). Antioxidant enzymes such as PO, PAL, and PPO, have been involved in defense reactions against biotic and abiotic stress (Chen et al. 2000). Although the induction of defense enzymes has been well studied in pathogen-plant interactions, little is known about PGPR ability to stimulate antioxidant enzymes and other defense-related compounds.

Summarizing, the stress caused by in vitro nutritional, hormonal and environmental conditions results in metabolic changes that are expressed by variations in the enzymatic profile of micropropagated plants. Also, the PGPR interaction may modify this profile. The aim of the present work was to study the variation of activities of PO, PAL and PPO during in vitro rooting of *H. impetiginosus* induced with different indole-3-butyric acid (IBA) concentrations in two culture media, and inoculated or not with the strains Cd or Az39 of *A. brasilense* and their correlation with rooting ability. In this sense, the study of biochemical indicators will contribute to the knowledge of the physiological processes that occur during in vitro rooting and the plant-PGPR interaction of woody species.

Materials and methods

Plant material and culture conditions

In vitro rooting of *H. impetiginosus* was achieved by the micropropagation protocol described previously (Larraburu and Llorente 2015a). Briefly, shoots with two to three nodes were excised from 4-week-old in vitro subcultures and transferred into two half-strength culture media: woody plant medium (½WPM) (Lloyd and McCown 1980) and Murashige and Skoog salts (1962) with Gamborg's vitamins (Gamborg et al. 1968) (½MSG). The media were supplemented with 100 mg L^{-1} myoinositol, 20 g L^{-1} sucrose, and 6 g L^{-1} agar (Britania, Argentina), and the pH adjusted to 5.8 before autoclaving. Flat-bottom glass tubes (100 × 25 mm) containing 15 mL of treatment media were used. The media were autoclaved at 121 °C for 20 min. One explant per tube was incubated in a growth chamber at 25 ± 2 °C with 55–60 % relative humidity under Phillips fluorescent daylight tubes (50 ± 5 µmol m^{-2} s^{-1}) with a 16-h photoperiod until the end of the experiment.

Bacterial strains and culture conditions

Bacterial inocula were prepared using *Azospirillum brasilense* Cd (ATCC 29710) and Az39 (locally isolated), as previously described in Larraburu and Llorente (2015a). Bacteria were grown in Okon´s liquid medium (Okon et al. 1977) and incubated for 72 h at 32 ± 1 °C (stationary phase of bacterial growth). Viable cell counts were evaluated on plaques with Congo red medium supplemented with 2 % agar (Rodríguez-Cáceres 1982) cultured at 37 °C for 48 h.

Rooting experiments

To induce rooting, each shoot was cultured for 3 days in tubes containing ½WPM or ½MSG with 0, 10, 30, or 50 µM IBA. Then, shoots were transferred to ½WPM or ½MSG auxin-free media for root development. Inoculation was carried out at the time of transferring induced shoots to the auxin-free media by adding 10^7 c.f.u. mL^{-1} of Cd or Az39 *A. brasilense* cultures at the base of the shoot. Controls consisted of treatments without bacterial inoculation. Absolute rooting for each week was determined as the relation between number of new rooted shoot and total shoots and was expresed as percentage. This parameter was determined weekly after inoculation until day 38 of culture.

Biochemical studies

Plant extract

H. impetiginosus shoots preserved in liquid nitrogen (−180 °C) were ground in a mortar, resuspended in 1 mL of 10 mM sodium phosphate buffer (pH 6), filtered and centrifuged at 4 °C and 12,000×*g* for 20 min. The supernatant was stored at −20 °C until analysis. Enzymatic determinations were performed according to Chen et al. (2000) with modifications, on days 0, 3, 6, 9, 12, and 15 on shoots from all treatments of rooting experiments.

Protein content

The soluble protein concentration was determined by Bradford's method (Bradford 1976) with bovine serum albumin as a standard.

Peroxidase (PO) activity (EC 1.11.1.7)

Enzymatic extract samples (100 µL) were diluted 20-fold with 10 mM sodium phosphate buffer (pH 6), and mixed with 20 µL of guaiacol and 50 µL of H_2O_2 0.88 M. The rate of increase in tetraguaiacol absorbance at 470 nm was measured at 30, 45, and 60 s. PO activity was expressed as the increase in absorbance per unit time and mg protein in each plant sample (ΔAbs_{470} min^{-1} mg^{-1} protein).

Phenylalanine ammonia-lyase (PAL) activity (EC 4.3.1.5)

The enzymatic extract (200 µL) was mixed with 200 µL of L-phenylalanine 12 mM and 4 mL 0.5 M Tris–HCl buffer. The mixture was incubated at 37 °C for 60 min. The amount of trans-cinnamic acid formed from L-phenylalanine was measured by spectrophotometry at 290 nm. PAL activity was expressed as the increase in absorbance per mg protein in each plant sample (ΔAbs_{290} mg^{-1} protein).

Polyphenol oxidase (PPO) activity (EC 1.14.18.1 o EC 1.10.3.2)

The enzymatic extract (100 µL) was mixed with 2000 µL of 10 mM sodium phosphate buffer (pH 6) and 200 µL of catechol 0.4 M. The mixture was incubated at 37 °C for 2 h and absorbance was determined at 420 nm. PPO activity was expressed as the increase in catechol-oxidation absorbance per mg protein in each plant sample (ΔAbs_{420} mg^{-1} protein).

Statistical analysis

A completely randomized design with three replicates and 15 to 20 shoots per treatment was conducted. The activities of PO, PPO, and PAL were determined in three shoots per replicate for each treatment. A repeated measures design to analyze the effect of IBA concentration (0, 10, 30, and 50 µM IBA), bacterization (non-inoculated and inoculated with *A. brasilense* Cd or Az39), and medium composition (½WPM and ½MSG) was used. Means were compared by Tukey's test. Pearson´s correlation analysis was performed between PO, PPO, and PAL activities (6, 9, 12, and 15 days) and final rooting percentage (38 days). Statistical analyses were performed using SPSS *v* 21 software. Antioxidant enzymes index (AEI) was performed by adding standardized PO and PAL activities as follows: $AEI(k) = \Sigma(X_i(k) - GM_i)/SG_i$; where: $X_i(k)$: value of enzymatic activity i for case k; GM_i: grand mean for enzymatic activity i; SG_i: overall standard deviation for enzymatic activity i; i: 1 = PO, 2 = PAL. Standardized PO and PAL activities allowed to compare enzymatic activity results to a normal distribution.

Results

Repeated measures analysis during the in vitro rooting of *H. impetiginosus* showed that PO, PPO, and PAL activities were significantly affected ($p \leq 0.08$) by the medium type × bacterization × IBA concentration triple interaction (Table 1).

Shoots induced with 0 and 50 µM IBA in ½WPM showed two PO activity peaks on days 3 and 12, whereas those induced in ½MSG showed only the first PO peak on day 3 (Fig. 1a, b). Maximum absolute rooting percentage (not cumulative) with 0 µM IBA occurred on day 17 in ½WPM (Fig. 2a) and on day 24 in ½MSG (Fig. 2b), whereas that with 50 µM IBA occurred on days 10–17 and 10–24, respectively (Fig. 2a, b).

The second PO peak in shoots induced with 50 µM IBA on ½WPM occurred earlier (3 days) upon inoculation with *A. brasilense* Cd and an increase of 66 % in absolute rooting on day 17 relative to non-inoculated shoots (Figs. 1a, c, 2a, c). In ½MSG, precocity was observed in all IBA induction

Table 1 Factorial repeated measure analysis of enzimatic activities and protein content of *Handroanthus impetiginosus* shoots during in vitro rooting with different medium types and indole butiric acid (IBA) concentrations, and with or without bacterization

Source	df	F			
		PO	PAL	PPO	Proteins
Medium type (M)	1	6.8*	1.3	5.4*	2.8
IBA concentration (I)	5	3.8*	10.9**	4.6**	12.9**
Bacterization (B)	2	2.2	6.6**	3.3*	1.9
M×I	5	11.8**	3.7**	1.6	4.8**
M×B	2	3.9*	0.6	1.7	4.7*
I×B	10	3.4**	4.3**	1.1	0.8
M×I×B	10	2.0	4.0**	3.2*	0.7

df degree of freedom, *M* two levels (½MSG and ½WPM), *I* six levels (0, 10, 30, and 50 µM IBA), *B* Three levels (non-inoculated, inoculated with *A. brasilense* Cd and inoculated with *A. brasilense* Az39), *PO* peroxidase, *PAL* phenylalanine ammonia-lyase, *PPO* polyphenol oxidase

*Significant differences at $p \leq 0.05$

**Significant differences at $p \leq 0.01$

treatments inoculated with *A. brasilense* Cd (Fig. 1b, d). Also, absolute rooting percentage increased by 37–93 % in ½ WPM and ½ MSG, respectively, on day 10 of culture in shoots induced with 30 µM IBA and inoculated with *A. brasilense* Cd (Fig. 2a–d). PO activity of IBA-non-induced *A. brasilense* Cd-inoculated shoots cultured in ½MSG increased by 3.4-fold on day 12 relative to controls, whereas PO activity of shoots induced with 10 µM IBA increased by 18-fold on day 9. These treatments increased absolute rooting percentage on day 17 by 322 and 457 %, respectively. PO activity was also increased by induction with 30 and 50 µM IBA in ½MSG (41 and 12-fold respectively) on days 9 and 12, respectively (Fig. 1b, d) with absolute rooting percentage increased 93 and 73 % on day 10 and 44 and 15 % on day 17, respectively (Fig. 2b, d).

Az39 strain inoculation in shoots induced with 10 µM IBA in ½MSG led to an increase of 33-fold in PO activity on day 9 and to an increase of fivefold in absolute rooting on day 17, relative to non-inoculated controls (Figs. 1b, f, 2b, f). Inoculation with this strain in shoots induced with 50 µM of IBA in ½MSG produced a wide PO activity peak between days 3 and 9, in correlation with an increase of 1.3-fold in absolute rooting on day 17 compared to non-inoculated controls (Figs. 1b, f, 2b, f).

Regarding PAL activity, most treatments showed a peak activity on day 3 with variable magnitude according to the concentration of IBA. The highest value was obtained in shoots induced with 10 µM IBA (Fig. 3). In ½WPM, non-inoculated shoots induced with 10 and 50 µM IBA showed a second and higher PAL peak activity on day 12 than those cultured in ½MSG with the same IBA concentration (Fig. 3a, b).

PPO activity of non-inoculated shoots induced with 50 µM IBA and grown in ½WPM quadrupled PPO activity on day 12 relative to those observed in the other treatments (Fig. 4a, b). Also, PAL and PPO activities differed between the culture media. In the absence of hormonal induction, shoots inoculated with the Cd strain in ½WPM showed high PAL and PPO activities. In this sense, enzymatic activity remained high between days 3–6 and 12 (Figs. 3c, 4c). Instead in ½MSG, non-induced shoots inoculated with this strain had a PPO activity peak on day 12 and two PAL activity peaks on days 3 and 12 (Figs. 3d, 4d). The Az39 strain in both culture media generally induced lower PPO activity than the Cd strain and two peaks of PAL activity on days 3 and 9, with higher values of the second peak in shoots induced with 10 and 50 µM IBA in ½MSG (Figs. 3c–f, 4c–f). This corresponded with increases of 5.6 and 1.3-fold, respectively, in the absolute rooting of Az39-inoculated shoots in ½MSG, relative to controls (Fig. 2b, f).

The correlation analysis showed that PO and PAL activities on day 9 were positively correlated with final rooting percentage ($p \leq 0.05$) (Table 2). In this sense, the antioxidant enzymes index was positive when the final rooting percentage was higher than 80 % (Fig. 5). In addition, PAL and PPO activities were positively correlated at all the times evaluated (Table 2). Although some treatments showed high PO, PAL, and PPO activities, no darkening or tissue necrosis were observed during rhizogenesis of pink lapacho (Fig. 5).

Discussion

Some physiological, biochemical, and molecular markers have been associated with developmental and stress processes. In this sense, the evolution of the antioxidant enzymes during culture allows using them as markers of the cell rhizogenic competence and of the sensitivity of cultured shoots to in vitro stress conditions (Bagnoli et al. 2001; Rout 2006; Fu et al. 2011).

Repeated measures analysis allows evaluating the effects of the factors studied (medium type, hormonal induction,

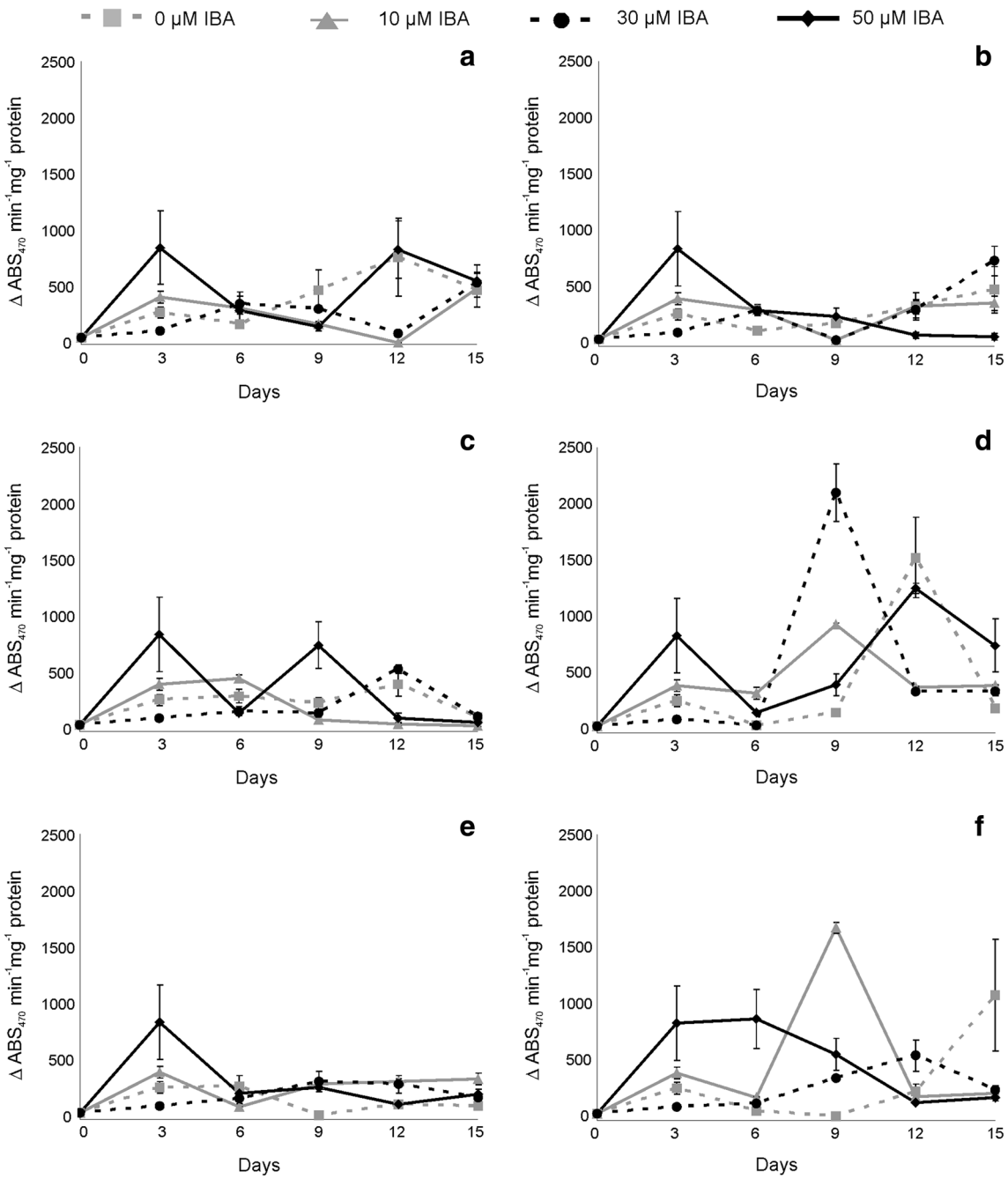

Fig. 1 Peroxidase activity as a function of time for in vitro shoots of *Handroanthus impetiginosus* considering the IBA concentration (0, 10, 20, 30, 40, or 50 µM), the culture media (½MSG or ½WPM), and the bacterization level (uninoculated or inoculated with *Azospirillum brasilense* Cd or Az39 strains). **a, c, e** ½WPM medium; **b, d, f** ½MSG medium; **a**–**b** uninoculated shoot; **c**–**d** shoots inoculated with *A. brasilense* Cd; **e**–**f** shoots inoculated with *A. brasilense* Az39

and *A. brasilense* inoculation) by using parameters sequentially measured in the same subject over time. The advantage of a repeated measures ANOVA over an independent ANOVA is that it is generally more powerful and accurate (Kuehl 2000). In the present study, this analysis allowed determining the significant effect of the triple interaction between culture medium composition, IBA concentration and bacterization on PO, PAL, and PPO activities. The interaction was correlated with changes in the enzymatic expression patterns of the different treatments.

The characteristics of the PO activity peak have been correlated with subsequent rooting performance (Gaspar et al. 1992, 1997, 2002). The positive correlation between PO activity and rooting percentage of *H. impetiginosus*

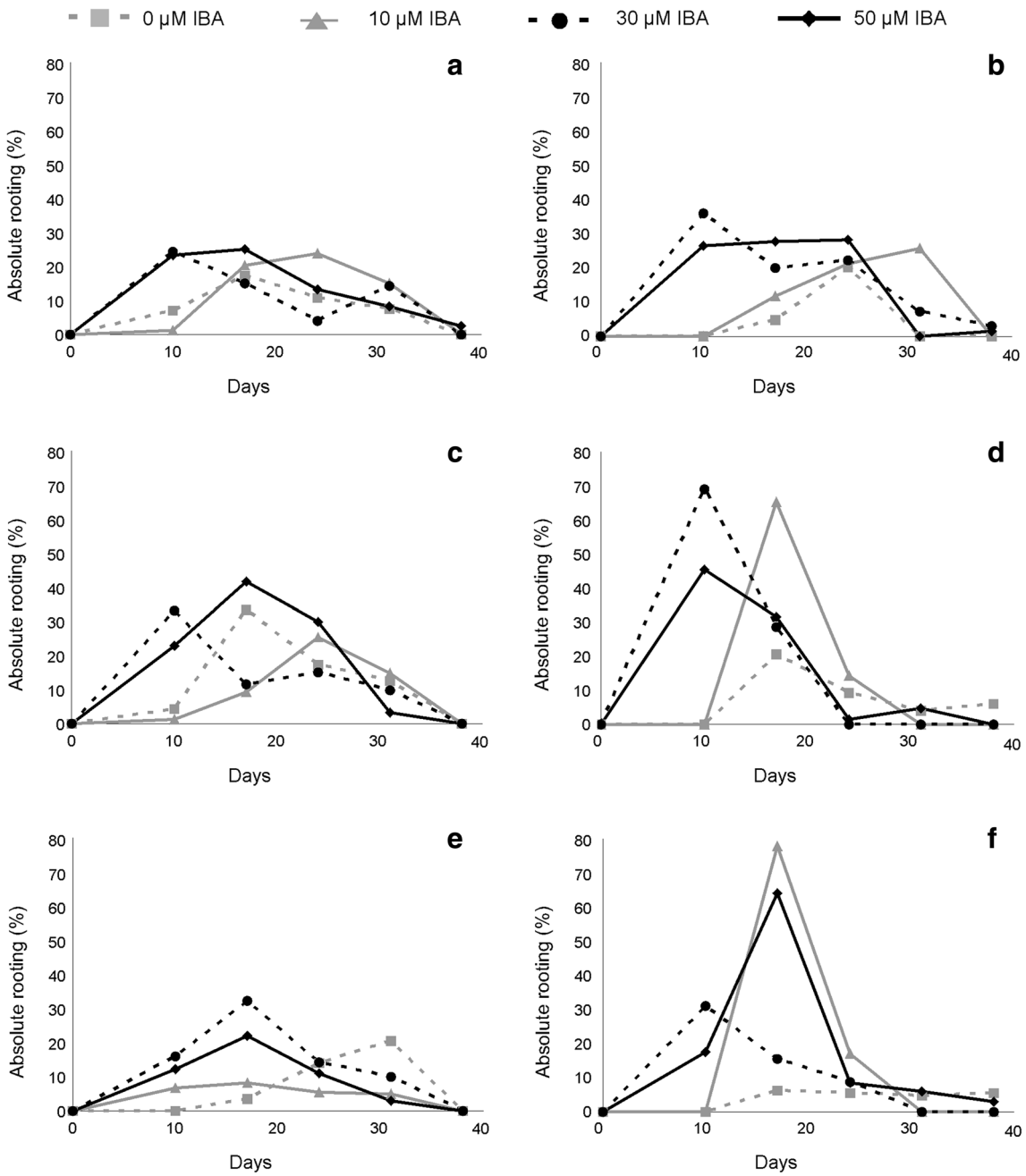

Fig. 2 Absolute rooting percentage (not cumulative) as a function of time for in vitro shoots of *Handroanthus impetiginosus* considering the IBA concentration (0, 10, 20, 30, 40, or 50 µM), the culture media (½MSG or ½WPM), and the bacterization level (uninoculated or inoculated with *Azospirillum brasilense* Cd or Az39 strains). **a, c, e** ½WPM medium; **b, d, f** ½MSG medium; **a**–**b** uninoculated shoot; **c**–**d** shoots inoculated with *A. brasilense* Cd; **e**–**f** shoots inoculated with *A. brasilense* Az39

observed in the present study is in agreement with that reported for *Prunus dulcis* (Caboni et al. 1997), *Taxus baccata* (Metaxas et al. 2004), *Gardenia jasminoides* (Hatzilazarou et al. 2006), and *Mucuna pruriens* (Li et al. 2009). Also, the PO activity of pink lapacho generally showed changes similar to those described by Gaspar et al. (1992, 2002): a low value in the induction phase, an increase (peak) in the initiation phase and a gradual decrease in the expression phase. The duration of these phases is affected by the plant species and culture conditions (Hatzilazarou et al. 2006; Metaxas et al. 2004). The increase in PO activity in the induction phase may be explained by cell protection against the overproduction of ROS induced by the stressful environmental conditions of in vitro culture. In addition, high PO activity is required to decrease IAA (indol-3-acetic acid) concentration during root expression because

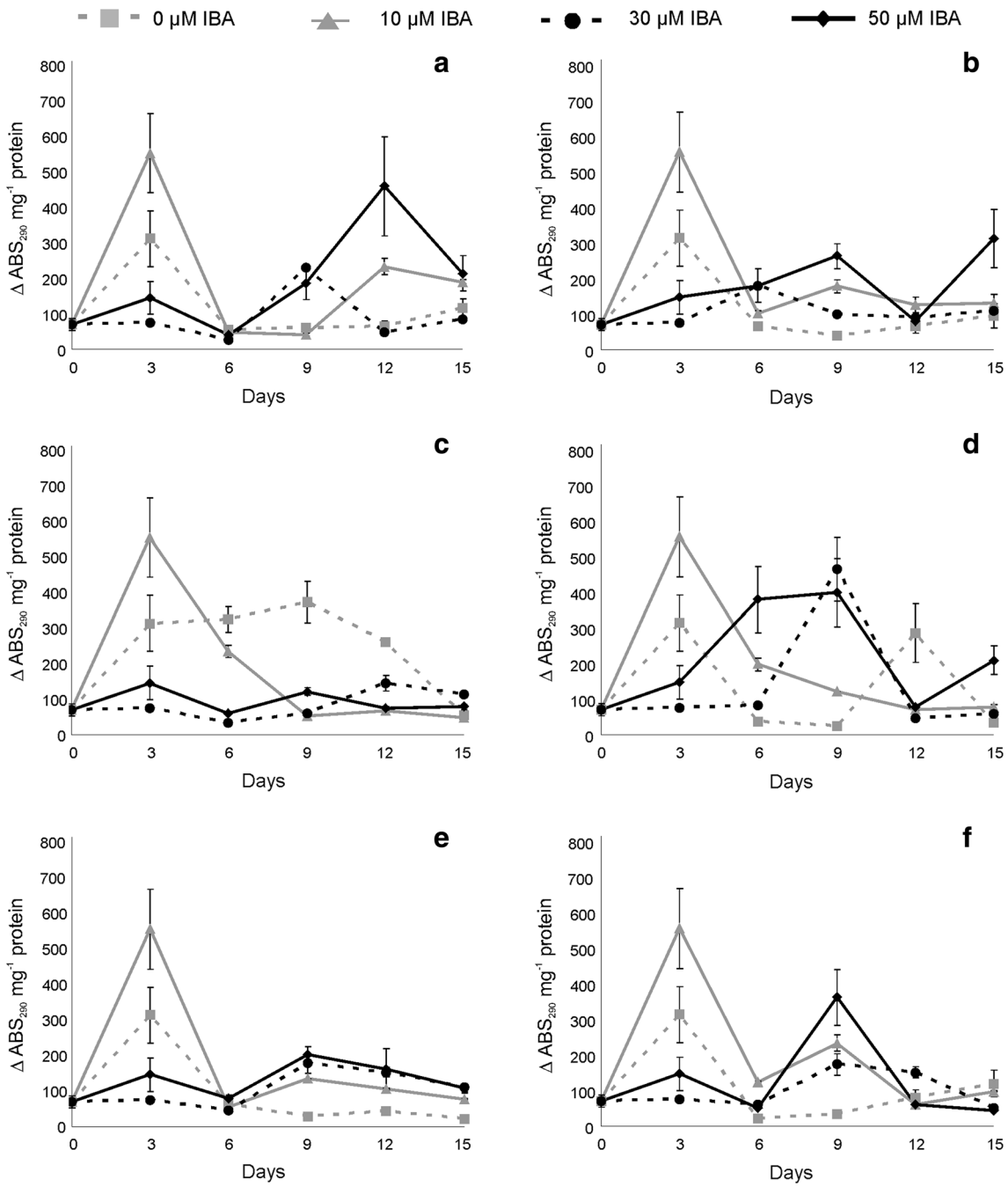

Fig. 3 Phenylalanine amonia-lyase activity as a function of time for in vitro shoots of *Handroanthus impetiginosus* considering the IBA concentration (0, 10, 20, 30, 40, or 50 μM), the culture media (½MSG or ½WPM), and the bacterization level (uninoculated or inoculated with *Azospirillum brasilense* Cd or Az39 strains). **a, c, e** ½WPM medium; **b, d, f** ½MSG medium; **a–b** uninoculated shoot; **c–d** shoots inoculated with *A. brasilense* Cd; **e–f** shoots inoculated with *A. brasilense* Az39

continuous auxin exposure may inhibit root emergence (Gaspar et al. 2002; Kevers et al. 2009; Li et al. 2009). *H. impetiginosus* rooting showed two PO activity peaks in non-induced shoots cultured in ½WPM, which were correlated with precocity of maximum absolute rooting percentage relative to shoots cultured in ½MSG, which did not show the second peak. The second PO activity peak at the beginning of the root expression phase correlates with root emergence (Kevers et al. 2009) and may be linked with the positive correlation observed in *H. impetiginosus* between PO activity on day 9 and final rooting percentage. In addition, the overproduction of the PO has been linked with precocity in rhizogenesis (Gaspar et al. 1992). Shoots inoculated with Cd or Az39 *A. brasilense* generally showed

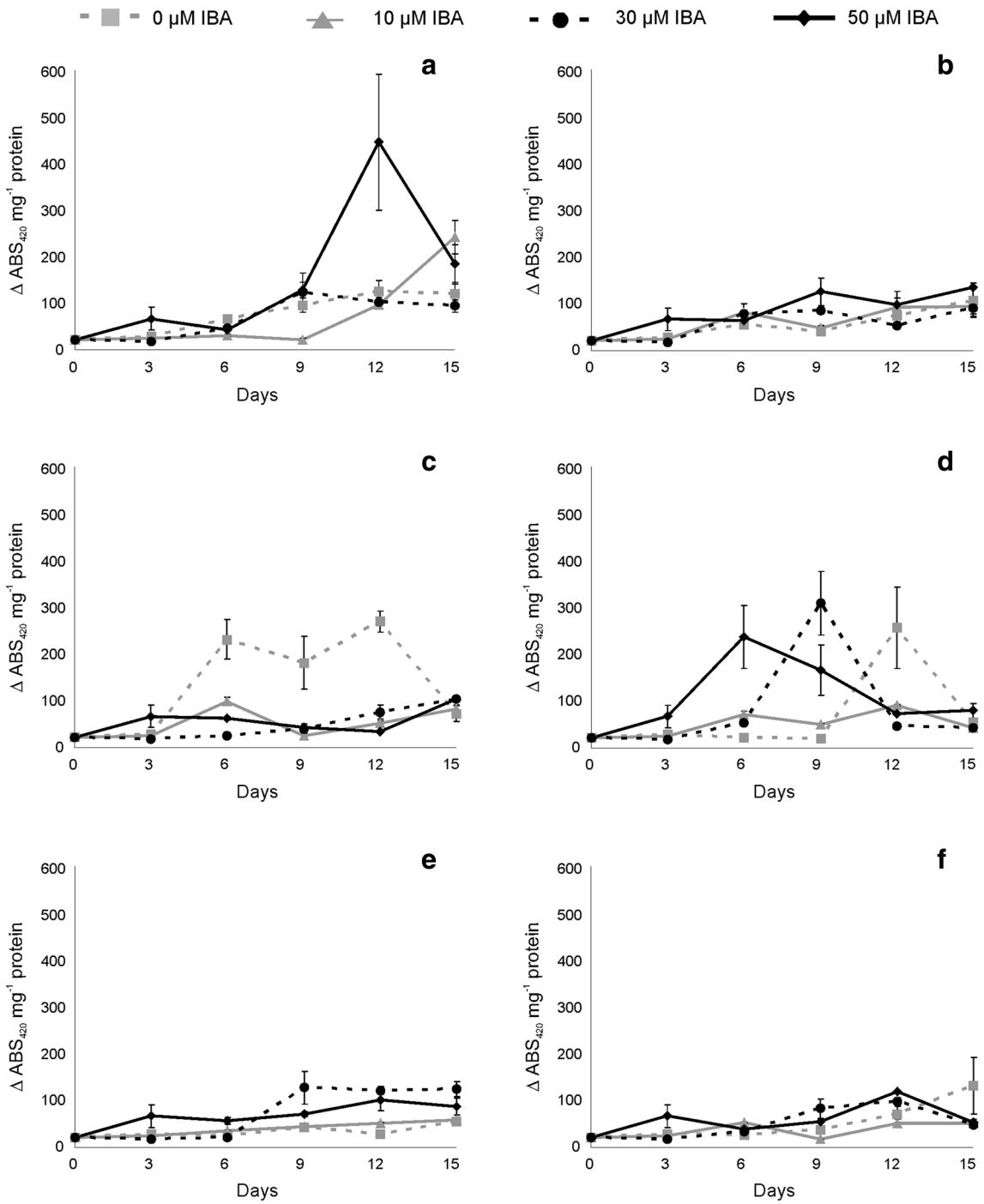

Fig. 4 Poliphenoloxidase activity as a function of time for in vitro shoots of *Handroanthus impetiginosus* considering the IBA concentration (0, 10, 20, 30, 40, or 50 μM), the culture media (½MSG or ½WPM), and the bacterization level (uninoculated or inoculated with *Azospirillum brasilense* Cd or Az39 strains). **a, c, e** ½WPM medium; **b, d, f** ½MSG medium; **a–b** uninoculated shoot; **c–d** shoots inoculated with *A. brasilense* Cd; **e–f** shoots inoculated with *A. brasilense* Az39

higher and earlier appearance of the second PO peak than non-inoculated ones, showing increases of up to 565% in absolute rooting at 17 days of culture, with some differential responses according to the strain, culture medium, and IBA concentration. The increase in PO activity after inoculation with a PGPR has been described in several plant-bacteria interactions. In this sense, *Bacillus sphaericus* significantly increases PO activity in roots of in vitro culture of *Musa paradisiaca* (Mahmood et al. 2010) and different strains of *Pseudomonas* sp. increase PO activity relative to controls in chili pepper, tomato, and cucumber (Chen et al. 2000; Sharma et al. 2007).

Table 2 Pearson's correlation coefficients between peroxidase (PO), phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO) activities (6, 9, 12, 15 days) and final in vitro rooting percentage of *Handroanthus impetiginosus* after 38 days in culture, considering all treatments

Days	PO	PAL	PPO
PAL			
6	0.13	1	
9	0.53**	1	
12	0.42*	1	
15	0.26	1	
PPO			
6	0.07	0.92**	1
9	0.43*	0.77**	1
12	0.48*	0.90**	1
15	0.29	0.66**	1
Final rooting percentage			
6	0.35	0.31	0.22
9	0.57**	0.62**	0.39
12	−0.10	−0.08	−0.02
15	−0.22	0.19	−0.11

*Significant differences at $p \leq 0.05$

**Significant differences at $p \leq 0.01$

The higher PAL activity peaks observed on day 3 in non-induced pink lapacho shoots treated with low concentrations of IBA could be due to the stress of in vitro initiation culture because PAL activates some pathways of electron scavenger molecules, such as flavonoid and anthocyanins (Balen et al. 2009). Some studies have described that auxin is involved in adaptive responses to biotic and abiotic stress (Wang et al. 2009) and could explain the decrease in PAL activity observed in pink lapacho shoots treated with higher IBA concentrations. The second PAL activity peak observed in some treatments could be linked to the involvement of PAL in lignin formation during rooting, as observed in micropropagated walnut shoots (Bisbis et al. 2003). This could be linked to the positive correlation observed by us between PAL activity on day 9 and final rooting percentage. In addition, high PAL activity may be explain the highest secondary stem growth observed by Larraburu and Llorente (2015b) in pink lapacho inoculated with *A. brasilense*.

PPO catalyzes the oxidation of a wide variety of phenolic compounds needed for root differentiation (Rout 2006). The excessive increase in PPO may cause browning and death of tissue by oxidation of phenolic compounds, which could be attenuated by decreasing the substrates or cofactors necessary for oxidation. For example, lower salt concentration in the culture medium reduces the darkening and wilting of *Pinus sylvestris, Begonia venous, Hevea brasiliensis*, and *Musa sp* tissues (Azofeifa 2009). In the present study, no darkening or tissue necrosis were observed during rhizogenesis of pink lapacho, indicating that the half salt concentration of the culture media used was suitable to prevent the oxidation of phenolic compounds.

Azospirillum brasilense increased PO, PAL, and PPO activities of *H. impetiginosus* shoots, similarly to that observed in cucumber roots inoculated with some *Pseudomonas* strains (Chen et al. 2000). PO and PAL activities have also been found to be higher in micropropagated *Camellia sinensis* plants treated with microbial inoculants (*Pseudomonas fluorescens* and *Trichoderma harzianum*) than in controls (Thomas et al. 2010). The increased activity of these enzymes triggered by some non-pathogenic rhizobacteria has been linked to the production of induced systemic resistance, which increases defense capability without causing symptoms of necrosis in the host plant (Podile and Kishore 2006). The differential enzymatic kinetics of PO, PPO, and PAL when inoculating with the Cd or Az39 strains of *A. brasilense* could be linked to differences in the secretion of phytohormones of each strain. IAA production by *A. brasilense* Cd, cultured in chemically defined medium, is almost four times higher than that by Az39 under the same conditions (Perrig et al. 2007). In addition, in the present study, differences between PO curves of inoculated *H. impetiginosus* shoots induced with IBA were higher than those of non-inoculated shoots, a result that could be correlated with an increase in auxin sensitivity due to bacterial inoculation. These facts are in agreement with a decrease in IBA concentration requirement for maximum rooting and increase of root hair development due to *A. brasilense* inoculation (Larraburu and Llorente 2015a, b).

In this study, in some treatments, the culture medium composition affected PO, PAL, and PPO activities, rhizogenesis and precocity. Differences in PO activity have also been observed in *Kalmia latifolia* cultured in different media, a result attributed to variations in the NH_4NO_3

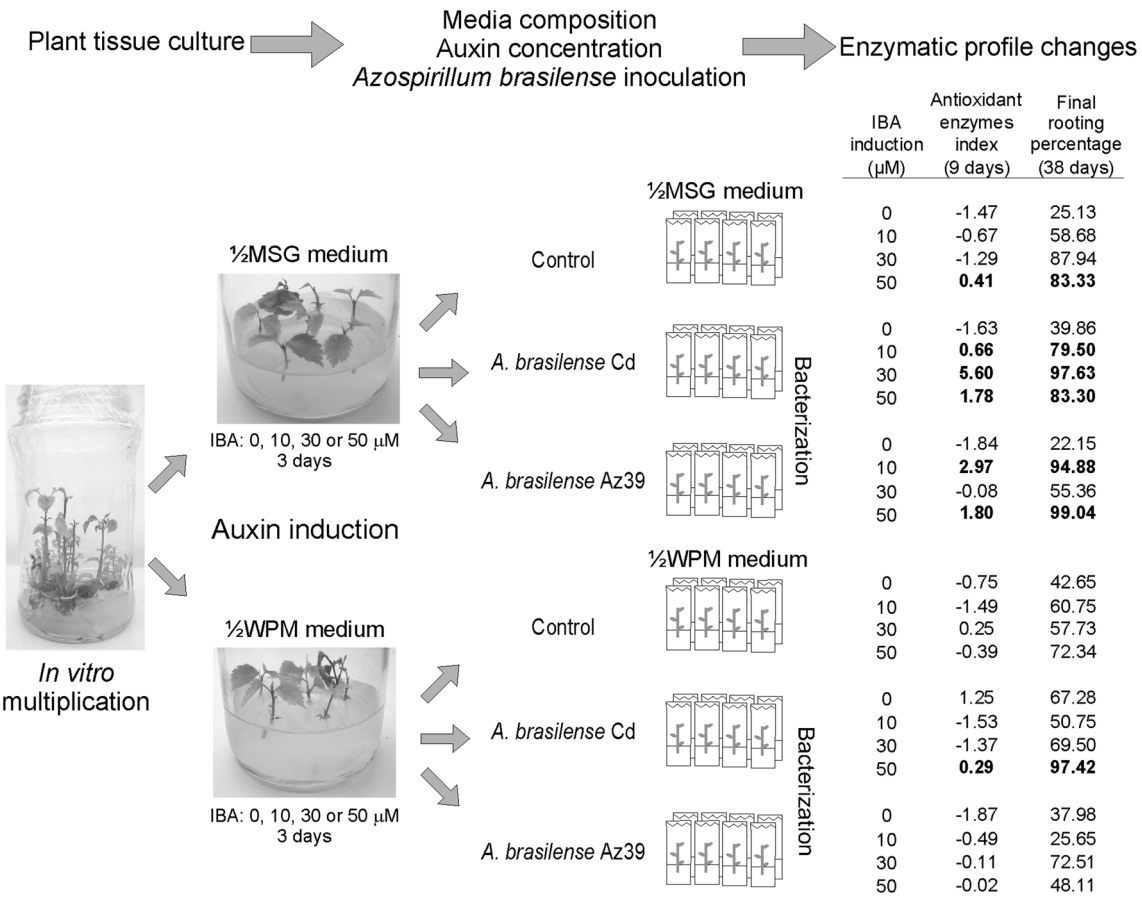

Fig. 5 Antioxidant enzymes index of *Handroanthus impetiginosus* in vitro rooting performed by adding standardized PO and PAL activities after 9 days of cultures and final rooting percentage after 38 days, considering all treatments. In *bold* positive enzymatic activity index correlated with final rooting percentage ≥80 %

and KNO_3 concentrations of the culture media used (Gaspar et al. 1992). The higher PAL activity peaks in ½WPM could be attributed to the presence of copper in ½WPM and its absence in ½MSG, in agreement with the strong PAL activity obtained by exposing *Matricaria chamomilla* roots to copper (Kováčik et al. 2010). Copper also increases PO and PPO activities in *Panax ginseng* (Ali et al. 2006).

The use of in vitro cell and tissue culture is a remarkable tool to understand the physiological, biochemical, and molecular regulation of plant development and stress response phenomena. In this sense, the biochemical studies carried out by us in pink lapacho allowed detecting an interaction between IBA concentrations, *A. brasilense* inoculation and nutritional conditions during in vitro rooting, which was reflected in variations in the enzymatic profile and rooting percentage. Our results suggest that changes in the biochemical status of plants, such as an increase in the levels of PO, PAL, and PPO, mitigate adverse effects of in vitro culture and development process. The evaluation of biochemical parameters as PO and PAL activities on day 9 as markers of rhizogenesis would allow enhancing the micropropagation of *H. impetiginosus* because these activities and successful in vitro rooting are positively correlated. Early determination of rooting performance allows reducing the selection time of easy-to-root clonal lines. In addition, the biochemical determinations carried out in this study contribute to better understanding how auxin induction, culture media and PGPR inoculation affect the physiology of woody plants.

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Author contributions EL and BL conceived and designed research idea. EL and MY executed the experiments. EL and BL analyzed the obtained data, supervised the work and wrote the manuscript. All authors approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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