Pak. J. Bot., 49(6): 2295-2299, 2017.

DYNAMICS OF ENDOGENOUS HORMONES, ANATOMICAL STRUCTURE DURING THE CUTTING PROPAGATION OF WILD *RHODODENDRON SACBRIFOLIUM* FRANCH

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Abstract

To establish a feasible commercial cutting propagation system of mountain *Rhododendron*, the physiological changes during cutting propagation of *Rhododendron scabrifolium* Franch were investigated. The semi-hardwood stems of *Rhododendron scabrifolium* were collected and treated with KNAA and Hormodin. Contents of endogenous hormones indoleacetic acid (IAA), gibberellin (GA), zeatin riboside (ZR), and abscisic acid (ABA) were measured during the rooting period, the structural and number changes of starch grains and crystal in the middle scales were observed by scanning electron microscope (SEM). The results showed that the treatments of hormone significantly affected the growth rate of the root and its number. Higher IAA levels in cuttings induced formations of root better; GA levels showed a positive correlation with the cuttings' root formation, but ABA levels showed a negative correlation with the rooting rates; Cuttings needed ZR in early-stage of rooting process, but ZR levels decreased in late-stage of rooting process. At the early-stage of rooting, there is no starch grain and crystal in the cell chamber of xylem and myeloid cells. Along with adventitious root formation and differentiation, starch grains and crystals were also formed. Starch grains are mainly round and the type of crystal cluster-like calcium oxalate crystals.

Key words: Cutting propagation, *Rhododendron scabrifolium*, Endogenous hormones.

Introduction

Rhododendron is the largest genus in the family Ericaceae containing about 1000 species, and the taxonomy has been historically complex (Chamberlain, 2003; Gillian *et al*., 2006). Their distribution is mostly in Asia, including 571 species in China (Fang *et al*., 2005). Chinese *Rhododendron* species are cultivated as ornaments worldwide, and the majority of these species have showy flowers. Throughout the century-old tradition, people have discovered and adapted native plant *Rhododendron* for alimentary, flavoured and medicinal use. Some species of *Rhododendron* contain toxic diterpenes, which are harmful to human beings (Li *et al*., 2013). Previous studies were conducted an in-depth study on the members of *Rhododendron* family using DNA sequence data, morphology and anatomy (Kron & Powell 2009; Bush *et al*., 2009; Gillespie & Kron 2013), moreover, ITS sequences were also studied (Gao e*t al*., 2002; Gillian *et al*., 2006; Miyano *et al*., 2013; Zhao *et al*., 2015). Baili *Rhododendron* National Forest Park is the only region on earth where there are undisturbed *Rhododendron* distributions in broad-leaved evergreen forests. This unbroken distribution has led to the formation of rich *Rhododendron* associations rarely seen elsewhere in the world (Li *et al*., 2015).

Rhododendron scabrifolium Franch (Ericaceae) is an evergreen understory shrub found in abundance on xeric, southeastern slopes on hillsides. Young shoots are densely gray-white-pubescent and spreading-hispid. *Rhododendron scabrifolium* typically forms a dense understory in mixed *Pinus yunnanensis* Franch stands and occasionally in association with *Rhododendron simsii* Planch (Fang *et al*., 2005). It is a dominant species in mountainous of Guizhou Province, especially in the Baili *Rhododendron* National Forest Park.

Transplant survival rate and cutting survival rate is low under natural conditions; these characteristics impede the cultivation of *Rhododendron scabrifolium*. To meet the market demand for propagation material, research on its reproduction is imperative. Hormones have a positive effect on cutting propagation, but there still exist several difficulties in rooting of wild *Rhododendron*. High levels of hormones may stimulate to cultivate many hard-to-root species (Jetton *et al*., 2005). This work was aimed to investigate endogenous hormones, including IAA, GA, ZR, ABA, and the starch grains and crystal in stem cells and also to study the rooting mechanism and promote cloning techniques of *Rhododendron*.

Materials and Methods

Plant material and cultivation: The study was carried out for three months from August to October 2014 in the Key Laboratory of Plant Physiology and Development Regulation at Guizhou Normal University campus, Guiyang, southwest China. Semi-hardwood cuttings (with or without heels) were collected in August 2014 from Baili *Rhododendron* National Forest Park, and transported with moist containers to the lab. Cuttings were prepared by standard procedures and kept under full-light greenhouse with intermittent mist.

Cuttings were treated with 1-Naphthylacetic acid potassium salt (KNAA) and Hormodin rooting powder (Merck Chemical Division, PA, USA). The treatments used were as follows: the KNAA levels were 3,000 mg·L^{-1}, and the Hormodin＃2 (3,000 mg·L^{-1} IBA-talc). The design was employed in this experiment with 20 pseudo-replicates per treatment. The control was tap water. The application method was quick dip, and the medium was peat moss and perlite, and the air relative was humidity ≥85%, removing the leaves from the lower one-third to one-half of the cutting, tapping the base of stems with scissor to facilitate cuttings and producing callus for a better absorption of exogenous hormones, inserting the cuttings one-third their length into the medium and maintaining the vertical orientation of the stems. The determination of rooting periods: Early-stage in rooting process (0-30d), late-stage in rooting process (30-60d). Endogenous hormones IAA, GA, ZR, and ABA determinated by enzyme-linked immune sorbent assay (ELISA), the results are the means ±SD of at least three replicates (Yang *et al*., 2001). Central stems with 2 cm long pieces before and after rooting, was sliced on a microtome, the thickness is 25 μm. Stems anatomical structure were examined by scanning electron microscopy (SEM, JSM-6490LV, Japan Electronics Co., Japan) and energy dispersive spectroscopy (EDS, Inca450, Oxford Co., UK). The starch grains in xylem and myeloid under the microscope, randomly selected five horizons, each horizon selected three cells the statistical, calculated the average number of single cell starch grains was calculated.

Statistical analysis of the data: All the data analysis and graphing were performed using the SPSS 13.0 software (SPSS Inc., Chicago, IL, USA), and the Origin 8.0 software (OriginLab Corporation, Northampton, MA, USA).

Results

Rooting efficiency of cuttings with different hormone treatments: The hormone significantly improved the rooting of *Rhododendron scabrifolium* cuttings. The variance analysis and multiple comparisons, the treatment of hormone have been better and the root number and rooting rates, and has significant differences with the control (Fig. 1A and B).The exogenous hormones that promote the rooting rate of cuttings have been reported by many researchers (Wang *et al*., 2013). Exogenous hormones are closely related to concentration of endogenous hormone under stages of the rooting process (Satisha *et al*., 2008; Rout, 2006).

Variation of endogenous hormone content in stems after cutting propagation: In the rooting process IAA content changes trend generally rendered as "rise-fall-rise" model (Fig. 2A). The contents IAA in cuttings increased during the beginning period, and this, probably, due to the effect of exogenous hormone treatment. IAA content increased compared with the control. It shows that a higher endogenous IAA content is conducive to root primordia differentiation and rooting. A great number of work suggested that IAA was the main hormone to promote adventitious root formation (Wiesman *et al*., 1989; Verstraeten *et al*., 2013), and part of them indicated that only IAA seemed to be closely associated with the potential of root development in cuttings of wild *Rhododendron* species (Yoo *et al*., 2008).

The changing trend of GA content is similar with IAA (Fig. 2B), the late-stage of cutting, GA showed a significant downward trend might, be new roots break through the epidermis and consume a certain amount of GA content, GA plays an important role in root primordia differentiation. The endogenous ZR content of cuttings change trend is first rising slowly, and then sharp decline, and then rise slowly, while the ZR content of control cuttings changed little (Fig. 2C). Thus, the treatment of exogenous hormone increased endogenous ZR synthesis of cuttings, consumed part of endogenous ZR when the callus was generated. ZR showed a downward trend after September.

ABA contents of treated cuttings were significantly lower than the control, indicating that low concentrations of ABA played an important role in the callus formation of cuttings (Fig. 2D). Low concentration of ABA is beneficial for IAA and other substance transport to the base of cuttings, cuttings through the treatment of exogenous hormone can enhance this effect.

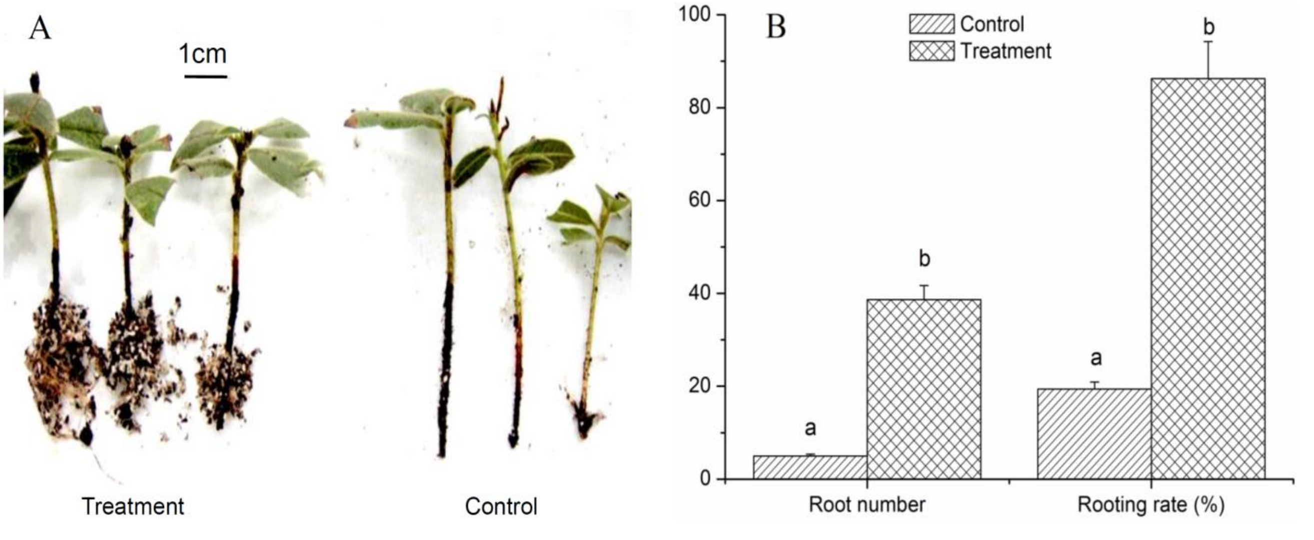

Fig. 1. Rooting efficiency with different treatments (A), and average root number, rooting rate of *R.scabrifolium* (B).

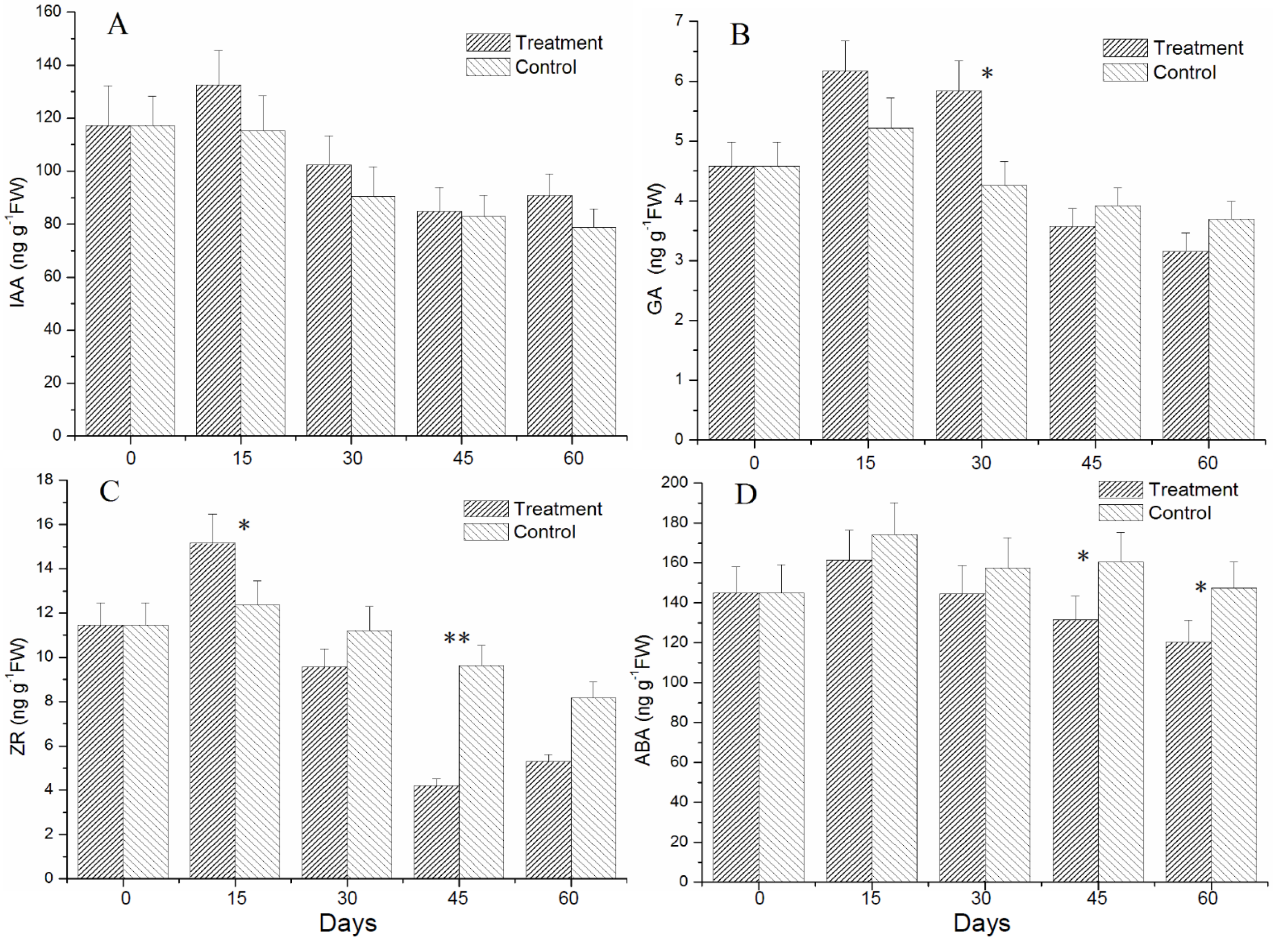

Fig. 2. Changes in contents of indol acetic acid (A), gibberellin (B), zeatin riboside (C), and abscisic acid (D) at different periods of root development from cuttings pretreated.
Means (n=3 per treatment ± SD). * and **, significant difference at 5 and 1%, respectively.

Anatomical structure of cuttings during different rooting process: The adventitious root of *R. scabrifolium* cuttings is derived from a type of induced root primordium (Fig. 3A and 3C). Starch grains and crystals in stem tissue cells are obviously changed at different stages after cutting. In the early-stage, stem xylems have no starch grains, starch grains are generated with the callus formed, while a large number of crystal clusters are generated. In the early-stage, carbohydrate consumption from respiration and growth is far more than produced by photosynthesis. When adventitious roots are generated, enhanced photosynthesis and accumulated carbohydrate, starch grains appeared in large numbers. At the same time, crystal generated with the plants absorb mineral nutrition (Fig. 3B and 3D).

Three elements, including C, O and Ca in the crystal were also analyzed in the present research, and these are 38.94%, 44.15% and 16.91%, respectively. The crystal type is calcium oxalate (CaOx) cluster-like crystals (Fig. 3E). The main component of calcium oxalate crystals is oxalic acid from endogenous synthesis and Ca^{2+} absorption from outside. The root system architecture of the plant is also dependent on the availability of calcium, and calcium is essential and important for the plant (Bellini *et al*., 2014). Crystals generated in the stems of *Rhododendron scabrifolium* probably consistent with the characteristics of the *Rhododendron* acidophilus environment. *Rhododendron* must be grown in acidic soils; most soils have a native acid reaction (Erfmeier & Bruelheide, 2010). Calcium oxalate crystals are the most prevalent and widely distributed mineral deposits in plants (Franceschi & Nakata, 2005). Calcium oxalate crystals may be produced by oxalic acid, and calcium ions abundant accumulation in a specific area in the plant, and oxalic acid is one of poison or the final product metabolism (Libert & Franceschi, 1987).

Discussion

Propagation by stem cuttings is the most commonly used methods to propagate many woody ornamental plants (Adeoluwa *et al*., 2014; Wang *et al*., 2014);cutting propagation technology is complex system involves many factors and the plant can easily root with exogenous auxin treatments (Dirr & Hwuser, 1987; Henrique *et al*., 2006).Cuttings of Bald cypress treated with K-IBA yielded an optimum root quantity and rooted cutting quality (King *et al*., 2011), but in the rooting of *Cryptomeria* cuttings, IBA had no effect on softwood cuttings (Jull *et al*., 1994). Wild *Rhododendron scabrifolium* is difficult to root with cuttings, different growth regulators and concentrations have a significant influence on the rooting rate. The cuttings root effect is best with quick dipped into liquid KNAA, and then the powder Hormodin has a significant affection. Exogenous auxin treatments can effectively improve the rooting cuttings of azalea consistent with previous research (Hartmann *et al*., 2007; Slade *et al*., 2012; Wang *et al*., 2013; Gehlot *et al*., 2015). To date, there has been no direct experimental test of the endogenous hormones, starch grains and crystal on rooting in *Rhododendron scabrifolium* stem cuttings. The results of this trial are encouraging and indicate that semi-hardwood stems from *Rhododendron* can be rooted at reasonably high rates with exogenous auxin treatments.

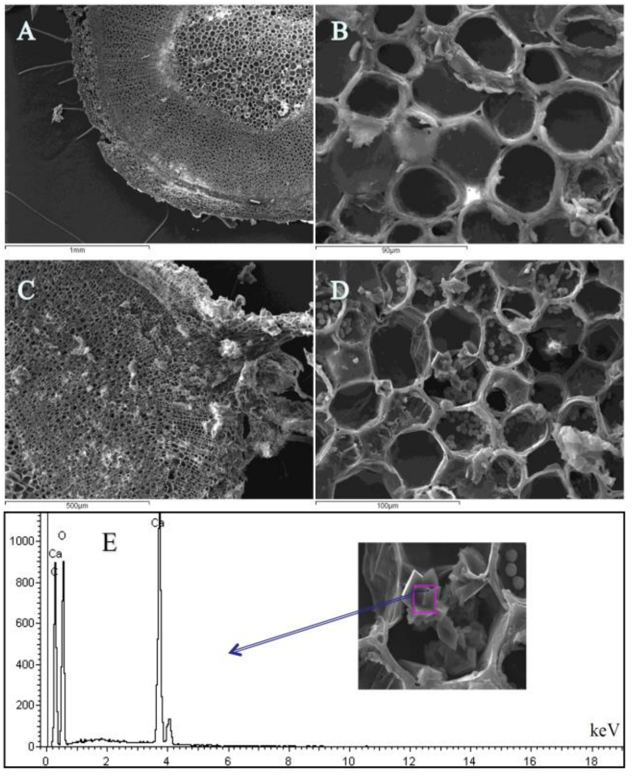

Fig. 3. (A) Stem anatomical structure of in early-stage rooting process; (B) Stem cell structure of stem in early-stage rooting process; (C) Stem anatomical structure of in late-stage rooting process; (D) Stem cell structure of stem in late-stage rooting process; and (E) Energy-dispersive X-ray spectroscopy component spectra of crystal.

IAA, GA, ZR and ABA content of cuttings are closely related with adventitious root formation. Higher concentrations of endogenous IAA promoted rooting rate in general (Sagee *et al.*, 1992; Kelen& Ozkan, 2003); however, rooting could occur by decreasing concentrations of endogenous IAA (Hausman, 1993). Also, previous studies have indicated that hormones play important and diverse roles in plant cutting propagation (Nag *et al*., 2001; Niemi *et al*., 2002; Rout, 2006).

Plant stems having a large number of calcium oxalate crystals show that calcium regulation mechanisms are formed. Crystals generation can help plants in the calcium regulation, plant protection, detoxification mechanisms. Starch grains are mainly round and nearly round and the type of crystal cluster-like calcium oxalate crystals (Konyar *et al*., 2014). Ca^{2+} plays a key role as an essential nutrient in plants; it also participates in root and stem elongation (White & Broadley, 2003). The formation of calcium oxalate crystal also requires a lot of calcium source; calcium ions can through the osmotic regulation are absorbed by the root xylem, and are accumulated in the cell (Bouropoulos *et al*., 2001; Xiong *et al*., 2006). At present, the mechanism of the formation of calcium oxalate crystal is unclear (Webb, 1999).

Conclusions

Rhododendron scabrifolium is one of the mountain flower species in East Asia, which deserve vegetative propagation for commercialization. Cutting propagation through stems might be one of the effective methods of clone for this species. The higher rooting rate of *Rhododendron scabrifolium* was using semi-hardwood cuttings with a heel, quick dip 3,000 mg·L^{-1} K-NAA and then dip Hormodin ＃2 powder, and rooting with perlite: peat moss (3:1) media.

The rooting mechanism of plant cuttings is complex, the physiological and biochemical factors which affect cuttings rooting of *Rhododendron scabrifolium* is also of wide range, the other physiological and biochemical indexes need the further research. This is the earlier research of cutting propagation and mechanism rooting of *Rhododendron*, and it's helpful for protection, expands breeding and further popularized of wild *Rhododendron*.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (NSFC, No. 31460136), the Science and Technology Foundation of Guizhou Province (Grant No. QianKeHe SY [2015] 3022, Qian KeHeLH [2016] 7203; Qian KeZhongYinDi [2017] 4006), and the Forestry Science and Technology Project of Guizhou Province (No. Qian LinKeHe [2016] 09).

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(Received for publiation 26 December 2016)