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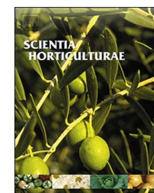

The effect of humic acid on endogenous hormone levels and antioxidant enzyme activity during in vitro rooting of evergreen azalea

Mohamed S. Elmongy[a,b], Hong Zhou[a], Yan Cao[a], Bing Liu[a], Yiping Xia[a,*]

[a] *Physiology and Molecular Biology Laboratory of Ornamental Plants, Department of Horticulture, College of Agriculture and Biotechnology, Zhejiang University, Hangzhou, PR China*
[b] *Department of Vegetable and Floriculture, Faculty of Agriculture, Mansoura University, Mansoura 35516, Egypt*

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ABSTRACT

It is important to produce roots in vitro for woody plants, such as azaleas. The influence of humic acid (HA) on histological development, antioxidant enzyme changes and endogenous hormone levels was evaluated during adventitious root formation in evergreen azalea microshoots. Explants (microshoots) were transferred to Anderson rooting medium supplemented with HA at 0, 0.5, 1, 2 and 5 mg L^{-1} for 56 days. HA at 1 and 2 mg L^{-1} improved the morphological root character of microshoots, such as root length, root number and rooting percentage compared with other treatments. The data collected during anatomical evaluation indicated that the cell division occurred on the third day of culture in the phloem adjacent to the cambium, which led to differentiation of the root primordium after ten days. Both treatments of HA (1 and 2 mg L^{-1}) increased the endogenous hormone levels of indole-3-acetic acid (IAA) and gibberellic acid (GA) in rooted shoots, especially at the first period of root development. However, the increase of zeatin riboside (ZR) and isopentenyl adenosine (iPA) levels was shown during the in vitro rooting process. Moreover, HA contributed to higher activities in peroxidase (POD), superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), polyphenol oxidase (PPO) and total soluble protein compared with other treatments at the same concentration. The results demonstrated that HA is effective for rooting in evergreen azaleas, and this effect was related to physiological and metabolic changes during adventitious root formation. Therefore, this information could help in developing a new type of rooting stimulator to reduce the high cost of plant growth hormones that are used for micropropagation.

1. Introduction

The azalea represents a small section of the *Rhododendron* genus, which includes nearly one thousand described cultivars and thousands more commercial cultivars (Meijón et al., 2011; Meijón et al., 2009). Recently, in China, the area devoted to azaleas cultivated for commercial production was extended to cover more than 2500 ha to meet the landscape requirements and home cultivation (Zhou, 2010). Stem cutting is the conventional method to propagate most of the *Rhododendron* species, including azaleas. However, rooting cuttings for seedlings is not too easy, depending on the weather and the variety. Tissue culture or micropropagation is a popular method of producing large numbers of azaleas and rhododendrons for commercial production because of the advancements in shoot-tip culture and developments in producing roots in vitro (Hsia and Korban, 1997). The formation of an adventitious root is a decisive step during in vivo and in vitro propagation of woody plants related to physiological, anatomical and biochemical factors (Ilczuk and Jacygrad, 2016). Additionally, most tissue culture protocols depend on a method that is successful for the root induction (Davies et al., 1994).

In the last years, restrictions have been imposed on the use of plant chemical hormones, including auxins, in plant production (Wiszniewska et al., 2016). Therefore, using root-promoting substances and natural root stimulators is one of the strategies aimed to improve the rooting efficiency and to reduce the duration of the use of exogenous auxins (Arthur et al., 2004; Pacholczak et al., 2012; Montero-Calasanz et al., 2013). Furthermore, application of natural rooting stimulators may reduce the losses caused by the poor quality of the root system that resulted from auxin usage in the rooting induction (De Klerk et al., 1999; Kakani et al., 2009). Humic acid (HA) is the most common natural polymeric material distributed worldwide (Stevenson, 1994). HA promotes the plant growth and development through improving nutrient uptake (Chang et al., 2012; Nikbakht et al., 2008) and mediating the physiological, biochemical and metabolic processes in plants (Canellas et al., 2012; Tahiri et al., 2015). Moreover, HA enhances root growth morphological traits (Canellas et al., 2002;

* Corresponding author.
E-mail address: ypxia@zju.edu.cn (Y. Xia).

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Zandonadi et al., 2007; Mora et al., 2012; Baldotto and Baldotto, 2014), such as root initiation and root length (Mylonas and McCants, 1980), and root fresh and dry weight (Nikbakht et al., 2008), since HA has an express auxinic effect and high hormonal activity in the plant (auxin-like activity) (Canellas et al., 2002; Muscolo et al., 1999; Nardi et al., 1994; Quaggiotti et al., 2004). HA is also involved in photosynthesis, amino acids, carbohydrates, protein content, synthesis of nucleic acids, and enzyme activities (Vaughan and Malcolm, 1985). HA also increases the signaling of endogenous auxin for root development and enhances root development in vitro, such as the effect of indole-3-acetic acid (IAA) (Nardi et al., 1994).

A relationship was observed between the effect of HA and the response of antioxidant enzymes (Chen and Aviad, 1990; Pinton et al., 1992), such as superoxide dismutase and peroxidase, in plants (Haghighi et al., 2010; Kaldenhoff and Fischer, 2006). Previous studies on production of adventitious roots have highlighted the relationship between antioxidant enzymes and their role in the process of in vitro rooting (Moncousin and Gaspar, 1983; Naija et al., 2008; Rout et al., 1999). Thus, it was demonstrated that changes in antioxidant enzyme activities could be used as analytical measures in the root initiation process (Cheniany et al., 2010; Syros et al., 2004).

The objectives of our study were to evaluate the efficiency of HA as a potential rooting promoter on the evergreen azalea (*Rhododendron* subgenus *Tsutusi*) during in vitro rooting. The effect of HA supplements was also tested in relation to endogenous hormone levels, anatomical changes and biochemical responses in process of rhizogenesis. Another aim was to develop a new protocol enabling the limitation of using exogenous auxin hormones for in vitro rooting of azaleas.

2. Materials and methods

2.1. Plant material and culture conditions

The experiments were carried out at the Physiology and Molecular Biology Laboratory of the Ornamental Plants and Tissue Culture Laboratory of Ornamental Plants, Department of Horticulture, Zhejiang University, Hangzhou, China. The cultivar of azalea (Zihudie) was collected from a campus nursery at Zhejiang University. Plant materials were taken from the multiplication medium of evergreen azalea plants that were cultured on the Anderson media (Anderson, 1984) supplemented with 5.7 μM indole-3-acetic acid, 4.56 μM zeatin, 30 g L − 1 sucrose and 8 g L − 1 agar (Difco Bacto TM Agar), and transferred to elongation media fortified with 14.76 μM 2-isopentenyladenine (2iP). Microshoots with 8–10 leaves (2–3 cm length) were cultured on rooting Anderson medium (Fig. 1A) fortified with HA (aladdin®, H108498, China) at concentrations of 0, 0.5, 1, 2, and 5 mg L^{-1} without adding any phytohormones to the media. HA was supplemented to the medium prior to autoclaving. All of the cultures were incubated at 25 ± 1 °C under a 16 h photoperiod of 2500 lx light intensity. Explants were cultured inside the culture cabinet (hood flow). Media were prepared before the culturing and poured into 250 mL jars. These media were sterilized at 121 °C with an overpressure of 0.1 MPa for 20 min in the autoclave. The pH was adjusted to 5.8 with 0.1 N NaOH or 0.1 N HCl prior to autoclaving. After 56 days of the root induction process, the ability of HA for rooting was evaluated, and data on the number of rooted microshoots (rooting percentage), the number of produced roots per microshoot and root length were taken. Endogenous hormone levels, physiological changes and biochemical traits were determined after 7, 14, 21, 28, 42 and 56 days after transfer to media. The shoots were harvested at 9:00 am on each sampling date. Microshoot samples were thoroughly washed and immediately frozen in liquid nitrogen (≥30 min), then stored at −75 °C until further use.

2.2. Histological analysis

Basal segments 1 cm in height from the base of the explant were collected at 0, 3, 5, 7, 10, 15, 18, 20, 26, 28, 30, 40 and 45 days after culturing on media. The sections were fixed on a mixture of formaldehyde/ethanol/acetic acid (FAA) 10:85:5 (v/v/v), respectively. Dehydration was thoroughly graded ethanol − xylene series, then the sections were embedded in paraffin wax (Jensen, 1962). The sections were cut into 15 μm lengths using a microtome Leica (RM2016, Leica Biosystems Nusstoch GmbH, Germany) and stained with hematoxylin for examination. The sections were then checked by using a Nikon ECLIPSE CI microscope (Japan) equipped with a high digital camera (Nikon DS-U3 Japan).

2.3. Determination of endogenous hormones levels

Measurements of endogenous hormones including indole acetic acid (IAA), gibberellic acid (GA), zeatin riboside (ZR), and isopentenyl adenosine (iPA) were performed by using the enzyme-linked immunosorbent assay (ELISA) according to You-Ming et al. (2001). The ELISA test kits for each plant hormone were obtained from the College of Crop Sciences, China Agricultural University, Beijing, China. Fresh tissue of in vitro-cultured explants (200 mg, approximately 4–6 whole shoots) was used for measuring the endogenous hormone levels. Samples were freeze-dried, homogenized and extracted for 24 h at 4 °C in 10 mL of cold 80% methanol containing butylhydroxytoluene (1 mM) as an antioxidant. The extracts were centrifuged for 10 min at 10,000*g* at 4 °C (Avanti 30 centrifuge, Beckman), then passed through a C_{18} Sep-Pak cartridge to purify (Waters, Milford, MA) and dried in N_2. The residues were extracted one more time with 2 mL of cold methanol for 12 h, and then the supernatants were combined and refined using Sep-Pak C-18. Then, all supernatants were moved to another flask. To remove the methanol remnant, the samples were vacuum-dried with a rotary evaporator at 37 °C. Then, the residues were dissolved in a buffer solution (0.05 mM Tris, 1 mM $MgCl_2$, 150 mM NaCl, 0.1% gelatin, and 0.1% Tween 20). Microtitration plates were coated with synthetic IAA, GA, ABA, iPA and ZR-ovalbumin conjugates in $NaHCO_3$ buffer (50 mol/L, pH 9.6) and incubated overnight at 37 °C. After incubation for 30 min at 37 °C, standard IAA, GA, ABA, iPA, ZR samples and antibodies were added and incubated for another 45 min at 37 °C. Next, peroxidase-labeled goat anti-rabbit immunoglobulin was added to each well and incubated for 1 h at 37 °C. After that step, the buffered enzyme substrate (ortho-phenylenediamine) was added, and the enzyme reaction was performed in the dark at 37 °C for 15 min and subsequently terminated using 2 mol/L H_2SO_4. The absorbance was recorded at 490 nm. In this experiment, the percentage recovery of each hormone was above 90%, and all sample extract dilution curves paralleled the standard curves, indicating the absence of nonspecific inhibitors in the extracts.

2.4. Measurements of antioxidant enzyme activities

Whole microshoots (0.5 g per treatment and approximately 8–10 shoots) were taken to measure the antioxidant enzyme activities (SOD, CAT, APX and POD). The samples were homogenized and suspended in 8 mL of 50 mM of ice-cold potassium phosphate buffer (pH 7.8). The homogenate was centrifuged at 10,000*g* for 20 min at 4 °C, and the supernatants were used to determine the antioxidant enzyme activities.

Peroxidase activity (POD, EC 1.11.1.7) was measured with guaiacol as the substrate in a total volume of 3 mL (Zhang, 1992). The reaction mixture contained 2.7 mL phosphate buffer (25 μM, pH 7.0) with 0.1 mL H_2O_2 (0.4%), 0.1 mL guaiacol (1.5%), and 0.1 mL of enzyme extract. Increase in the absorbance due to the oxidation of guaiacol ($E = 25.5\ mM^{-1}\ cm^{-1}$) was measured at 470 nm. The enzyme activity was calculated in terms of l M of guaiacol oxidized g^{-1} FW min^{-1} at 25 ± 2 °C.

Superoxide dismutase (SOD, EC 1.15.1.1) enzyme activity was checked by measuring its inhibition of the amount of nitro blue tetrazolium (NBT) photochemical reduction according to Sheteiwy et al. (2017). The total volume of the reaction mixture was 3.1 mL, including

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