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The possible bottleneck effect of polyamines' catabolic enzymes in efficient adventitious rooting of two stone fruit rootstocks

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ABSTRACT

Adventitious rooting is an important plant physiological response utilized in cutting propagation, a procedure with high financial significance. Many endogenous factors are involved, such as plant growth regulators, carbohydrates, minerals, polyamines etc. The objective of the present study was to investigate the role of polyamines and polyamine catabolic enzymes in the bases of softwood cuttings of two *Prunus* rootstocks, during the early phases of rhizogenesis. An easy-to-root and a difficult-to-root rootstock were studied, concerning their polyamine content (in free, soluble conjugate and insoluble bound form), polyamine catabolic enzyme activities (polyamine oxidase, PAO and diamine oxidase, DAO) and catalase activity, with and without the effect of indole-3-butyric acid as rooting hormone, during the early phases of rhizogenesis. Putrescine, spermine and their catabolic product, H_2O_2, were applied to test their function to rescue the rooting percentage of the recalcitrant species. Spermine was not detected in the difficult to root rootstock, which exhibited higher titer of putrescine and spermidine, PAO and catalase activity, but lower DAO activity compared to the easy-to-root one. The rooting percentage of the recalcitrant species was doubled under spermine and H_2O_2 application. The results obtained, highlighted the role of polyamine catabolic enzymes and indirectly the role of the polyamine catabolic product H_2O_2 as more significant than the polyamine content *per se* in adventitious rooting of the specific stone fruit rootstocks.

1. Introduction

Cutting propagation is broadly used in nurseries for propagating important horticultural crops (Hartmann et al., 2001) and it is based on adventitious root formation (ARF) property of the cutting. ARF in the stem base of cuttings involves sequential phases (induction, initiation and expression phase). It starts with the reprogramming of adventitious roots (AR) source cells and ends with the formation of new AR primordia and includes changes in cells structure, division and differentiation (Druege et al., 2019). However, there are differences in rooting capacity among species or even cultivars within the same species (Denaxa et al., 2014). Endogenous factors such as phenolic compounds, carbohydrates, auxins, minerals (da Costa et al., 2013) and polyamines (PAs) (Cristofori et al., 2010) can affect the behavior and rooting capacity of the cutting.

PAs are ubiquitous low molecular weight aliphatic compounds found in almost all living organisms (Abbasi et al., 2017). The main PAs in plants are the diamine putrescine (Put), the triamine spermidine (Spd) and the tetramine spermine (Spm). PAs are involved in many physiological processes such as morphogenesis, organ development, stress responses, growth, leaf senescence, cell signaling and proliferation, programmed cell death etc. (Lutts et al., 2012; Kusano et al., 2008) and are considered as plant growth regulators during ARF (Geny et al., 1997). They have been proposed as rooting markers since their accumulation has been positively correlated with the initial rhizogenesis stages (Neves et al., 2002; Couee et al., 2004).

Rooting induction is mainly related to increased level of free PAs (Kevers et al., 1997; Uribe et al., 2004), whereas bound PAs have been also reported to have a possible role in rhizogenesis (Biondi et al., 1990), which is not fully clarified yet (Burtin et al., 1990). Experiments on olive cuttings indicated that cultivars with increased total PAs content rooted in higher percentages than those with low one (Denaxa et al., 2014). Nag et al. (2001) working with mung bean, found indication that Put has a significant role among the PAs, since Spd and Spm endogenous levels did not change significantly throughout the induction and initiation phases. Similarly, Bonneau et al. (1995)

Abbreviations: Put, putrescine; Spd, spermidine; Spm, Spermine; PAO, polyamine oxidase; DAO, diamine oxidase; CAT, catalase; PAs, polyamines; DAP, days after planting; IBA, indole-butyric acid; Con, conjugate; Ins, insoluble

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reported increased Put levels during rooting initiation phase.

However, the effectiveness of exogenous PAs on inducing cuttings' rooting remains unclear. in vitro experiments have indicated a positive effect of PAs on microshoots rooting in some species, such as hazelnut and MM109 apple rootstock (Rey et al., 1994; Naija et al., 2009) irrespectively of the exposure duration of the explants to PAs. Dipping of the base of olive cuttings into a PUT solution had a positive effect on the difficult-to-root 'Kalamata' cultivar, as they increased rooting percentage (Denaxa et al., 2014), but Spd and Spm applications were ineffective (Rugini et al., 1990; Denaxa et al., 2014). Put application was also effective on promoting rooting of hazelnut cuttings after base dipping in the PA solutions (Cristofori et al., 2010), whereas Friedman et al. (1982) reported that all three Put, Spd, Spm failed to promote ARF in *Vigna radiata* after cutting immersion for 24 h.

PAs catabolism is mediated by polyamine oxidase (PAO; EC 1.5.3.11) and copper-containing amine oxidase (CuAO; DAO EC 1.4.3.6) (Tsaniklidis et al., 2016). The first one (PAO) is responsible for Spm and Spd catabolism and the back-conversion of Spm to Spd and then to Put (Shelp et al., 2012). On the other hand, DAO mediates the Put levels, while hydrogen peroxide (H_2O_2) is produced by the activity of both enzymes (Kusano et al., 2008; Moschou and Roubelakis-Angelakis, 2011; Tsaniklidis et al., 2016). Hydrogen peroxide has been involved in adventitious root formation (Li and Xue, 2010) and has been used for improving adventitious rooting in various species, such as olive and mung bean (Sebastiani and Tognetti, 2004; She et al., 2010). Catalase (EC 1.11.1.6) (CAT) catalyzes the breaking down of H_2O_2 to H_2O and O_2 and its role in the rooting process has not been fully clarified (Molassiotis et al., 2004).

The aim of the present study was: a) to assess the fluctuations of all forms of PAs concentration and DAO, PAO and CAT activities during the early phases of rhizogenesis between an easy- and a difficult-to root stone fruit rootstocks and b) to examine if the rooting of the difficult-to-root rootstock can be enhanced by PAs treatment based on the results of the prementioned biochemical analysis.

2. Materials and methods

2.1. Plant material and sampling

The experiment was conducted at the orchard of Agricultural University of Athens. The rootstocks selected were the easy-to-root 'Myrobalan 29C' (*Prunus cerasifera*) and the difficult-to-root 'GF 677' (*Prunus persica* x *Prunus amygdalus*). The mother stock plants were heavily pruned every year in order to induce the production of juvenile shoots. During early September softwood cuttings, 15–18 cm long, were collected from 7 year old mother plants. The cuttings were defoliated leaving only the upper two leaves. The base of each cutting was dipped in 2000 mg L^{-1} IBA (diluted in an aqueous solution of 30% ethanol) for 5 s, whereas cuttings dipped for 5 s in 30% aqueous ethanol served as control. Cuttings were then placed under mist system in a glasshouse using perlite as a substrate. The rooting percentage, the number and length of roots per rooted cutting were recorded two months after planting, in a total of 35 randomly selected cuttings per replication. Cuttings were exposed to diffused light due to the reflectance coverage of the glasshouse, while temperatures ranged from 24 °C to 32 °C.

For the polyamines assessment, samples of 25 cuttings' bases (1–1.5 cm long) per replication were collected at day 0 (day of cutting excision and planting-before auxin application), 1, 3, 5, 7 and 15 days after planting (DAP), whereas for DAO, PAO and CAT activity assays, seven cuttings' bases per replication were used. The samples were instantly frozen in liquid nitrogen and transferred to the laboratory. The samples for PAs analysis were lyophilized, ground into a fine powder and stored in a freezer (−25 °C). The samples used for the enzyme activity determination were stored at −80 °C for a few days until their assessment.

2.2. PAs extraction

The extraction of PAs was performed according to Pedrol and Tiburcio (2001). Approximately 100 mg of dried tissue was used. PAs were extracted with 1 ml 5% v/v cold perchloric acid (PCA) in water using 1.6 hexanediamine as internal standard. The mixture was let stand for 1 h at 4 °C in the dark after vortexing. Samples were centrifuged at 4000 rpm for 6 min. Both the supernatant and the pellet fractions were collected. Supernatant fraction was used for the estimation of free (F) and soluble conjugate (SC) polyamines whereas pellet fraction for the estimation of insoluble bound (IB) polyamines. For free PAs determination in 0.2 ml of supernatant 0.2 ml Na_2CO_3 (21 g 100 ml^{-1} H_2O) and 0.2 ml dansyl chloride (7.5 mg ml^{-1} acetone) were added and vortexed. After 24 h in the dark under room temperature, 0.1 ml proline (100 mg ml^{-1} H_2O) was added and the mixture was left to stand for 15 min under darkness. Then, 0.5 ml of toluene was added and the solution was vortexed for approximately 45 s. The two phases were separated and 0.4 ml of the upper phase was collected and evaporated to dryness under a stream of nitrogen in a water bath at 40 °C. The dry residues were dissolved in 0.5 ml HPLC grade methanol, filtered through a 0.45 μm nylon syringe filter and analyzed by HPLC. Aliquots of 0.2 ml supernatant and pellet fractions were hydrolyzed with 0.2 ml of HCl 12 N for 18 h at 110 °C, after pellet washing thrice with 1 ml PCA 5% v/v in water each time. After hydrolysis, the solution was evaporated in an oven at 70 °C. The residue was dissolved into 0.4 ml PCA 5% v/v in water and followed the same procedure as for free polyamines derivatization.

2.3. Separation and quantification of polyamines by HPLC

Polyamines analysis was performed by HPLC equipped with a fluorescence detector. The mobile phase consisted of 82% acetonitrile and 18% water; the following flow rate gradient was used: at 0 min till 7 min 0.8 ml min^{-1}; at 7.8 min till 11 min 1.0 ml min^{-1}; at 11.5 min till 28 min 1.8 ml min^{-1} and at 29 min 0.8 ml min^{-1}. The derivatized polyamines were separated using an Inertsil ODS-3 reverse phase column and detected by fluorescence detector HP 1048A adjusted at excitation wavelength of 360 nm and emission wavelength of 510 nm. A five point calibration curve was constructed using derivatized mixtures of known PAs concentrations. The individual free and soluble conjugate PAs concentrations were calculated using the relative response factor (RRF) obtained by the internal standard concentration, whereas the insoluble bound PAs concentration was estimated by external calibration curve. Final concentrations were expressed at mg g^{-1} d.w.

2.4. Extraction and assay of diamine oxidase (DAO) and polyamine oxidase (PAO) activities

PAO and DAO activity were estimated according to Nag et al. (2001) with some modifications. Briefly, cutting base tissue was homogenized in 4 ml of extraction buffer (consisted of 10^{-2} M catechol in phosphate buffer 50 mM, pH 7.0) using Ultra Turrax at 4 °C. Afterwards, the extraction mixture was centrifuged at 4.000 rpm for 5 min. The reaction mixture contained 3.48 ml of reaction phosphate buffer (40 mM, pH 7.4), 300 μl of extract and 120 μl of putrescine or spermine (100 mM) as substrate depending on the enzyme assayed (DAO or PAO respectively). The mixture was incubated at 37 °C for 45 min and the reaction was terminated by adding 0.1 ml $TiSO_4$ (0.5% w/v in 23% v/v sulphuric acid in water) forming a yellowish colour indicating the presence of H_2O_2. After 3 min, the mixture absorbance was measured at 410 nm. Each sample was self-blanked by adding $TiSO_4$ before enzyme addition forming a colourless solution. The enzyme activities were expressed as unit per min mg^{-1} of protein. One unit of DAO and PAO activity was defined as the amount of enzyme that caused an increase in absorbance of 0.01 per minute at 410 nm.

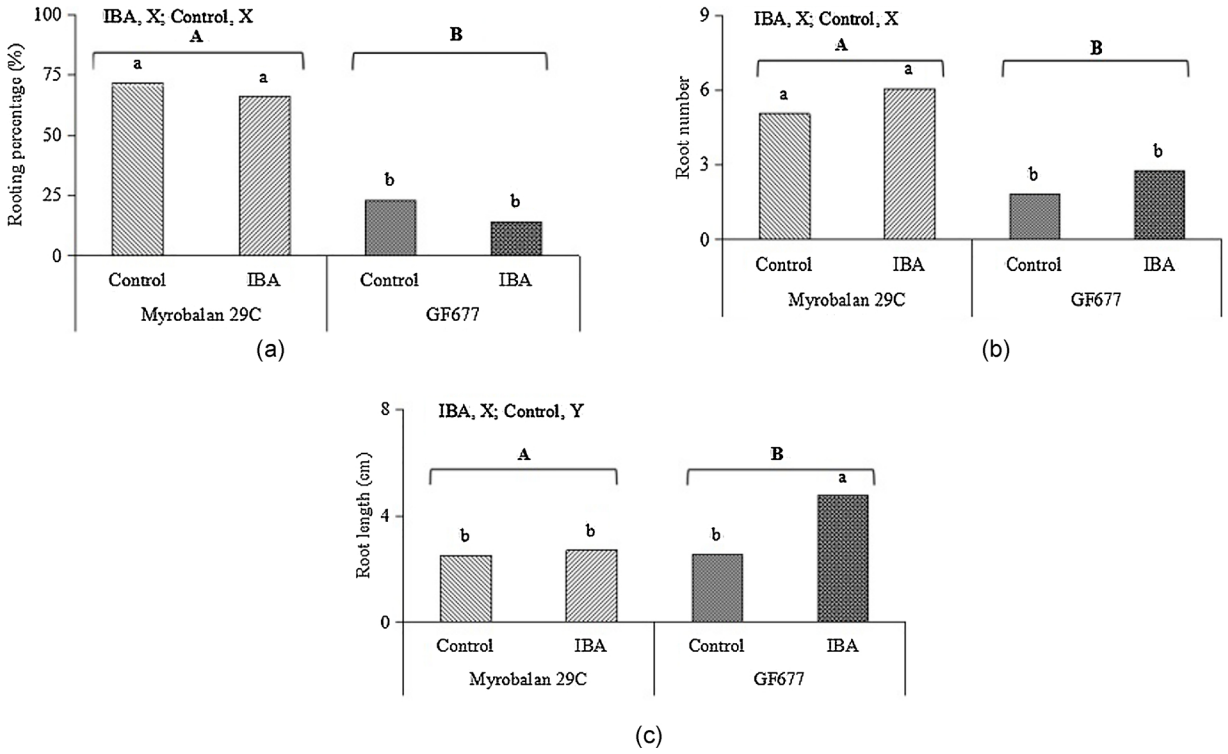

Fig. 1. Effect of rootstock, auxin treatment and their interaction in rooting parameters (rooting percentage, root number and root length per rooted cutting) of 'Myrobalan 29C' and 'GF 677' rootstocks two months after planting. Different lower-case letter above each column denote statistical difference between the treatments for the same rootstock according to Tukey's HSD test at $\alpha = 0.05$. Different upper-case letter above each rootstock columns denote statistical difference between the rootstocks according to Student's *t*-test at $\alpha = 0.05$. Different upper-case letter beside treatment levels (IBA, control) denote statistical difference between them according to Student's *t*-test at $\alpha = 0.05$.

2.5. Extraction and assay of catalase (CAT) activity

CAT extraction was performed according to Kochhar et al. (2008). Briefly, the cutting bases were homogenized using an Ultra-Turax with 100 mM phosphate buffer (pH 7.0 containing 2 mM EDTA, 1% w/v PVPP and 1 mM ascorbic acid). Catalase activity was estimated according to Góth (1991) with some modifications. Briefly, 200 µl of enzyme extract was incubated in 2 ml of reaction buffer (65 pmol per ml H_2O_2 in 60 mmol l^{-1} sodium-potasium phosphate buffer, pH 7.4) at 37 °C for 1 min. The enzymatic reaction was stopped with 1.0 ml of 32.4 mmol l^{-1} ammonium molybdate and the yellow complex of molybdate and H_2O_2 was measured at 405 nm (A_{sample}) against blank (reaction buffer without H_2O_2). The absorbance of reaction mixture containing H_2O_2 was also measured (A_{max}). The enzyme activity was expressed as unit per min mg^{-1} of protein. One unit of CAT activity was estimated from the difference in absorbance between A_{max} and A_{sample} and defined as 0.01 per minute at 405 nm between A_{max} and A_{sample}.

2.6. Determination of protein concentration

Protein content was determined by Bradford (1976) method. 0.1 ml from the enzyme extract was added in 5 ml of Coomassie brilliant blue G250 solution followed by addition of 0.9 ml of phosphate buffer (0.1 M, pH 7.2) and vortexed. After 3 min the absorbance was read at 595 nm. Known concentrations of bovine serum albumin (BSA) were used to construct the calibration curve.

2.7. Evaluation of PAs and H_2O_2 efficiency to inducing 'GF 677' cutting's rooting

In order to assess any possible effect of PAs and H_2O_2 on inducing rooting response in the cuttings of difficult to root 'GF 677', 15–18 cm long shoots were collected from the same mother plants. The cuttings were defoliated leaving two leaves at the top of each cutting. The base of each cutting was dipped into either a) Put or Spm solutions (0.5, 1, 10 and 20 mM) for 20 min or b) H_2O_2 solutions (of 1%, 2% and 3% v/v in water) for 20 s. Afterwards, the cuttings bases were dipped in 2000 mg L^{-1} IBA ethanolic solution (30% v/v in water) and planted in perlite substrate in the mist unit. Cuttings treated only with auxin served as control. Two months later, the rooting percentage, the number of roots and the length of roots produced were measured.

2.8. Statistical analysis

Statistical analysis was performed using JMP 8.0 statistical software (SAS Institute, NC, U.S.A.). Rooting percentage and PAs concentration were transformed to arcsin and log respectively.

In order to assess the effect of rootstock and auxin application on rooting parameters, the data were analyzed as two-way ANOVA with the factors being the rootstock ('Myrobalan 29C' and 'GF 677') and treatment (IBA 2000 mg L^{-1} and control).

The experiments on PAs concentration and enzyme activities fluctuations were arranged as completely randomized design with three replications. Data were analyzed as three-way ANOVA with the factors being the rootstock, treatment (auxin and control) and day after planting (DAP). Significant differences were determined by Tukey's HSD test ($\alpha = 0.05$). SE were calculated from the residual variances of the Multi Factor ANOVA and used to determine significant differences among means when factors' effect was significant.

For PAs and H_2O_2 cuttings treatments the experiment was arranged as completely randomized design with three replications of at least ten cuttings per plot. Raw data were analyzed as one-way ANOVA and the significant differences were determined by Tukey's HSD test ($\alpha = 0.05$).

Principal component analysis (PCA) based on all raw data derived in

the present experiment was performed in order to describe the overall rootstocks' performance by a short number of parameters and to find possible relation between the measured parameters and the rooting capacity of the rootstocks

3. Results

3.1. Rooting parameters with and without the effect of IBA on rooting of Prunus rootstocks

The rooting percentage, root number and root length differed significantly between the two rootstocks (Fig. 1). The rooting percentage of 'Myrobalan 29C' cuttings was higher than that of 'GF 677', reaching 71.88% and 23% respectively for the untreated cuttings (Fig. 1a). IBA application did not have any significant effect on rooting efficiency in both rootstocks (Fig. 1a) but increased the root length in 'GF 677' cuttings (Fig. 1c). The number of roots formed was not affected by auxin application in both genotypes assessed (Fig. 1b). On the other hand, rootstock effect was significant since 'Myrobalan 29C' presented higher number of roots than 'GF 677' (Fig. 1b). As far as the root length is concerned, the highest root length was observed in 'GF 677' cuttings treated with IBA (Fig. 1c).

3.2. Initial polyamine content and polyamines detected

The main PAs detected were Put, Spd and Spm in both rootstocks (Fig. 2). Spm was not or rarely detected in 'GF 677' in free and conjugate form (Fig. 2a). Spd was the predominant PA in both rootstocks in free and conjugate form (Fig. 2a). On the other hand, insoluble Put was the predominant among the insoluble PA forms (Fig. 2b). 'GF 677' presented higher content in free Spd, conjugated Put and Spd (Fig. 2a) and insoluble Spd (Fig. 2b). 'GF 677' was also characterized by higher total conjugated PAs and total Spd content (Fig. 2b). The initial total PA content i.e. the sum of all the PAs and their forms was also higher in 'GF 677' compared to 'Myrobalan 29C' (Fig. 2b).

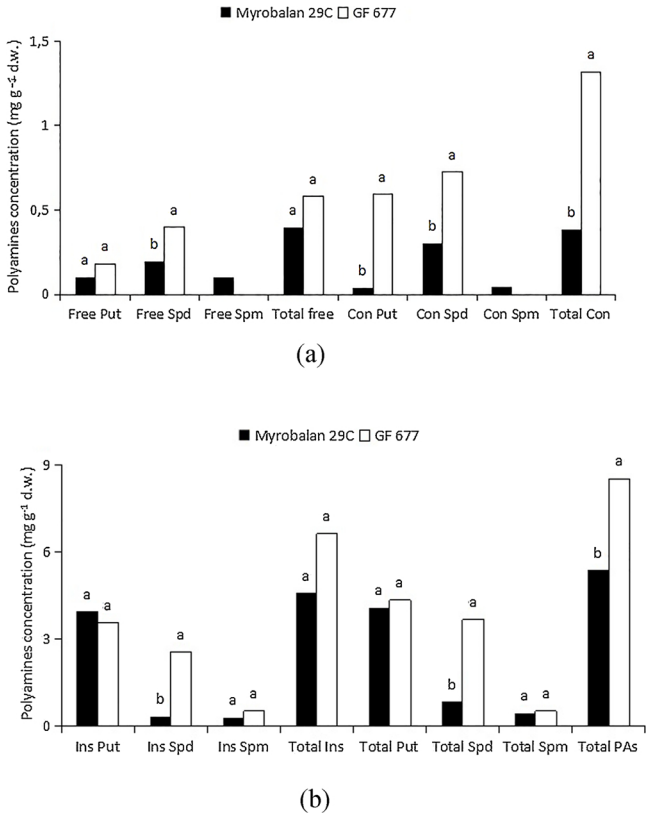

Fig. 2. Initial contents (before planting) of polyamines fractions and total PAs in the base of 'Myrobalan 29C' and 'GF 677' cuttings. Different letters above each column within the same PA form denote statistical difference between rootstocks according to Tukey's HSD test, at $\alpha = 0.05$.

The percentage of free Put out of the initial total free content was similar in both rootstocks (Fig. 3a), whereas free Spd percentage was higher in 'GF 677' than in 'Myrobalan 29C' (Fig. 3b). Free Spm was not detected in 'GF 677' (Fig. 3b).

3.3. Enzymes fluctuations during early phases of rhizogenesis. Effects of rootstock, auxin treatment and DAP

Both rootstock and DAP significantly influenced DAO, PAO and CAT activities, whereas auxin treatment had no significant effect (Table 1). There were also significant interactions of the rootstock x DAP on all three enzymes (Table 1). 'Myrobalan 29C' was characterized by lower PAO and CAT activity but higher DAO activity than 'GF 677' (Fig. 4). 'GF 677' presented higher CAT activity compared to 'Myrobalan 29C' (Fig. 4c).

The difficult-to-root rootstock 'GF 677' exhibited higher PAO activity until DAP 5 (Fig. 4a). Then, PAO activity decreased and stabilized at DAP 7 at similar levels as those of 'Myrobalan 29C' (Fig. 4a). The enzyme activity in the easy-to-root rootstock 'Myrobalan 29C' at DAP 3 was the lowest. As far as the DAO activity is concerned, the easy-to-root rootstock 'Myrobalan 29C' exhibited a small peak, which, however, was strongly enhanced by IBA application at DAP 7 (Fig. 4b). CAT activity was higher in the difficult-to-root rootstock 'GF 677' compared to 'Myrobalan 29C' until DAP 3 (Fig. 4c). At DAP 15 enzyme activity was similar between the two rootstocks (Fig. 4c).

3.4. Polyamines fluctuations during early phases of rhizogenesis. Effects of rootstock, auxin treatment and DAP

Rootstock affected the content of the majority of PAs assessed in the bases of the *Prunus* leafy cuttings (Table 2). Free Put was the only Put fraction affected by IBA-treatment unlike the other soluble PAs (Table 2). Insoluble bound and total Spd and Spm were also significantly affected by auxin treatment (Table 2). There were also only a few significant interactions of the various factors concerning the content of the measured PAs.

Free Put concentration was increased in the bases of 'Myrobalan 29C' cuttings after IBA application (Fig. 5a). 'Myrobalan 29C' presented significant lower free Put, Spd and total free PAs titer than 'GF 677' (Fig. 5a, b and d). Free Spm was not detected in 'GF 677' cuttings bases during the early phases of rhizogenesis. In 'Myrobalan 29C' the total free PA titer remained almost unchanged during the sampling period and was lower than 'GF 677' titer (Fig. 5d). 'GF 677' presented significant higher conjugate Put than 'Myrobalan 29C' (Fig. 6a). Conjugate Put content was decreased in the bases of 'Myrobalan 29C' cuttings by the IBA treatment whereas in 'GF 677' the opposite was observed (Fig. 6a). On the other hand, conjugate Spd content was increased by the auxin treatment only in 'Myrobalan 29C', while no difference was observed in 'GF 677' (Fig. 6b). Conjugated Put fluctuation was similar in both rootstocks until DAP 3 (Fig. 6a). As far as conjugated Spd is concerned, it was reduced in 'GF 677' between DAP 3 and 5 and then increased again but such pattern was not observed in 'Myrobalan 29C' (Fig. 6b). Conjugate Spm was generally not detected or occasionally detected in 'GF 677' cuttings bases during the early phases of rhizogenesis.

Insoluble bound Spd content was decreased by IBA-treatment in the cutting bases of both rootstocks (Fig. 7b). 'Myrobalan 29C' presented significant lower insoluble Spd titer than 'GF 677' (Fig. 7b). Control treatments exhibited higher insoluble Spm content whereas 'GF 677' had higher insoluble Spm content than 'Myrobalan 29C' (Fig. 7c).

All total PAs content was affected by rootstock (Table 2). 'GF 677' exhibited higher total PAs content than 'Myrobalan 29C' (Fig. 8). Total

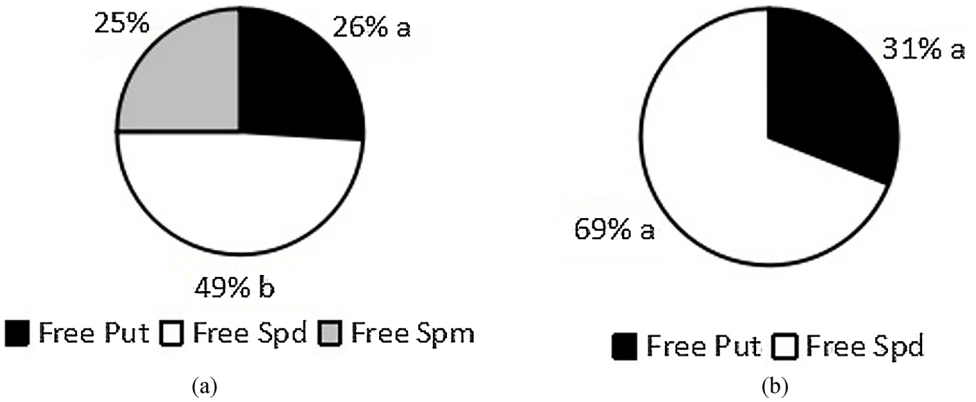

Fig. 3. Initial free Put and free Spd percentage per (out of) total free PAs in 'Myrobalan 29C' (a) and 'GF 677' (b) rootstocks. Different letters for the same PA between the two rootstocks denote statistical difference according to Tukey's HSD test, at $\alpha = 0.05$.

Table 1
Probabilities of the effects of rootstock, days after planting (DAP) and auxin treatment on enzymes activities of 'Myrobalan 29C' and 'GF 677' *Prunus* rootstocks.

Factors	DAO	PAO	CAT
Rootstock (R)	***	***	***
DAP	**	***	**
Treatment (T)	ns	ns	ns
R x DAP	***	***	***
R x T	ns	ns	ns
DAP x T	ns	ns	ns
R x DAP x T	ns	ns	ns

ns Not significant differences, x denotes interaction.
DAO diamine oxidase; PAO polyamine oxidase; CAT catalase.
*p < 0.05; ** p < 0.01; ***p < 0.001.

Spd and Spm contents (Fig. 8b and c) were decreased by auxin treatment irrespectively of rootstock and DAP, whereas total PAs content was affected by the interaction between rootstock and IBA-treatment (Table 2).

The principal component analysis revealed six components with eigenvalue above 1.0, accounting altogether to almost 82,9% of the variability of the original data. The first two components presented (Fig. 9) explained together 49.5% of the original variation. Components were comprised by Free Spd, Total Ins, Ins Spm, Total Con, Con Spd and Free Put. As indicated in the scree plot, 'Myrobalan 29C' and 'GF 677' can be distinguished (Fig. 9a). The easy-to-root 'Myrobalan 29C' was characterized by high DAO activity, free Spm, conjugated Spm and total Spm content (Fig. 9a and b). On the other hand, 'GF 677' was characterized by high CAT, PAO and Free Put (Fig. 9a and b). As presented by loading plot, DAO and Spm (Free, Con and Total) were negatively related to Free Put, PAO and CAT (Fig. 9b).

3.5. Effect of applied PAs and H_2O_2 on rooting ability of cuttings

Spm and H_2O_2 applied at concentrations of 0.5 mM and 2% v/v respectively, combined with IBA (2000 mg L^{-1}), significantly increased rooting percentage compared to IBA (2000 mg L^{-1}) application alone

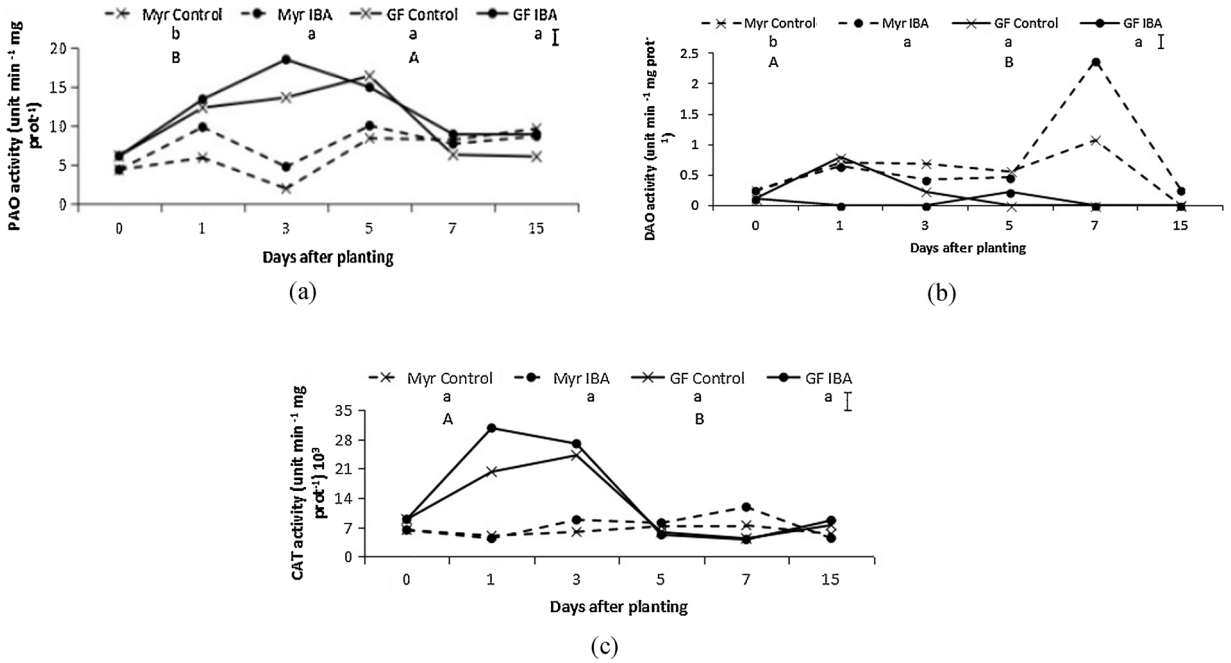

Fig. 4. Effect of rootstock, DAP and treatment (control and IBA 2000 mg L^{-1}) on PAO, DAO and CAT activities during the early phase of rhizogenesis. The vertical bars at the right side of each diagram represent the SE of the multi-factor ANOVA. Different lower-case letters within the same rootstock indicate statistical difference between control and IBA treatments according to Tukey's HSD test at $\alpha = 0.05$. Different upper-case letters indicate statistical difference between the two rootstocks under control treatment according to Tukey's HSD test at $\alpha = 0.05$.

Table 2
Probabilities of the effects of rootstock, days after planting (DAP) and auxin treatment on cutting polyamine concentrations.

Factor	Free				Conjugate				Insoluble bound				Total			
	Put	Spd	Spm	Total	Put	Spd	Spm	Total	Put	Spd	Spm	Total	Put	Spd	Spm	Total
Rootstock (R)	**	***	–	***	***	ns	–	***	ns	***	*	ns	*	***	**	***
DAP	ns	***	ns	**	**	***	ns	***	ns	***	ns	**	ns	***	ns	***
Treatment (T)	*	ns	ns	ns	ns	ns	ns	ns	ns	***	*	ns	ns	**	**	ns
R x DAP	ns	ns	–	ns	ns	**	–	*	ns	***	ns	ns	ns	***	ns	ns
R x T	*	ns	–	ns	*	*	–	ns	ns	ns	ns	ns	ns	ns	ns	*
DAP x T	**	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns
R x DAP x T	ns	**	–	ns	*	ns	–	ns	ns	ns	ns	ns	ns	ns	ns	ns

ns Not significant differences, Total total PAs, x denotes interaction.
Put Putrescine, Spd Spermidine, Spm Spermine.
*p < 0.05; **p < 0.01; ***p < 0.001.

(Table 3). Spm application almost doubled (94.8%) the rooting percentage whereas the increase achieved by H_2O_2 was 74.2% (Table 3). Higher concentrations of Spm resulted in lower rooting percentages for the difficult-to-root 'GF 677' (Table 3). Put treatments did not improve rooting ability any further, compared to control for the same rootstock (Table 3). The number and length of roots did not differ among experimental treatments (Table 3).

4. Discussion

4.1. The effect of auxin on rooting efficiency

IBA treatment at the concentration applied, did not increase the rooting percentage neither of 'GF 677' (*P. persicae* x *P. amygdalus*) nor 'Myrobalan 29C' (*P. ceracifera) Prunus* rootstocks compared to control, while the rooting ability differed significantly between the two rootstocks (Fig. 1a). Based on previous observations, the roots usually emerge at about 30–45 DAP, with 'Myrobalan 29C' being more precocious compared to 'GF 677'. Even if treating cuttings with IBA is a widely used application, it has been found to be ineffective in some species or cultivars (Tonon et al., 2001; Denaxa et al., 2014). The rooting percentage of 'Myrobalan 29C' ranged between 66% and 71.88%, whereas that of 'GF 677' between 14% and 23%, classifying 'GF 677' as a more difficult-to-root rootstock. Similar results have been reported by AI-Tamimi and QrunBeh (1996) and Zilkah et al. (2006) characterizing 'GF 677' as recalcitrant due to low rooting rates irrespectively of the type of cutting used (softwood or hardwood).

4.2. Polyamines detected and initial polyamine content

Free Spd was found to be the predominant free PA form in both 'GF 677' and 'Myrobalan 29C' (Fig. 2). Spd has been also found to be the dominant PA in olive (Denaxa et al., 2014) and peach (Fraga et al., 2004). However, in species such as *P. avium,* populus and *Fraxinus angustifolia,* low amounts of free Spd have been detected, as well as domination of free Put (Biondi et al., 1990; Tonon et al., 2001). On the other hand, in most cases, free and conjugate Spm were not detected in 'GF 677', similarly to that reported in *P. avium* (Biondi et al., 1990). These results indicate that both the predominant PA and the presence of Spm are species or even cultivar dependent.

The majority of the experiments conducted on the relation between rooting and PAs, focus on biosynthesis and exogenous application of Pas, with Put (especially the free fraction), emerging as a significant factor for increased rooting performance (Burtin et al., 1990; Hausman et al., 1994, 1995; Denaxa et al., 2014).

Put, applied exogenously, can increase rooting (Rugini et al., 1997;

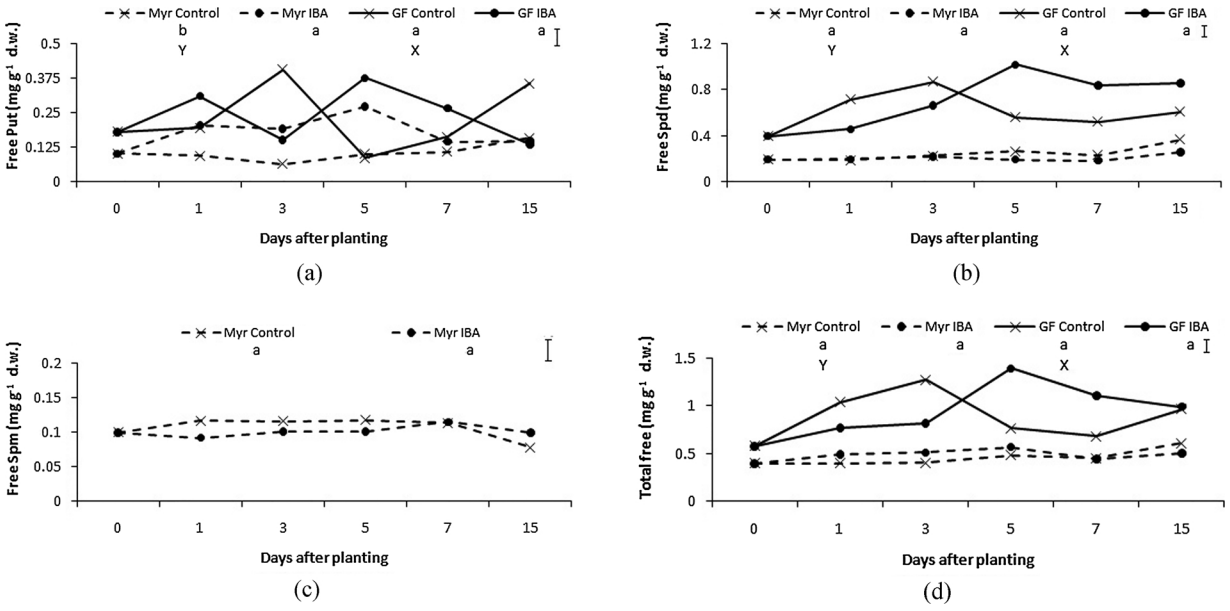

Fig. 5. Effect of rootstock, DAP and treatment (control and IBA 2000 mg L^{-1}) on titer of free polyamines: putrescine, spermidine, spermine and total free polyamines. The vertical bars at the right side of each diagram represent the SE of the multi-factor ANOVA. Different lower-case letters within the same rootstock indicate statistical difference between control and IBA treatments according to Tukey's HSD test at α = 0.05. Different upper-case letters indicate statistical difference between the two rootstocks under control treatment according to Tukey's HSD test at α = 0.05.

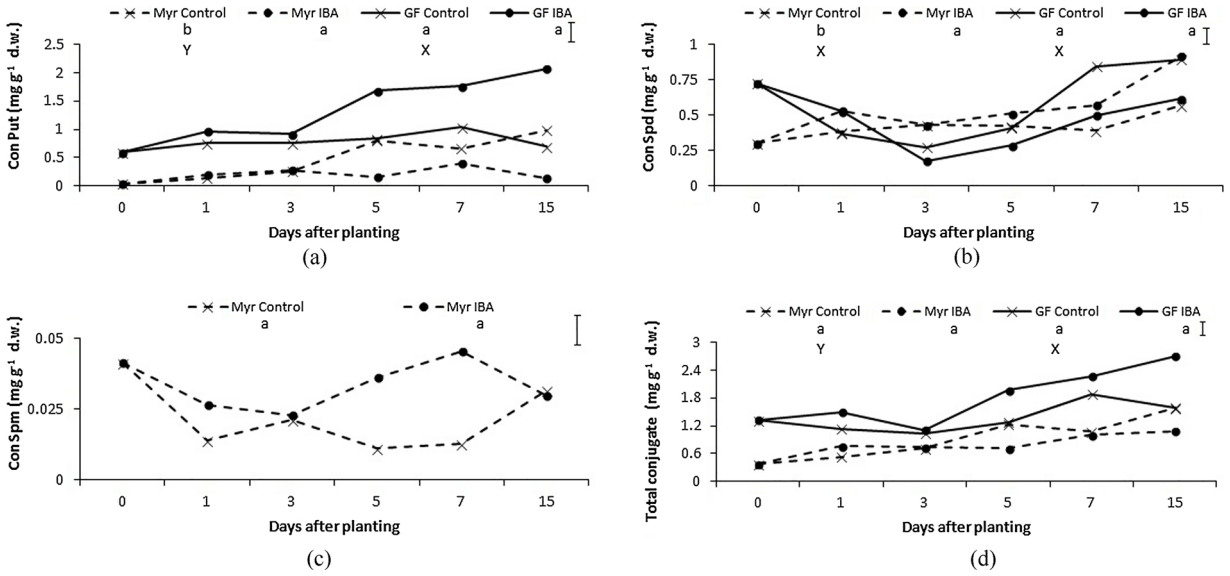

Fig. 6. Effect of rootstock, DAP and treatment (control and IBA 2000 mg L^{-1}) on titer of conjugated polyamines: putrescine, spermidine, spermine and total conjugated polyamines. The vertical bars at the right side of each diagram represent the SE of the multi-factor ANOVA. Different lower-case letters within the same rootstock indicate statistical difference between control and IBA treatments according to Tukey's HSD test at $\alpha = 0.05$. Different upper-case letters indicate statistical difference between the two rootstocks under control treatment according to Tukey's HSD test at $\alpha = 0.05$.

Matam and Parvatam, 2017), whereas its high initial endogenous content has been correlated with high rooting percentages in various tree species (Hausman et al., 1995; Bartolini et al., 2008; Denaxa et al., 2014). Thus, a lower free Put concentration was expected for the difficult-to-root 'GF 677'. Interestingly, the previous expectation was not confirmed as no significant difference was detected among the initial Put titer (in free, insoluble bound and total fraction) between the two rootstocks (Fig. 2a and b). Moreover, the percentage of free Put out of the initial total free PAs content was similar in both rootstocks (Fig. 3). These results may indicate that a) the presented difference in rooting performance between the two rootstocks cannot be attributed to the initial absolute content of Put, and b) Put cannot be considered as a potential key factor explaining the difference on rooting ability between the two rootstocks. Similar conclusions have been reported by Kevers et al. (1997) in walnut. The fact that exogenous Put application did not improve the rooting percentage of 'GF 677' (Table 3) further supports the previous assumptions, while similar results after Put application have been reported by Biondi et al. (1990) on *P. avium*.

On the contrary, 'GF 677' was characterized by higher initial Spd concentration (of all fractions) (Fig. 2a and b) and higher free Spd percentage, out of the initial total free Pas, compared to 'Myrobalan 29C' (Fig. 3). According to Hausman et al. (1995), the application of Spd may counteract the endogenous auxin elevation during the induction phase of rhizogenesis, resulting in low rooting percentages, being thus undesirable for the root primordia formation (de Klerk et al., 1999). Furthermore, in vitro application of Spd inhibited the rooting of Gisela 6 (*Prunus cerasus* x *Prunus canescens*) cherry rootstock explants (Sarropoulou et al., 2017), suggesting a possible inhibitory role of Spd

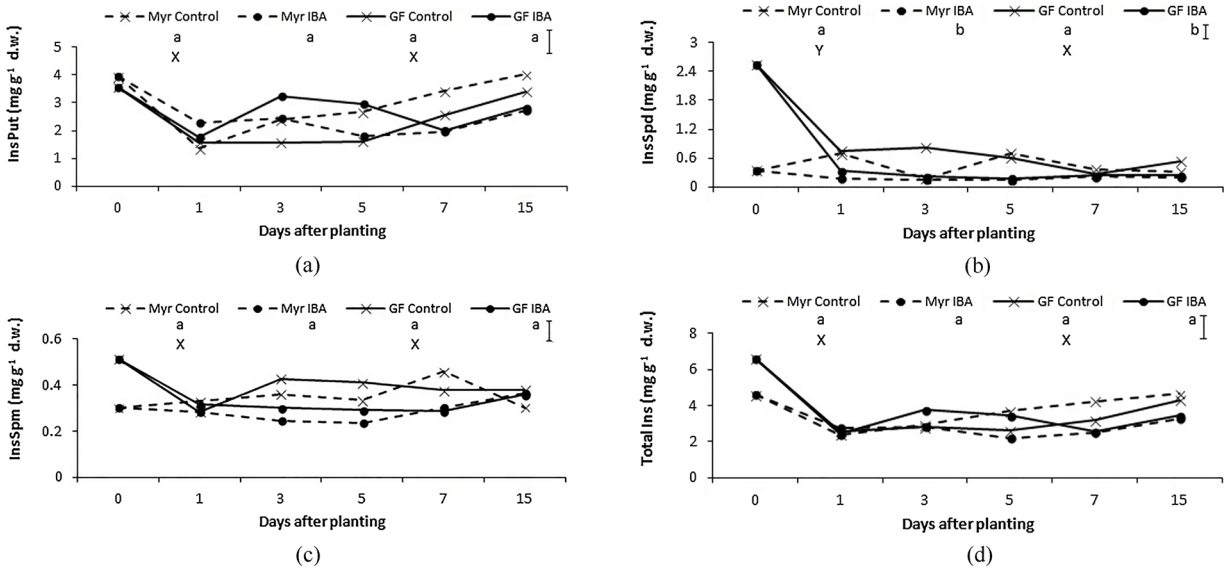

Fig. 7. Effect of rootstock, DAP and treatment (control and IBA 2000 mg L^{-1}) on titer of insoluble bound polyamines: putrescine, spermidine, spermine and total insoluble bound polyamines. The vertical bars at the right side of each diagram represent the SE of the multi-factor ANOVA. Different lower-case letters within the same rootstock indicate statistical difference between control and IBA treatments according to Tukey's HSD test at $\alpha = 0.05$. Different upper-case letters indicate statistical difference between the two rootstocks under control treatment according to Tukey's HSD test at $\alpha = 0.05$.

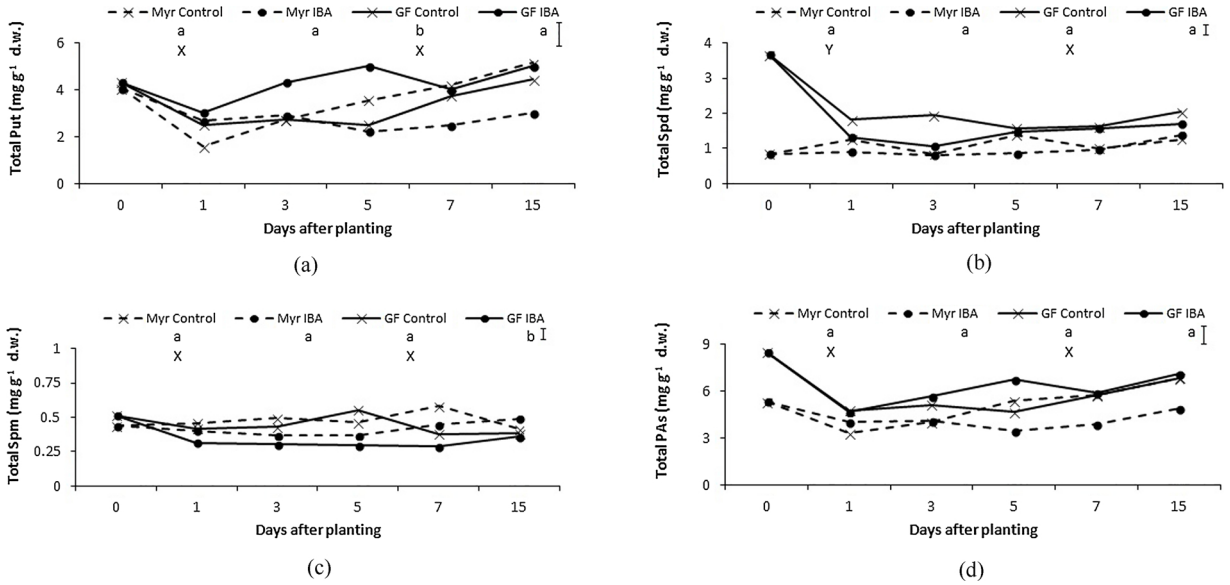

Fig. 8. Effect of rootstock, DAP and treatment (control and IBA 2000 mg L^{-1}) on titer of total individual polyamines: putrescine, spermidine, spermine and total polyamines (of all fractions). The vertical bars at the right side of each diagram represent the SE of the multi-factor ANOVA. Different lower-case letters within the same rootstock indicate statistical difference between control and IBA treatments according to Tukey's HSD test at $\alpha = 0.05$. Different upper-case letters indicate statistical difference between the two rootstocks under control treatment according to Tukey's HSD test at $\alpha = 0.05$.

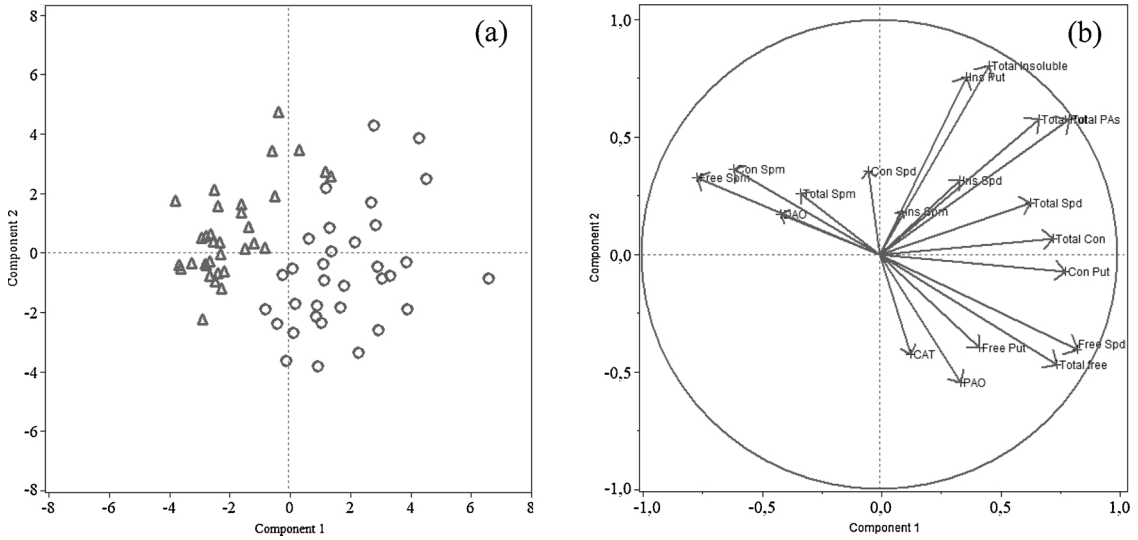

Fig. 9. Scree (a) and loading plots (b) presentation of the principal component analysis and rootstock classification based on the measured parameters. Cycle marks represent 'GF 677' and triangle marks represent 'Myrobalan 29C'. CAT, catalase; DAO, diamine oxidase; PAO, polyamine oxidase; Free Put, free putrescine; Free Spd, free spermidine; Free Spm, free spermine; Con Put, conjugate putrescine; Con Spd, conjugate spermidine; Con Spm, conjugate spermine; Ins Put, insoluble putrescine; Ins Spd, insoluble spermidine; Ins Spm, insoluble spermine; Total free, total free polyamines, Total con, total conjugate polyamines; Total Ins, total insoluble polyamines; Total Put, total putrescine; Total Spd, total spermidine; Total Spm, total spermine.

in the rooting of 'GF 677' cuttings too.

In the present study, Spm was not detectable in the cuttings of difficult-to-root 'GF 677' in most cases, in contrast to those of 'Myrobalan 29C' (Fig. 2a and b), indicating a potential promotive role of Spm in rooting. According to Galston and Flores (1991), Spm is correlated with the activity of meristematic centers and the development of root initials, while it has been correlated with enhanced rooting percentage in vitis rootstock 'Ruggeri 140' (Bartolini et al., 2009). Moreover, it has been found that Spm enhances the expression of the alternative oxidase (AOX) gene (Tang and Newton, 2005b), an enzyme, which has important functions in scavenging of reactive oxygen species and has been proposed as genetic marker for high-rooting olive genotypes (Macedo et al., 2009; Druege et al., 2019). On the other hand, Spm has been reported to inhibit rooting in some species, such as walnut (Kevers et al., 1997) and poplar (Hausman et al., 1994). In the present experiment, Spm exogenously applied in combination with IBA, acted in a dose dependent way (Table 3). Low concentrations resulted in increased rooting potential in the difficult – to – root 'GF 677' rootstock, whereas the highest concentration inhibited rooting (Table 3) as Tang and Newton (2005a) and Mendes et al. (2011) noted in other species. The promotive potential of Spm has been reported in walnut hardwood cuttings (Mc Kenna and Sutter, 1997) and in olive leafy cuttings (Denaxa et al., 2014), highlighting further Spm as a potent rooting promotive factor in some species and especially in those endogenously "deficient" in Spm (Denaxa et al., 2014).

Overall, 'GF 677' presented higher endogenous PA content than 'Myrobalan 29C' and simultaneously lower rooting capacity. These results directly challenge the opinion that a high endogenous PA content

Table 3
Effect of exogenously applied PAs and H_2O_2 followed by IBA (2000 mg L^{-1}) treatment on adventitious root formation in recalcitrance to root 'GF 677' *Prunus* rootstock.

Treatment	Rooting percentage (%)	Number of roots	Length of roots (cm)
Mock	15.3 bc	2.2 a	2.8 a
Put 0.5 mM	6.6 de	1.5 a	0.7 a
Put 1 mM	13.3 bcd	6.3 a	1 a
Put 10 mM	10 cde	8 a	1 a
Put 20 mM	10 cde	6.75 a	0.9 a
Spm 0.5 mM	30.2 a	6.8 a	1.45 a
Spm 1 mM	10.4 cde	4.1 a	1.3 a
Spm 10 mM	4.3 e	5.5 a	1.2 a
Spm 20 mM	4 e	2.5 a	1.75 a
H_2O_2 1%	5.9 e	1 a	2.3 a
H_2O_2 2%	27 a	2.9 a	3.1 a
H_2O_2 3%	17.8 b	2.3 a	2 a

Mock, IBA Indole-butyric acid 2000 mg L^{-1} treatment; Put Putrescine; Spm Spermine.
Means within the same column followed by the same letter do not differ significantly according to Tukey's HSD test, at $\alpha = 0.05$.

is positively related to efficient rooting (Denaxa et al., 2014; Rugini et al., 1991, 1993).

4.3. Effect of rootstock and auxin treatment on enzyme activity and polyamine content during the early phases of rhizogenesis

Rootstock significantly affected the majority of PAs titer, while auxin treatment had a significant effect on free Put level (Table 2).

As Nag et al. (2001) indicated in mung bean, IBA application increased free Put level in 'Myrobalan 29C', but such response was not observed in 'GF 677' (Fig. 5a). On the contrary, in 'GF 677' only the conjugated Put titer was increased under IBA treatment (Fig. 6a), as has also been indicated by Burtin et al. (1990) in tobacco leaf explants in vitro. According to Martin-Tanguy (1997), Put and its conjugate form can be accumulated in explants with low DAO activity under auxin presence. Thus, the present results indicate that IBA enhances Put biosynthesis, but its form (free or conjugate) depends strongly on DAO activity.

Free Put peaked in the IBA treated cuttings in both rootstocks at DAP 1 and DAP 5 (Fig. 5a), which has been related to the beginning (Gaspar et al., 1997; Uribe et al., 2008; Denaxa et al., 2014) and completion of the inductive phase in various species, (Denaxa et al., 2014; Nag et al., 2001; Tonon et al., 2001. However, 'GF 677" rooting efficacy was lower than that of 'Myrobalan 29C', indicating that free Put concentration was not crucial for increased rooting efficiency. Free Spd concentration in 'Myrobalan 29C' was lower than in 'GF 677' during the first days of rhizogenesis (Fig. 5b). The lack of free Spm in 'GF 677' and the increased level of Spd could be possibly attributed to the back-conversion activity of PAO to form Spd from Spm (Tavladoraki et al., 2016), since PAO activity in 'GF 677' was higher than that in 'Myrobalan 29C' until DAP 5 (Fig. 4a). The high levels of Spd may probably act as an inhibitor factor of rhizogenesis, as has been earlier reported.

IBA treatment, increased DAO's and PAO's activity in 'Myrobalan 29C' (Fig. 4a and b). Similar results have been reported by Nag et al. (2001) in *Vigna radiata* IBA treated cuttings. On the other hand, such response was not observed in 'GF 677' cuttings (Fig. 4a and b). The easy-to-root 'Myrobalan 29C' was characterized by higher DAO activity (Fig. 4b) resulting in potentially higher Put catabolism rate than 'GF 677'. Inhibition of DAO activity resulted in increased Put accumulation (Martin-Tanguy, 1997), with subsequent reduced rooting (Gaspar et al., 1997; She et al., 2010), strengthening the possible role of this enzyme and its catabolic activity in root induction. In the present study, the difficult-to-root 'GF 677' exhibited higher Put content than 'Myrobalan 29C', with almost no DAO activity and low rooting potential, in accordance with the previous literature. 'Myrobalan 29C' was characterized by high rooting capacity and DAO activity (Fig. 9), indicating a possible role of DAO in rhizogenesis, possibly through the enhanced Put catabolism rate (as it was shown by the low levels of Put found in its cuttings) and the subsequent production of the root promoting H_2O_2 as the catabolic product. Martin-Tanguy et al. (1997) reported similar DAO action in *Chrysanthemum morifolium* in vitro, while Nag et al. (2001) suggested that oxidation of Put may be more important than its biosynthesis in adventitious rooting formation and this may be the case in the present experiment.

She et al. (2010) related the CuAO (DAO) activity and the derived H_2O_2 with adventitious root formation, while IBA was found to induce the production of H_2O_2 through the catabolism of Put, increasing thus rooting of cuttings (Li et al., 2009). It can be proposed that the decreased activity of DAO and the resulting low production of H_2O_2 in 'GF 677' could probably be one of the causes for the reduced rooting capacity observed. This is further supported by the increase of rooting by H_2O_2, when it was exogenously applied in 'GF 677' leafy cuttings in the present experiment (Table 3). Low endogenous H_2O_2 in 'GF 677' could also result due to the high CAT activity determined, higher than that found in 'Myrobalan 29C' (Fig. 4c), as CAT is one of the enzymes responsible for H_2O_2 catabolic detoxification. This may indicate that whatever concentration of H_2O_2 may be produced, in 'GF 677', it can be detoxified more efficiently than in 'Myrobalan 29C'. CAT and other H_2O_2 scavengers such as ascorbic acid (Li et al., 2009) and N,N'-dimethylthiourea (Su et al., 2006) have been found to abolish the action of H_2O_2, reducing thus the rooting potential and the rooting-enhancing effect of H_2O_2 (Su et al., 2006; Li et al., 2009). Molassiotis et al. (2004) working with 'GF 677' in vitro, also noticed that decreased CAT activity increased rooting, intensifying the role of H_2O_2 in rooting response.

Thus, the high H_2O_2-detoxificating CAT activity combined with the decreased H_2O_2-producing DAO activity in 'GF 677' could probably be parts of the reasons for the potential low H_2O_2 action and consequently the poor rooting potential. This, is indirectly confirmed by the increase of rooting of the recalcitrant 'GF 677' under H_2O_2 treatment. Spm external application resulted in increased rooting as well, probably through its oxidation by PAO (its activity being higher in 'GF 677'), producing H_2O_2 which was found to enhance rooting when applied exogenously. Thus, even though 'GF 677' exhibited higher level of endogenous Put than 'Myrobalan 29C', this could not have a significant contribution to rooting without DAO activity and with simultaneously high CAT activity, scavenging the already low concentration of the produced H_2O_2. The role of H_2O_2 in rooting is further highlighted and related to auxin since, according to the current view, auxin homeostasis and auxin response are key factors determining the genetic variation in rooting of cuttings, with H_2O_2 as component of early auxin response (Druege et al., 2019). The present findings enhance the significance of PAs catabolism and partly elucidate the role of the PAs-DAO-PAO-CAT complex in rooting induction.

Author's contribution

Athanasios Tsafouros carried out the experiment and wrote the manuscript. Peter A. Roussos supervised this work and reviewed the manuscript. All authors read and approved the manuscript.

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