Humic Acid and Auxins Induced Metabolic Changes and Differential Gene Expression during Adventitious Root Development in Azalea Microshoots

Mohamed S. Elmongy

Department of Horticulture, College of Agriculture and Biotechnology, Zhejiang University, 866 Yuhangtang Road, Hangzhou 310058, P.R. China; and Department of Vegetable and Floriculture, Faculty of Agriculture, Mansoura University, Mansoura 35516, Egypt

Xiuyun Wang, Hong Zhou, and Yiping Xia

Department of Horticulture, College of Agriculture and Biotechnology, Zhejiang University, 866 Yuhangtang Road, Hangzhou 310058, P.R. China

Additional index words. Auxin response factor, phenolic compound, reactive oxygen species, rhododendron, soluble carbohydrate

Abstract. Auxins and humic acid (HA) were investigated for their roles in adventitious root induction in azalea microshoots in our previous study. To reveal the regulatory mechanisms of auxins and HA in this process, measurements of the levels of reactive oxygen species (ROS), carbohydrates, and phenolic compounds and gene expression during in vitro root development were performed. During the adventitious root induction process, ROS levels in shoots treated with auxins and HA increased compared to untreated shoots, especially during the earliest period after transfer to the media. Media supplemented with NAA experienced increases in H₂O₂ contents by 480% and 250%, respectively, after 7 and 14 days of culture. The phenolic compound levels were also enhanced in the shoots treated with auxins and HA, reflecting the different rooting-promoting abilities of both auxins and HA. The highest levels of total phenolic [68.6 mg·g⁻¹ fresh weight (FW)], polyphenolic acids (121.72 µg caffeic acid/g FW), and total flavonols (162.42 µg quercetin/g FW) were recorded after 21 days for NAA media, but the maximum levels of anthocyanins (49.76 µg cyanidin/g FW) were recorded after 21 days for IBA medium. Soluble carbohydrate, starch, and soluble protein levels were increased in the shoots treated with all treatments; however, the influence of NAA treatments was stronger than that of other treatments for most investigated parameters. The NAA significantly enhanced soluble carbohydrates by 30%, 37%, and 25%, respectively, at 14, 21, and 28 days compared with untreated microshoots. Expression of the *POD1* gene increased in the shoots submitted to HA treatment media. Expression levels of auxin response factors (*ARFs*) increased with IBA- and NAA-treated explants, suggesting that *ARFs* may have diverse regulatory roles in adventitious root induction in evergreen azalea. Moreover, the profiles of the *IAA1*, *IAA9*, *IAA14*, and *IAA27* transcripts were analyzed to reveal their roles in the adventitious rooting of evergreen azalea microshoots. These results indicate that auxins and HA promote adventitious root induction in *Rhododendron* plants through their impact on ROS, carbohydrate contents, phenolic compound levels, and expression levels of different genes related to root development in evergreen azalea plants.

Azalea plants, which belong to the genus *Rhododendron* L. (family *Ericaceae*), are some of the most popular ornamental shrubs worldwide. The in vitro production of *Rhododendron*

plants is becoming commercially significant (Eeckhaut et al., 2010; Wong, 1982). Additionally, great efforts have been made to find efficient protocols and functional media to reduce the costs of in vitro micropropagation because commercial production of tissue culture plants still entails increased costs (Wang et al., 2015). In vitro adventitious rooting is an important process in tissue culturing of azalea plants (Elmongy et al., 2018b). However, the success of micropropagation depends on the development of efficient protocols for the induction of adventitious root formation (Davies et al., 1994).

The roles of exogenously applied auxins and HA in the formation of adventitious roots

have been previously described (Zandonadi et al., 2007). In our previous study, we demonstrated the effectiveness of HA and auxins in influencing the sprouting and vitality of microshoots by controlling the morphological traits and endogenous and physiological changes that occurred during the rooting stages, which were associated with different concentrations of auxins and HA (Elmongy et al., 2018a, 2018b).

HA-mediated root induction has been studied in several plants under in vitro and in vivo conditions (Mora et al., 2012). Remarkable improvements were shown in various morphological, physiological, and biochemical aspects during root development (Nunes et al., 2019). This was clearly visible through the induction of lateral root formation, root hair initiation, and increased root number, root diameter, and root elongation (Pizzeghello et al., 2002). Such beneficial effects may have occurred because HA contains phytohormones such as auxins that could enhance root induction and alter the assimilation of partitioning patterns (Canellas et al., 2012). Furthermore, HA positively affects plant metabolism by controlling macro-elements absorption, carbon and nitrogen metabolism, and related biochemical pathways under normal and stressful conditions (Canellas et al., 2015; García et al., 2019; Nardi et al., 2017; Trevisan et al., 2010). It was reported that HA and fulvic acid enhanced the respiration in woody plants that supports their metabolic role (Vaughan and Malcolm, 1985). In addition, the metabolic influence of HA was not an individual effect, and the changes in biochemical parameters differed according to plant species, plant phenology, type of HA, and experimental conditions (Nardi et al., 2009).

The relationship between ROS and recalcitrance during the rooting process has been detected previously (Benson, 2000; Molassiotis et al., 2004). However, the accumulation of H₂O₂ is considered a stress signal, and H₂O₂ participates in gene expression regulation during rhizogenesis (Carvalho et al., 2006; Molassiotis et al., 2004). Therefore, H₂O₂ content can be used as an early indicator of microshoot root development, especially during the early root induction phase (Neves et al., 1998). Furthermore, the increase in O₂ content enhanced root initiation when the culture medium was supplemented with H₂O₂ (Weiser and Blaney, 1967). Auxin and HA promoted ROS levels at the cellular level and the expression of SOD-responsive genes (García et al., 2019; Ilczuk and Jacygrad, 2016). In this context, García et al. (2016) indicated that the HA stimulated the regulation of ROS in the growth areas during root elongation and differentiation. Furthermore, HA was able to regulate ROS homeostasis in rice roots during root development (Olaetxea et al., 2016). Phenolic compounds are considered secondary metabolites (Kefeli et al., 2003) that can enhance root development (Curir et al., 1990). They are used as physiological markers of the production of adventitious roots (de Klerk et al., 1999) because they are

Received for publication 24 Jan. 2020. Accepted for publication 13 Apr. 2020.

Published online 14 May 2020.

This work was supported by the Science and Technology Major Project of Zhejiang Province, China (projects 2016C02056-12) and National Natural Science Foundation of China (31800597). H.Z. and Y.X. are the corresponding authors. E-mail: ypxia@zju.edu.cn or lilyzhou@zju.edu.cn. This is an open access article distributed under the CC BY-NC-ND license (<https://creativecommons.org/licenses/by-nc-nd/4.0/>).

able to modify peroxidase activity (Ilczuk and Jacygrad, 2016). They are also considered one of the defense groups that inhibit stresses caused by wounding or adverse environmental conditions (Solar et al., 2006). However, the mechanisms of action of phenolic compounds and their accumulation during the induction of adventitious roots are not quite clear (Wiszniowska et al., 2016). Changes in carbohydrate levels have vital roles in plant developmental, physiological, and metabolic processes involved in plant formation (Mishra et al., 2009). Additionally, sugars have a signaling function and are considered regulatory molecules during plant development (Chu et al., 2010).

Adventitious root formation includes many complex stages that are regulated by a number of external or internal factors (Trevisan et al., 2010). *ARFs* are transcription factors that regulate the auxin signaling pathway (Zhang et al., 2017). They can promote or inhibit the expression of some genes related to the auxin response by binding to the promoters of auxin-responsive elements (AuxREs) (Guilfoyle and Hagen, 2007). In addition, auxins are considered a primary dominant signal during root development that promotes the mitotic activity of pericycle cells during the stage of primordia formation (De Smet et al., 2006). Based on the importance of IAA for rooting induction (Fu et al., 2011), and according to studies by Oono et al. (2003) and Yang et al. (2004) regarding the role of auxin-responsive genes during adventitious root formation, this study evaluated the auxin-responsive genes *IAA1*, *IAA9*, *IAA14*, and *IAA27* in conjunction with auxins and HA treatments.

To reveal the regulatory mechanisms of auxins and HA in root development, some physiological parameters and related genes in evergreen azalea (*Rhododendron* genus) microshoots were studied. To investigate the physiological changes, the levels of phenolics, lignin, ROS, soluble carbohydrates, starch, and soluble proteins were studied. At the molecular level, the differential gene expressions of *POD1*, *ARFs*, and early auxin-responsive genes related to IAA were analyzed after the application of different auxin concentrations and HA.

Materials and Methods

Plant material, rooting media, and culture conditions. Experiments were conducted at the Laboratory of the Ornamental

Plants, Department of Horticulture, Zhejiang University, Hangzhou, China. Explants were collected from plants grown at a nursery located on the campus of Zhejiang University. Microshoots of a single clone of the evergreen azalea cultivar 'Zihudie' (*Rhododendron* subgenus *Tsutsusi*), already taken during the in vitro multiplication stage, were used for all the experiments. The microshoots (10–12 leaves; height, 4 cm) were prepared for the in vitro rooting medium. To test rooting, all explants were placed in Anderson media (Anderson, 1984) supplemented with IBA, NAA, and HA (Aladdin, fulvic acid $\geq 90\%$, H108498, China) at a concentration of 2 mg/L. IBA, NAA, and HA were directly added to the medium before autoclaving. The glass flasks (250 mL) containing 30 mL culture medium with 8 g·L⁻¹ commercial agar-agar and 30 g·L⁻¹ sucrose were used to grow the plants. All cultures were incubated at day/night temperatures of 26 °C/23 °C (± 1 °C) with a 16-h photoperiod under cool white fluorescent lights at a photosynthetically active radiation (*PAR*) level of 2500 LUX light intensity. All glasses were planted with four microshoots, and a total of 80 microshoots were initially used for each treatment. ROS, phenolic group, major carbohydrate concentrations, and gene expression were determined after 7, 14, 21 and 28 d of culture. We harvested and checked the parameters every 7 d. The specific sampling dates were 5, 12, 19, and 26 Jan. 2018. The microshoots were collected at 9:00 AM on each sampling date. Microshoot samples were thoroughly washed and immediately frozen in liquid nitrogen (≥ 30 min) and then stored at -80 °C until further use.

Reactive oxygen species analysis. To measure the hydrogen peroxide (H₂O₂) content, whole microshoots (0.5 g each and 7–8 in vitro culture plants) were ground and then dissolved in 5.0 mL of 0.1% trichloroacetic acid. Then, the samples were centrifuged at 12,000 g_n for 20 min (Perveen and Anis, 2015). The supernatant was mixed with potassium phosphate buffer (10 mM) with 1 mL of 1 mM KI. Then, a spectrophotometer measurement was performed at 390 nm, and the H₂O₂ content was calculated by using a standard curve. The hydroxyl radical ($\cdot$ OH) concentration was estimated using the method of Halliwell et al. (1987). The microshoot samples (0.5 g each) were treated at 37 °C for 2 h with 15 mM 2-deoxy-D-ribose

(pH 7.4). Then, the mixture (0.7 mL) was combined with a reaction mixture containing 0.5% (w/v) thiobarbituric acid (1% stock solution made in 5 mM NaOH) and 1 mL glacial acetic acid. Finally, to perform the measurement, a heated water bath (100 °C) was used to heat the reaction mixture for 30 min; after which, the mixture was placed at 4 °C for 10 min to cool. The method of Elstner and Heupel (1976) with slight changes was used to measure the superoxide radical (O₂⁻) level. Then, 0.5 g of each microshoot sample was mixed with potassium phosphate buffer adjusted to pH 7.7, and the reaction was centrifuged at 4000 g_n for 12 min. Then, the reaction was left for 24 h at 25 °C, and the absorbance of the supernatant was determined at 530 nm.

Phenolic group and lignin content determination. Phenol levels were determined in whole microshoots that were sampled directly 7, 14, 21, and 28 d after being transferred to the media. Samples were collected and stored at -80 °C. The total phenols, flavonols, polyphenolic acids, and anthocyanins were measured by spectrophotometry using the method described by Fukumoto and Mazza (2000). The lignin content determination was performed according to the method of Bisbis et al. (2003). The solution absorbance was calculated at 280 nm.

Soluble carbohydrate, starch, and soluble protein extraction. The concentrations of major carbohydrates (total soluble sugars, sucrose, and starch) were determined by modified anthrone colorimetry according to the method of McCready et al. (1950). The microshoot samples (200 mg and ≈ 3 –4 in vitro culture plants) were mixed with ethanol by heating at 80 °C for 15 min, and the extraction mixture was sampled 4 times. The volume was calculated after centrifugation of the mixture at 1200 g_n for 5 min to determine the sucrose and soluble sugar contents. The total soluble carbohydrates and starch were measured at 620 nm according to the phenol-sulfur method. The soluble protein level was determined according to the method of Bradford (1976) by using bovine serum albumin as a standard. Finally, 52% (v/v) perchloric acid was used to extract starch.

Total RNA isolation and reverse transcription real-time quantitative polymerase chain reaction (RT-qPCR). Two to three frozen microshoots (≈ 100 mg) were ground in liquid nitrogen. The total RNA was isolated from

Table 1. Sequences of oligonucleotide primers used in the reverse-transcription polymerase chain reaction.

Gene name	Accession number	Forward primer	Reverse primer	Product length bp
18S rRNA	AB300708	CGCATCCCCACTGTATTAGAC	CGTAACAAGGTTTCCGTAGGTG	100
Ubiquitin	HE716984	TCCAAGACAAGGAGGGCATC	CCTTAGACGGAGACCAAAATG	135
POD1	XM_028265071	GCTTTCAGTCTCAGTGCCCTTAT	GGGAATCTATGTTGGTTTCGTTG	151
ARF3	MK075474	GCTTTTTTTACCTCCCCCG	GACGACGAACCTATCGCCAA	142
ARF5	XM_028217268	TAAGCCAGTTGACTTCTTTAGGGA	CAATCCGTGGTTTGGTTGAGT	112
ARF17	XM_028235285	GTTGGTTGTGCGGAAGGTAAT	TGAACATCAAGCCATCTCGTA	119
ARF18	XM_028235557	ACGAGAAGGGAGAAAGTGAGGC	ATCCTCAGTTTCAAAAGGGCATC	198
IAA1	XM_009340734	GAGCACCTTATCTCAGAAAAGTCG	CCGTTTACTGGGTCCATTAGC	139
IAA9	XM_028228198	TAGTGCCTCAACAAGTGTCTCAAGA	CAACCTGTGCCTTGGTAGCG	114
IAA14	XM_028269217	AACTCAATCTTCAGCAGCCAAAT	CGTTCCTCTGTACGACCTAACTG	181
IAA27	XM_028257802	AAGCTCTGACAAGATCTCC	TTTTCCTCCATAAAAAGA	145

Fig. 1. Effects auxins and humic acid treatments on hydrogen peroxide (H₂O₂) (A), superoxide radical (O₂⁻) (B), and hydroxyl (OH) (C) contents of evergreen azalea microshoots during adventitious root formation. Means express the average of three replicates $\pm$ SE, and means within each graph denoted by the same lowercase letter do not significantly differ according to the Duncan test at $P < 0.05$.

both the control shoots and shoots treated with 2 mg/L IBA, NAA, and HA using the RNeasy Pure polysaccharide polyphenol plant total RNA extraction kit (TIANGEN, Beijing, China). The RNA concentrations were calculated with a NanoDrop and cDNA

was synthesized with the PrimeScript RT reagent kit with gDNA Eraser (Perfect, Real-Time; Takara, Otsu, Japan). PCR was performed with TB Green Premix Ex Taq (Takara Bio, Mountain View, CA) on a CFX Connect Real-Time System (Bio-Rad, Hercules, CA).

Specificity of primers for *POD1*, *ARF3*, *ARF5*, *ARF17*, *ARF18*, *IAA1*, *IAA9*, *IAA14*, and *IAA27* was confirmed by checking the melting point. Genes were used at a concentration of 400 μ M. *Ubiquitin* and *18S* rRNA genes were used as reference genes for data normalization, and the expression of genes of interest was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Table 1 shows all primers used in RT-qPCR.

Statistical analysis. Significant differences among different time points were identified by using an analysis of variance with Duncan's multiple range test at a significance level of $P < 0.05$. Statistical analyses were performed using SPSS v16.0.

Results

Effects of auxin and humic acid treatments on reactive oxygen species levels. Azalea microshoots treated with auxins and HA treatment showed a large increase in H₂O₂ levels 7 and 14 d after transfer to media compared with the control (Fig. 1A). Within auxins, microshoots inoculated in media supplemented with NAA significantly increased the H₂O₂ contents by 480% and 250%, respectively, after 7 and 14 d of culture compared with the control. After that, H₂O₂ contents declined until the end of the rooting stage for both treatments with auxins and with HA. Furthermore, a slight increase in microshoots in the control was observed 21 and 28 d after transfer to media. A similar phenomenon was observed with changes in O₂⁻ levels, which increased at 7 and 14 d after transfer to media and then decreased until the experimental period. Additionally, the O₂⁻ contents were significantly increased in the azalea microshoots under NAA treatment at 7 and 14 d after inoculation in the culture media (Fig. 1B). During the rooting process, the levels of OH⁻ increased in treated microshoots for all treatments and were higher after 7 and 14 d compared with levels in untreated shoots (control) (Fig. 1C). However, the NAA treatment showed a significant increase compared to the other treatments at only 7 d of culture. It was also observed that the OH⁻ contents in the control microshoots gradually increased until 21 d of culture and then decreased at 28 d.

Changes in the phenolic compound content at different root induction stages. The total phenolic content in all treatments increased from the start of the experiment until day 21 and then declined on day 28 after transfer to media (Fig. 2A). The highest levels of total phenolic compounds were obtained from microshoots inoculated in culture media supplemented with auxins (NAA and IBA), followed by those inoculated in media containing HA. However, the auxin-free medium had the lowest values for total phenolic content throughout the rooting process. In addition, the peak in total phenolic content occurred after 21 d of culture; however, of all the treatments, the microshoots treated with NAA recorded the highest levels (68.6 mg·g⁻¹ FW) of total phenolic content.

Fig. 2. Effects of different auxins and humic acid mediums on the phenolic profile in microshoots of evergreen azalea (Zihudie cultivar) after 7, 14, 21, and 28 d of transferring to the rooting induction media: (A) total phenols, (B) polyphenolic acids, (C) flavonols, (D) anthocyanins, and (E) lignin. Means express the average of three replicates $\pm$ SE, and means within each graph denoted by the same lowercase letter do not significantly differ according to the Duncan test at $P < 0.05$.

Changes in the polyphenolic acid content were also similar to the changes that occurred in the total phenolic levels (Fig. 2B). After 7 d of culture, a slight increase in the polyphenolic acid content was observed in auxins and microshoots rooted in media with HA, and the contents gradually increased to their maximum values at day 21 and increased by 180%, 110%, and 100%, respectively, in the NAA, HA, and IBA treatments compared with the untreated plants. After 28 d, poly-

phenolic acid levels decreased in all treated microshoots except those in the control medium, which had slightly increased levels. Regarding the flavonol content, no significant difference was observed between treated and nontreated plants after 7 d of culture (Fig. 2C). The total flavonol content in all microshoots peaked initially 14 d after transfer to media. Additionally, in the microshoots treated with NAA, the flavonol content was two-times higher than the flavonol content in

the control microshoots; in shoots treated with IBA and HA, the flavonol content was 1.7-times higher than that in the control microshoots. There was another peak on day 21, when the highest flavonol content was recorded in the microshoots rooted in the media fortified with NAA. The concentration of anthocyanins was slightly higher in the azalea microshoots treated with IBA than in those in other treatments on most sampling days (Fig. 2D). Additionally, the maximum

Fig. 3. Effects of different auxins and humic acid mediums on major carbohydrates, sucrose content (A), total soluble sugars (B), starch content (C), and total soluble protein (D) during in vitro rooting of evergreen azalea (Zihudie cultivar). Means express the average of three replicates $\pm$ SE, and means within each graph denoted by the same lowercase letter do not significantly differ according to the Duncan test at $P < 0.05$.

contents of anthocyanins were recorded after 21 d of culture, and the highest value (49.76 μ g cyanidin/g FW) was obtained when IBA was applied to the medium. The application of auxins and HA in the rooting medium did not have a substantial effect on the lignin content during the first 21 d of culture (Fig. 2E). In contrast, a large increase in the lignin content was observed in all treatments after 14 d. The peak level occurred at 28 d. However, the lignin content increased by 100%, 80%, and 70% in the NAA, IBA, and HA treatments, respectively, compared with that in the control.

Changes in soluble carbohydrates, starch, and soluble protein during microshoot rooting. There were no significant effects on sucrose content in shoots treated with auxins or HA at all time points of the rooting process, but a significant effect was observed between the treated plants and nontreated plants (Fig. 3A). The highest levels of sucrose were found in the microshoots after 28 d of culture; the sucrose content gradually increased after 7 d of culture, reaching its highest values after 21 and 28 d of culture. The total soluble sugars showed almost the same trends as sucrose during the different rooting stages (Fig. 3B). However, the soluble sugars in microshoots treated with NAA increased significantly by 30%, 37%, and 25%,

respectively, at 14, 21, and 28 d compared with the control treatments.

Microshoots under the auxins and HA treatments showed differences in starch contents at almost all time points (Fig. 3C). After 7 d, the starch content was higher in microshoots transferred to media supplemented with IBA and HA, and the microshoots rooted in the control and NAA media had the same starch content. At 14 d after the inoculation, the starch levels were significantly increased in all treatments compared with their initial levels. Then, the starch contents continued to increase, reaching their maximum values after 21 d. After 28 d of treatment, the starch content decreased to 5% lower than the starch content at 21 d in all microshoots. The soluble protein content increased in treated microshoots from the time of transferring the plants until 14 d of treatment (Fig. 3D). After 14 d, the soluble protein contents decreased in all microshoots until the last day of the experiment. The highest content of soluble protein (241.86 μ g·g⁻¹ FW) was obtained in the microshoots treated with IBA after 14 d. Furthermore, the microshoots rooted in the control media produced the lowest contents of soluble protein on all tested days.

Gene expression profiles in rooted azalea microshoots. The expressions of certain genes described in the literature as being involved in auxin signaling were analyzed by RT-qPCR during adventitious root development (from day 7 to day 28) in evergreen azalea microshoots (Fig. 4). It was found that the expression of *POD1* increased after 7 d by 12% and 100% in the IBA-treated and HA-treated microshoots, respectively, compared with the control treatments. After 14 d, the expression of *POD1* increased only in the HA-treated shoots by 52% compared with the control treatments. Additionally, after 28 d, *POD1* expression increased by 120%, 100%, and 30% in plants rooted in HA, NAA, and IBA media, respectively, compared with untreated plants. Additionally, the effects of exogenous auxins and HA applications on the expression of *ARF* genes were also evaluated (Fig. 4). Four *ARF* genes (*ARF3*, *ARF5*, *ARF17*, and *ARF18*) showed differential expression for all treatments. The expression of *ARF3* was upregulated on all days in all auxin-treated microshoots compared with that in the control on the same days; in addition, the microshoots treated with NAA and IBA had the highest levels of expression of the *ARF3* gene compared with the control, but the shoots treated with HA had the lowest

Fig. 4. Effects of auxins and humic acid on expression patterns of *POD1* and ARFs in microshoots of evergreen azalea (*Rhododendron* genus) according to quantitative reverse-transcription polymerase chain reaction. Data are the means of three replicates $\pm$ SE shown by vertical bars.

values of *ARF3* expression. *ARF5* expression was slightly increased in azalea shoots when exposed to IBA and NAA medium 1 and 2 weeks after transfer to media compared with that in nontreated plants; in contrast, *ARF5* expression in HA-treated plants decreased. Then, *ARF5* expression increased in all treated microshoots at days 21 and 28 when the microshoots were rooted in NAA, HA, and IBA, respectively, compared with that in nontreated microshoots. *ARF17* expression increased by 30%, 5%, 50%, and 30%, respectively, at 7, 14, 21, and 28 d in the IBA application compared with that in the control, and NAA application enhanced *ARF17* expression at 14 and 21 d. The highest expression of *ARF18* in microshoots was obtained in IBA and NAA treatments, where it increased at 21 and 28 d. The transcript level of *ARF18* peaked after 21 d of adventitious root formation in the microshoots treated with

NAA. Similarly, the transcript levels of four auxin-responsive genes (*IAA1*, *IAA9*, *IAA14*, and *IAA27*) were evaluated (Fig. 5). All these genes showed differential expression for all types of treatments and throughout the rooting process. First, the expression of the *IAA1* gene increased by 100% and 50% after 7 and 28 d, respectively, of culture on media fortified with IBA compared with that in nontreated plants (Fig. 5). Furthermore, the expression of the same gene also increased in microshoots rooted in NAA media by 10%, 40%, and 95% at 7, 14, and 28 sampling days, respectively, compared with that in untreated plants. The data indicated that *IAA9* expression was only upregulated in azalea shoots treated with IBA during the whole period of root development compared with that in the control, except in shoots rooted in NAA media at 28 d. The expression level of *IAA14* significantly increased under NAA

treatment on all tested days. However, it increased under the IBA treatment only after the first 7 d, whereas the expression of *IAA27* increased in all treated plants more than it did in the control, and the IBA treatment had the highest levels of *IAA27* expression during the whole rooting period.

Discussion

It has been reported that many internal and external factors can control the process of adventitious root formation (Da Costa et al., 2013; Pacurar et al., 2014). In this experiment, we investigated how auxins (IBA and NAA) and HA signaling are implicated in adventitious root formation by studying the levels of ROS, carbohydrates, and phenolic compounds, as well as the transcript levels of differential genes in evergreen azalea microshoot rooting. The ROS production causes

Fig. 5. Effects of auxins and humic acid on expression patterns of *IAA1*, *IAA9*, *IAA14*, and *IAA27* in microshoots of evergreen azalea (*Rhododendron* genus) according to quantitative reverse-transcription polymerase chain reaction. Data are the means of three replicates $\pm$ SE.

oxidative stress and cell death in plants (Vatankhah et al., 2010). The relationship between ROS and cell resistance throughout rooting was detected by Benson (2000), who indicated that H_2O_2 has a role as a molecular signal involved in the auxin induction during adventitious roots formation (Li et al., 2009). In addition, a high level of H_2O_2 is required for the induction of adventitious roots (Neves et al., 1998; Su et al., 2006). In the present study, an increase in ROS levels (H_2O_2 , O_2^- and $^{\bullet}OH$) was observed during the first 7 d of adventitious root induction. Furthermore, treatments with NAA, IBA, and HA cause an increase in H_2O_2 in microshoots during the initiation phase of adventitious roots (Osterc et al., 2016; Yildiztugay et al., 2019). The data demonstrated that NAA, IBA, and HA promoted H_2O_2 production during the initiation phase of root development, possibly via increased peroxidase (POD) activity in this phase, because POD can participate in oxidative metabolism and in the production of H_2O_2 (Elmongy et al., 2018a, 2018b). Similarly, Li et al. (2007) proved that the activity of catalase during root development might be repressed by H_2O_2 . The increase in ROS levels after 7 d enhanced the formation of root primordia in the microshoots treated with auxins (Ilczuk and Jacygrad, 2016).

Our experiment was designed while considering the auxinic compounds NAA and IBA (Wiesman et al., 1988), and the auxin-like activity and high hormonal activity of HA (Canellas et al., 2002; Muscolo et al., 1998) added to the rooting medium. Therefore,

four phenolic compounds were determined during induction of adventitious root formation in evergreen azalea. It was reported that ferulic, gallic, and chlorogenic acid could enhance woody plant rooting (De Klerk et al., 2011). In addition, the levels of endogenous phenolics were considered as well-defined physiological indicators related to adventitious root formation (Curir et al., 1990; de Klerk et al., 1999; Wiszniewska et al., 2016). In this experiment, we observed that the total phenols, flavonols, polyphenolic acids, and anthocyanins in the studied plants increased in all treatments compared with those in the control until day 21 and then decreased at day 28. The increase in total phenolic levels until the end of the induction phase was in agreement with the study by Cheniany et al. (2010), who considered that this increase is related to the activity of phenylalanine ammonia-lyase (PAL), which is a major enzyme in phenolic biosynthesis. Additionally, the presence of phenolic compounds in the first stages of root development is involved in IAA oxidation, which can enhance the root primordia in the early rooting phases (Güneş, 2000). Furthermore, phenolics have a supporting role during the first stages of adventitious root formation (de Klerk et al., 1999). In this context, the previous study by Dash et al. (2011) of *Saraca asoka* indicated that primordia formation and elongation occurred due to high phenolic levels. The high levels of flavonoids (flavonols and anthocyanins) in azalea plants are connected to their rooting ability (Fu et al., 2011). Phenolic compounds

have a critical role in the process of lignin biosynthesis and serve as precursors of monolignols (Haissig, 1986). Our study demonstrated that total phenolic and polyphenolic acids increased to their highest values after 21 d of cultivation upon supplementation of the media with auxin and HA. The accumulation of phenolic compounds has been reported to stimulate cell differentiation into root primordia (Porfrio et al., 2016); therefore, this reflected the morphological development of adventitious roots (Supplemental Fig. 1). Based on this concept, the lignin contents during root development were determined, and an increase in the lignin content was found in all treatments after 14 d of culture. This increase was also confirmed by Bisbis et al. (2003), who found that the lignin content in rooted shoots of walnut increased from the beginning of the rooting induction phase. It was found that shoots rooted with IBA, NAA, and HA showed higher peroxidase activity, which supports that peroxidase works when bound to the cell wall (Elmongy et al., 2018b). Cell wall-bound peroxidase is considered to be involved in wall lignification (Pedreno, 1995). Furthermore, a significant relationship was found between IAA levels and secondary xylem development in the shoots of *Talipariti tiliaceum* (DeGroote and Larson, 1984).

The enhancement of adventitious roots in azalea microshoots may be attributed to the concentration of carbohydrates. The process of rooting induction requires a significant amount of energy (Wiszniewska et al.,

2016) so high levels of carbohydrates can support the formation of adventitious roots (Li and Leung, 2000). In addition, soluble carbohydrates are considered the main source of carbon in plants, which made their measurement essential, especially during rooting (Chu et al., 2010). The importance of starch reserves in *Arabidopsis* has been proven. Therefore, determination of carbohydrates during in vitro root induction in evergreen azalea is essential to understanding the physiological changes during the process to be able to develop an efficient protocol for adventitious root formation in this plant species. In our study, the presence of both auxins and HA in the media increased the sucrose, soluble sugars, and starch contents during adventitious root formation. A similar trend was found by Husen and Pal (2007) and Agulló-Antón et al. (2011); in their studies, carbohydrate accumulation was enhanced in the presence of IBA and NAA compared with that in untreated plants. Additionally, Wu et al. (2016) indicated that root development in lily plants was promoted by HA application. Our findings showed that all carbohydrate contents decreased after 7 d of culture and then gradually increased on the following sampling days during adventitious root formation, which may be due to the enhancement of hydrolytic enzymes accumulating carbohydrates in microshoots. Then, the carbohydrates were moved to the shoot rooting area and used as energy for cell division and root primordia formation (Haissig and Davis, 1994; Hartmann et al., 1997). However, the levels of soluble proteins were enhanced by IBA, NAA, and HA applications, but the protein concentration was high after 7 and 14 d, followed by a decrease on days 21 and 28 of treatment. These high levels of soluble protein during the early stages of root induction occur because auxin and HA are related to higher protein contents (Oliver et al., 1994; Pizzeghello et al., 2013). Additionally, the increase in protein content occurred during the initiation stage of the rooting phase, and it was synchronous with the changes in enzymatic proteins responsible for synthesis during the root development stages (Husen, 2008). Furthermore, it was suggested that the accumulation of the protein/polysaccharide mixture can help the organs to coalesce safely (Brighigna et al., 1990).

POD has been reported to participate in the process of auxin metabolism and lignification in plant cell walls (Quiroga et al., 2000). In this study, the presence of HA in the growth media increased the expression of *POD1* more than the presence of IBA or NAA, especially during the first period of root induction (Fig. 4). HA increases *POD* gene expression because HA has a practical role in which it works as an antioxidant and can activate auxin induction (Cordeiro et al., 2011). In addition, HA has the ability to regulate some genes that are in charge of the water and solute flow between the cytoplasm and vacuolar compartments (Kaldenhoff and Fischer, 2006). The decrease in *POD1* expression in the microshoots treated with IBA and

NAA after 14 d may have been due to the accumulation of endogenous IAA (Liu et al., 1996), and the low POD activity may have been due to the inhibition of the *de novo* synthesis of *POD1* synthesis during the adventitious root formation process (Yan et al., 2017). Additionally, we observed the lowest *POD1* expression in shoots rooted in NAA media, especially at 7 and 14 d. Lagrimini et al. (1992) suggested that this decrease occurred because NAA strongly repressed the regulation of the *POD* gene by binding between the *POD* promoter and some auxin response elements. Similarly, NAA suppressed the expression of the *POD* gene, resulting in an increase in the endogenous IAA content and stimulating cell division and elongation in the treated plants during root development (Aeschbacher et al., 1994; Chen et al., 2002). ARFs are considered essential in the auxin signaling pathway, where their functional role as transcriptional activators or inhibitors has been detected through their binding to auxin-responsive genes (Shen et al., 2015; Woodward and Bartel, 2005). Additionally, the analyses by Zhang et al. (2017) of hot pepper genes demonstrated the important roles that *ARFs* have during adventitious root formation. Similarly, Yang et al. (2014) found that 20 *ARF* genes participate in the development of adventitious roots in *Populus* shoots. Our results indicated that the expression of most *ARF* genes changed during the induction of adventitious roots, suggesting that they might have an important role during root formation in azalea by regulating the transcription of auxin-responsive genes. In our study, we found different levels of *ARF* gene expression on different rooting days; the expression of four genes (*ARF3*, *ARF5*, *ARF17*, and *ARF18*) was highly increased during the early period (7 and 14 d) of adventitious root formation. These transcription *ARF* levels showed that the roles of azalea *ARF* genes may vary at different times during the induction of adventitious roots (Zhang et al., 2017). Furthermore, a negative relationship was observed between the presence of HA and the expression of *ARF* genes; the expression of these genes was lower in HA-treated microshoots. This relationship may be because the auxinic effects of HA were lower than those of NAA and IBA.

To investigate the effects of IBA, NAA, and HA on different auxin-inducible genes, we analyzed the expression levels of *IAA1*, *IAA9*, *IAA14*, and *IAA27* during in vitro rooting. Most of the tested transcription was found to be upregulated by auxin treatments at different levels and at different time points. Our results suggest that transcription of *IAA1*, *IAA9*, *IAA14*, and *IAA27* genes may be considered a diagnostic marker for detecting the molecular changes caused by auxins and HA or others that are related to the indole acetic acid signal transduction pathway, in agreement with the study by Trevisan et al. (2010).

In conclusion, this work shows the impact of IBA, NAA, and HA during adventitious root formation, mostly through their influence on ROS changes, phenolic compound

levels, carbohydrate metabolic changes, and the expression of different genes related to auxin signaling. Overall, it was found that the presence of both auxins and HA affected ROS, phenolic groups, and carbohydrate metabolic changes during the formation of adventitious roots in evergreen azalea microshoots. At the same time, the application of additional HA upregulated *POD1* expression more than the application of auxins, but the expressions of *ARF* genes and auxin-responsive genes (*IAA1*, *IAA9*, *IAA14*, and *IAA27*) were increased more by auxins treatment than by HA treatment.

Literature Cited

- Aeschbacher, R.A., J.W. Schiefelbein, and P.N. Benfey. 1994. The genetic and molecular basis of root development. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 45:25–45.
- Agulló-Antón, M.Á., J. Sánchez-Bravo, M. Acosta, and U. Druege. 2011. Auxins or sugars: What makes the difference in the adventitious rooting of stored carnation cuttings? *J. Plant Growth Regul.* 30:100–113.
- Anderson, W.C. 1984. A revised tissue culture medium for shoot multiplication of rhododendron. *J. Amer. Soc. Hort. Sci.* 109:343–347.
- Benson, E.E. 2000. Do free radicals have a role in plant tissue culture recalcitrance? *In Vitro Cell. Dev. Biol. Plant* 36:163–170.
- Bisbis, B., C. Kevers, M. Crèvecoeur, J. Dommes, and T. Gaspar. 2003. Restart of lignification in micropropagated walnut shoots coincides with rooting induction. *Biol. Plant.* 47:1–5.
- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
- Brighigna, L., A.C. Fiordi, and M. Palandri. 1990. Structural comparison between free and anchored roots in *Tillandsia* (*Bromeliaceae*) species. *Caryologia* 43:27–42.
- Canellas, L.P., L.B. Dobbss, A.L. Oliveira, J.G. Chagas, N.O. Aguiar, V.M. Rumjanek, E.H. Novotny, F.L. Olivares, R. Spaccini, and A. Piccolo. 2012. Chemical properties of humic matter as related to induction of plant lateral roots. *Eur. J. Soil Sci.* 63:315–324.
- Canellas, L.P., F.L. Olivares, N.O. Aguiar, D.L. Jones, A. Nebbioso, P. Mazzei, and A. Piccolo. 2015. Humic and fulvic acids as biostimulants in horticulture. *Scientia Hort.* 196:15–27.
- Canellas, L.P., F.L. Olivares, A.L. Okorokova-Façanha, and A.R. Façanha. 2002. Humic acids isolated from earthworm compost enhance root elongation, lateral root emergence, and plasma membrane H⁺-ATPase activity in maize roots. *Plant Physiol.* 130:1951–1957.
- Carvalho, L.C., B.J. Vilela, P. Vidigal, P.M. Mullineaux, and S. Amâncio. 2006. Activation of the ascorbate-glutathione cycle is an early response of micropropagated *Vitis vinifera* L. explants transferred to ex vitro. *Intl. J. Plant Sci.* 167:759–770.
- Chen, L.-M., J.-T. Cheng, E.-L. Chen, T.-J. Yiu, and Z.-H. Liu. 2002. Naphthaleneacetic acid suppresses peroxidase activity during the induction of adventitious roots in soybean hypocotyls. *J. Plant Physiol.* 159:1349–1354.
- Cheniany, M., H. Ebrahimzadeh, A. Masoudinejad, K. Vahdati, and C. Leslie. 2010. Effect of endogenous phenols and some antioxidant enzyme activities on rooting of Persian walnut (*Juglans regia* L.). *Afr. J. Plant Sci.* 4:479–487.

- Chu, E.P., A.R. Tavares, S. Kanashiro, P. Giampaoli, and E.S. Yokota. 2010. Effects of auxins on soluble carbohydrates, starch and soluble protein content in *Aechmea blanchetiana* (Bromeliaceae) cultured *in vitro*. *Scientia Hort.* 125:451–455.
- Cordeiro, F.C., C. Santa-Catarina, V. Silveira, and S.R. de Souza. 2011. Humic acid effect on catalase activity and the generation of reactive oxygen species in corn (*Zea mays*). *Biosci. Biotechnol. Biochem.* 75:70–74.
- Curir, P., C.F. VanSumere, A. Termini, P. Barthe, A. Marchesini, and M. Dolci. 1990. Flavonoid accumulation is correlated with adventitious roots formation in *Eucalyptus gunnii* Hook micropropagated through axillary bud stimulation. *Plant Physiol.* 92:1148–1153.
- Da Costa, C.T., M.R. De Almeida, C.M. Ruedell, J. Schwambach, F.D.S. Maraschin, and A.G. Fett-Neto. 2013. When stress and development go hand in hand: Main hormonal controls of adventitious rooting in cuttings. *Front. Plant Sci.* 4:133.
- Dash, G.K., S.K. Senapati, and G.R. Rout. 2011. Effect of auxins on adventitious root development from nodal cuttings of *Saraca asoka* (Roxb.) de Wilde and associated biochemical changes. *J. Hort. For.* 3:320–326.
- Davies, F.T., T.D. Davis, and D.E. Kester. 1994. Commercial importance of adventitious rooting to horticulture, p. 53–59. In: T.D. David and B.E. Haissig (eds.). *Biology of adventitious root formation*. Springer, New York.
- De Klerk, G.-J., H. Guan, P. Huisman, and S. Marinova. 2011. Effects of phenolic compounds on adventitious root formation and oxidative decarboxylation of applied indoleacetic acid in *Malus* 'Jork 9'. *Plant Growth Regulat.* 63:175–185.
- de Klerk, G.-J., W. van der Krieken, and J.C. de Jong. 1999. Review the formation of adventitious roots: New concepts, new possibilities. In *Vitro Cell. Dev. Biol. Plant* 35:189–199.
- De Smet, I., S. Vanneste, D. Inzé, and T. Beeckman. 2006. Lateral root initiation or the birth of a new meristem. *Plant Mol. Biol.* 60:871–887.
- DeGroote, D.K. and P.R. Larson. 1984. Correlations between net auxin and secondary xylem development in young *Populus deltoides*. *Physiol. Plant.* 60:459–466.
- Eeckhaut, T., K. Janssens, E. De Keyser, and J. De Riek. 2010. Micropropagation of rhododendron, p. 141–152. In: S.M. Jain and S.J. Ochatt (eds.). *Protocols for in vitro propagation of ornamentals plants*. Humana Press Inc, Totowa, NJ.
- Elmogy, M.S., Y. Cao, H. Zhou, and Y. Xia. 2018a. Root development enhanced by using Indole-3-butyric acid and naphthalene acetic acid and associated biochemical changes of *in vitro* azalea microshoots. *J. Plant Growth Regul.* 37:813–825.
- Elmogy, M.S., H. Zhou, Y. Cao, B. Liu, and Y. Xia. 2018b. The effect of humic acid on endogenous hormone levels and antioxidant enzyme activity during *in vitro* rooting of evergreen azalea. *Scientia Hort.* 227:234–243.
- Elstner, E.F. and A. Heupel. 1976. Inhibition of nitrite formation from hydroxylammonium-chloride: A simple assay for superoxide dismutase. *Anal. Biochem.* 70:616–620.
- Fu, Z., P. Xu, S. He, J.A.T. da Silva, and M. Tanaka. 2011. Dynamic changes in enzyme activities and phenolic content during *in vitro* rooting of tree peony (*Paeonia suffruticosa* Andr.) plantlets. *Maejo Intl. J. Sci.* 5:252–265.
- Fukumoto, L. and G. Mazza. 2000. Assessing antioxidant and prooxidant activities of phenolic compounds. *J. Agr. Food Chem.* 48:3597–3604.
- García, A.C., L.A. Santos, L.G.A. de Souza, O.C.H. Tavares, E. Zonta, E.T.M. Gomes, J.M. García-Mina, and R.L.L. Berbara. 2016. Vermicompost humic acids modulate the accumulation and metabolism of ROS in rice plants. *J. Plant Physiol.* 192:56–63.
- García, A.C., T.A. van Tol de Castro, L.A. Santos, O.C.H. Tavares, R.N. Castro, R.L.L. Berbara, and J.M. García-Mina. 2019. Structure–property–function relationship of humic substances in modulating the root growth of plants: A review. *J. Environ. Qual.* 48:1622–1632.
- Guilfoyle, T.J. and G. Hagen. 2007. Auxin response factors. *Curr. Opin. Plant Biol.* 10:453–460.
- Güneş, T. 2000. Peroxidase and IAA-oxidase activities during rooting in cuttings of three poplar species. *Turk. J. Bot.* 24:97–102.
- Haissig, B.E. 1986. Metabolic processes in adventitious rooting of cuttings, p. 141–189. In: M.B. Jackson (ed.). *New root formation in plants and cuttings*. Springer, New York.
- Haissig, B.E. and T.D. Davis. 1994. A historical evaluation of adventitious rooting research to 1993, p. 275–331. In: T.D. David and B.E. Haissig (eds.). *Biology of adventitious root formation*. Springer, New York.
- Halliwell, B., J.M. Gutteridge, and O.I. Aruoma. 1987. The deoxyribose method: A simple “test-tube” assay for determination of rate constants for reactions of hydroxyl radicals. *Anal. Biochem.* 165:215–219.
- Hartmann, H.T., D.E. Kester, F.T. Davies, and R.L. Geneve. 1997. *Plant propagation: Principles and practices*. Prentice-Hall Inc., Upper Saddle River, NJ.
- Husen, A. 2008. Clonal propagation of *Dalbergia sissoo* Roxb. and associated metabolic changes during adventitious root primordium development. *New For.* 36:13–27.
- Husen, A. and M. Pal. 2007. Metabolic changes during adventitious root primordium development in *Tectona grandis* Linn. f. (teak) cuttings as affected by age of donor plants and auxin (IBA and NAA) treatment. *New For.* 33:309–323.
- Ilczuk, A. and E. Jaczygrad. 2016. The effect of IBA on anatomical changes and antioxidant enzyme activity during the *in vitro* rooting of smoke tree (*Cotinus coggygria* Scop.). *Scientia Hort.* 210:268–276.
- Kaldenhoff, R. and M. Fischer. 2006. Functional aquaporin diversity in plants. *Biochim. Biophys. Acta* 1758:1134–1141.
- Kefeli, V.I., M.V. Kalevitch, and B. Borsari. 2003. Phenolic cycle in plants and environment. *J. Cell Mol. Biol.* 2:13–18.
- Lagrimini, L.M., J. Vaughn, J. Finer, K. Klotz, and P. Rubaihayo. 1992. Expression of a chimeric tobacco peroxidase gene in transgenic tomato plants. *J. Amer. Soc. Hort. Sci.* 117:1012–1016.
- Li, M. and D.W. Leung. 2000. Starch accumulation is associated with adventitious root formation in hypocotyl cuttings of *Pinus radiata*. *J. Plant Growth Regul.* 19:423–428.
- Li, S.-W., L. Xue, S. Xu, H. Feng, and L. An. 2009. Hydrogen peroxide acts as a signal molecule in the adventitious root formation of mung bean seedlings. *Environ. Exp. Bot.* 65:63–71.
- Li, S., L. Xue, S. Xu, H. Feng, and L. An. 2007. Hydrogen peroxide involvement in formation and development of adventitious roots in cucumber. *Plant Growth Regulat.* 52:173–180.
- Liu, Z.-H., I.-C. Hsiao, and Y.-W. Pan. 1996. Effect of naphthaleneacetic acid on endogenous indole-3-acetic acid, peroxidase and auxin oxidase in hypocotyl cuttings of soybean during root formation. *Bot. Bull. Acad. Sin.* 37:247–253.
- Livak, K.J. and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25:402–408.
- McCready, R., J. Guggolz, V. Silviera, and H. Owens. 1950. Determination of starch and amylose in vegetables. *Anal. Chem.* 22:1156–1158.
- Mishra, B.S., M. Singh, P. Aggrawal, and A. Laxmi. 2009. Glucose and auxin signaling interaction in controlling *Arabidopsis thaliana* seedlings root growth and development. *PLoS One* 4:e4502, doi: 10.1371/journal.pone.0004502.
- Molassiotis, A., K. Dimassi, G. Diamantidis, and I. Therios. 2004. Changes in peroxidases and catalase activity during *in vitro* rooting. *Biol. Plant.* 48:1–5, doi: 10.1023/B:BIOP.0000024267.68394.96.
- Mora, V., R. Baigorri, E. Bacaoa, A.M. Zamarreno, and J.M. García-Mina. 2012. The humic acid-induced changes in the root concentration of nitric oxide, IAA and ethylene do not explain the changes in root architecture caused by humic acid in cucumber. *Environ. Exp. Bot.* 76:24–32.
- Muscolo, A., S. Cutrupi, and S. Nardi. 1998. IAA detection in humic substances. *Soil Biol. Biochem.* 30:1199–1201.
- Nardi, S., P. Carletti, D. Pizzeghello, and A. Muscolo. 2009. Biological activities of humic substances, p. 309–339. In: N. Senesi, B. Xing, and P.M. Huang (eds.). *Biophysicochemical processes involving natural nonliving organic matter in environmental systems*. Wiley, Hoboken, NJ.
- Nardi, S., A. Ertani, and O. Francioso. 2017. Soil-root cross-talking: The role of humic substances. *J. Plant Nutr. Soil Sci.* 180:5–13.
- Neves, C., M.C. Sá, and S. Amâncio. 1998. Histochemical detection of H_2O_2 by tissue printing as a precocious marker of rhizogenesis in grapevine. *Plant Physiol. Biochem.* 36:817–824.
- Nunes, R.O., G.A. Domiciano, W.S. Alves, A.C.A. Melo, F.C.S. Nogueira, L.P. Canellas, F.L. Olivares, R.B. Zingali, and M.R. Soares. 2019. Evaluation of the effects of humic acids on maize root architecture by label-free proteomics analysis. *Sci. Rep.* 9:1–11, doi: 10.1038/s41598-019-48509-2.
- Olaetxea, M., V. Mora, A.C. García, L.A. Santos, R. Baigorri, M. Fuentes, M. Garnica, R.L.L. Berbara, A.M. Zamarreno, and J.M. García-Mina. 2016. Root-shoot signaling crosstalk involved in the shoot growth promoting action of rhizospheric humic acids. *Plant Signal. Behav.* 11:e1161878, doi: 10.1080/15592324.2016.1161878.
- Oliver, M.J., I. Mukherjee, and D.M. Reid. 1994. Alteration in gene expression in hypocotyls of sunflower (*Helianthus annuus*) seedlings associated with derooting and formation of adventitious root primordia. *Physiol. Plant.* 90:481–489.
- Oono, Y., C. Ooura, A. Rahman, E.T. Aspuria, K.-I. Hayashi, A. Tanaka, and H. Uchimiya. 2003. p-Chlorophenoxyisobutyric acid impairs auxin response in *Arabidopsis* root. *Plant Physiol.* 133:1135–1147.
- Osterc, G., M.M. Petkovšek, and F. Stampar. 2016. Quantification of IAA metabolites in the early stages of adventitious rooting might be predictive for subsequent differences in rooting response. *J. Plant Growth Regul.* 35:534–542.
- Pacurar, D.I., I. Perrone, and C. Bellini. 2014. Auxin is a central player in the hormone cross-

- talks that control adventitious rooting. *Physiol. Plant.* 151:83–96.
- Pedreno, M. 1995. The polyfunctionality of cell wall peroxidases avoids the necessity of an independent H₂O₂-generating system for phenolic coupling in the cell wall. *Plant Perox. Newslett.* 5:3–8.
- Perveen, S. and M. Anis. 2015. Physiological and biochemical parameters influencing *ex vitro* establishment of the *in vitro* regenerants of *Albizia lebbbeck*. *Agrofor. Syst.* 89:721–733.
- Pizzeghello, D., O. Francioso, A. Ertani, A. Muscolo, and S. Nardi. 2013. Isopentenyladenosine and cytokinin-like activity of different humic substances. *J. Geochem. Explor.* 129:70–75.
- Pizzeghello, D., G. Nicolini, and S. Nardi. 2002. Hormone-like activities of humic substances in different forest ecosystems. *New Phytol.* 155:393–402.
- Porfírio, S., M.D.R. Gomes da Silva, M.J. Cabrita, P. Azadi, and A. Peixe. 2016. Reviewing current knowledge on olive (*Olea europaea* L.) adventitious root formation. *Scientia Hort.* 198: 207–226.
- Quiroga, M., C. Guerrero, M.A. Botella, A. Barceló, I. Amaya, M.I. Medina, F.J. Alonso, S.M. de Forchetti, H. Tigier, and V. Valpuesta. 2000. A tomato peroxidase involved in the synthesis of lignin and suberin. *Plant Physiol.* 122:1119–1128.
- Shen, C., R. Yue, T. Sun, L. Zhang, L. Xu, S. Tie, H. Wang, and Y. Yang. 2015. Genome-wide identification and expression analysis of auxin response factor gene family in *Medicago truncatula*. *Front. Plant. Sci.* 6:73.
- Solar, A., M. Colarić, V. Usenik, and F. Stampar. 2006. Seasonal variations of selected flavonoids, phenolic acids and quinones in annual shoots of common walnut (*Juglans regia* L.). *Plant Sci.* 170:453–461.
- Su, G.X., W.H. Zhang, and Y.L. Liu. 2006. Involvement of hydrogen peroxide generated by polyamine oxidative degradation in the development of lateral roots in soybean. *J. Integr. Plant Biol.* 48:426–432.
- Trevisan, S., D. Pizzeghello, B. Ruperti, O. Francioso, A. Sassi, K. Palme, S. Quaggiotti, and S. Nardi. 2010. Humic substances induce lateral root formation and expression of the early auxin-responsive *IAA19* gene and *DR5* synthetic element in *Arabidopsis*. *Plant Biol.* 12:604–614.
- Vatankhah, E., V. Niknam, and H. Ebrahimzadeh. 2010. Activity of antioxidant enzyme during *in vitro* organogenesis in *Crocus sativus*. *Biol. Plant.* 54:509–514.
- Vaughan, D. and R.E. Malcolm. 1985. Influence of humic substances on growth and physiological processes, p. 37–75. In: D. Vaughan and R.E. Malcolm (eds.). *Soil organic matter and biological activity*. Springer Netherlands, Dordrecht.
- Wang, L., C. Ba, H. Cao, C. Zong, and H. Yao. 2015. Study on multiplication, rooting and transplanting of tissue culture plantlets of *Rhododendron chrysanthum* Pall. *Agr. Sci. Tech.* 16:1348–1373.
- Weiser, C. and L. Blaney. 1967. The nature of boron stimulation to root initiation and development in beans. *Proc. Amer. Soc. Hort. Sci.* 90:191–200.
- Wiesman, Z., J. Riov, and E. Epstein. 1988. Comparison of movement and metabolism of indole-3-acetic acid and indole-3-butyric acid in mung bean cuttings. *Physiol. Plant.* 74:556–560.
- Wiszniewska, A., B. Nowak, A. Kolton, E. Sitek, K. Grabski, M. Dziurka, O. Długosz-Grochowska, K. Dziurka, and Z. Tukaj. 2016. Rooting response of *Prunus domestica* L. microshoots in the presence of phytoactive medium supplements. *Plant Cell Tiss. Org.* 125:163–176.
- Wong, S. 1982. Direct rooting of tissue-cultured rhododendrons into an artificial soil mix. *agris.-fao.org* 31:36–39.
- Woodward, A.W. and B. Bartel. 2005. Auxin: Regulation, action, and interaction. *Ann. Bot.* 95:707–735.
- Wu, Y., Y.-p. Xia, J.-p. Zhang, F. Du, L. Zhang, and H. Zhou. 2016. Low humic acids promote *in vitro* lily bulblet enlargement by enhancing roots growth and carbohydrate metabolism. *J. Zhejiang Univ. Sci. B* 17:892–904.
- Yan, S.P., R.H. Yang, F. Wang, L.N. Sun, and X.S. Song. 2017. Effect of auxins and associated metabolic changes on cuttings of hybrid aspen. *Forests* 8:117, doi: 10.3390/f8040117.
- Yang, C., M. Xu, L. Xuan, X. Jiang, and M. Huang. 2014. Identification and expression analysis of twenty *ARF* genes in *Populus*. *Gene* 544:134–144.
- Yang, X., S. Lee, J.h. So, S. Dharmasiri, N. Dharmasiri, L. Ge, C. Jensen, R. Hangarter, L. Hobbie, and M. Estelle. 2004. The *IAA1* protein is encoded by *AXR5* and is a substrate of SCFTIR1. *Plant J.* 40:772–782.
- Yildiztugay, E., C. Ozfidan-Konakci, F. Elbasan, A. Yildiztugay, and M. Kucukoduk. 2019. Humic acid protects against oxidative damage induced by cadmium toxicity in wheat (*Triticum aestivum*) roots through water management and the antioxidant defence system. *Bot. Serb.* 43:161–173.
- Zandonadi, D.B., L.P. Canellas, and A.R. Façanha. 2007. Indolacetic and humic acids induce lateral root development through a concerted plasmalemma and tonoplast H⁺ pumps activation. *Planta* 225:1583–1595.
- Zhang, H., C. Ning, D. Chunjuan, and Q. Shang. 2017. Genome-wide identification and expression of ARF gene family during adventitious root development in hot pepper (*Capsicum annuum*). *Hort. Plant J.* 3:151–164.

Supplemental Fig. 1. Evergreen azalea plants grown for 30 d in Anderson free medium (A) and media supplemented with IBA (B), NAA (C), and HA (D) at a concentration of 2 mg/L.