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Full Paper

Dynamic changes in enzyme activities and phenolic content during in vitro rooting of tree peony (*Paeonia suffruticosa* Andr.) plantlets

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Abstract: The dynamic changes of phenolic content and peroxidase (POD), polyphenol oxidase (PPO), indole-3-acetic acid oxidase (IAAO) and phenylalanine ammonia lyase (PAL) activities were assessed during the in vitro rooting process of three cultivars of tree peony (*Paeonia suffruticosa* Andr.). These changes in enzyme-related activity and phenolic content—observed at the level of the whole plant—differed during the first 20 days of the rooting process in easy-to-root 'Feng Dan Bai' cultivar and difficult-to-root 'Wu Long Peng Sheng' and 'Tai Ping Hong' cultivars, and in most cases they were actually opposite. The ease with which 'Feng Dan Bai' was able to root was closely related to the activity of all four enzymes (POD, PPO, IAAO, PAL) as well as to the phenolic content.

Keywords: tree peony, *Paeonia suffruticosa*, rooting, enzyme activities, phenolic content, dynamic changes

INTRODUCTION

Tree peony (*Paeonia suffruticosa* Andr, Paeoniaceae) is a perennial woody flowering ornamental plant and a famous traditional flower in China [1-2]. Tree peony tissue culture is in vogue since traditional breeding methods have limitations [3]. However, it has not yet been able to meet the requirements for mass production due to difficulty in rooting and the low root quality of plantlets in vitro [4]. Therefore, solving the problem of rooting in vitro may be a key for a breakthrough in tree peony tissue culture.

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There is a close relationship between the occurrence of adventitious roots in plants and their peroxidase (POD, EC 1.11.1.7), polyphenoloxidase (PPO, EC 1.10.3.1), indole-3-acetic acid oxidase (IAAO, no EC number) and phenylalanine ammonia lyase (PAL, EC 4.3.1.5) activities as well as their phenolic content. These enzymes have different functions during rooting [5-6]. Increasing POD activity is a rooting signal in the induction and formation of root primordia [7-8]. POD is involved in auxin metabolism and in the lignification of cell walls in the presence of phenolic compounds [9]. PPO is the main enzyme causing the oxidation of phenolic compounds; it can also catalyse phenolic compounds and IAA to form IAA-phenolic complexes, which promote the occurrence and development of adventitious roots [10]. IAAO affects the occurrence of adventitious roots in plants by oxidising IAA and changing its levels [11-12]. PAL is a key enzyme involved in the synthesis of phenolic compounds [13-14]. PAL activity and total phenolic content of lettuce plants and *Phalaenopsis* orchids were significantly correlated with browning [5-6,15]. There is a yet-to-be-disproved train of thought that an important difference between easy-to-root and difficult-to-root cultivars lies in the difference in the content of phenolic compounds: the former is thought to contain less polyphenols than the latter [16].

In order to provide a theoretical and mechanistic basis for the establishment of efficient rooting during the tissue culture of tree peony, this experiment aims to determine the dynamic changes in POD, PPO, IAAO and PAL activities and total phenol content during the in vitro rooting culture of tree peony cultivars in order to establish whether a relationship between the activities of these enzymes and the phenolic metabolism exists. If a correlation between rooting ability and enzyme activity could be established, this would open up a gateway for molecular manipulation of the plants to increase the level of those enzymes that would permit greater and more successful in vitro rooting, and hence for a successful micropropagation of this ornamental plant. Since rooting and root formation are both poorly understood and equally poorly achieved for this ornamental, an understanding of the enzymatic and biochemical dynamics is expected to provide vital clues to how better to try and improve its in vitro rooting.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals and reagents used in this study were of tissue culture or HPLC grade. Murashige and Skoog (MS) medium [17] and woody plant medium (WPM) [18] were made fresh from MS stocks and WPM stocks respectively. Other more important chemicals and reagents were purchased from the following companies: Phytagel, Folin-Ciocalteu's reagent and agar powder from Solarbio Science and Technology Co. Ltd., Beijing, China; 1-naphthaleneacetic acid (NAA) from Huixing Chemical Reagents Ltd., Shanghai, China; 6-benzylaminopurine (6-BA), indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) from Boao Biotech Co. Ltd., Shanghai, China; guaiacol and L-phenylalanine from Guangfu Chemical Institute, Tianjin, China; catechol, gallic acid and polyvinylpyrrolidone-30 (PVP-30) from Ke Miou Chemical Reagents Development Centre, Tianjin, China; trichloroacetic acid, ferric chloride and sucrose from Guanghua Chemical Reagents Ltd., Guangzhou, China; manganese chloride from Xilong Chemical Factory, Shantou, China; 2,4-

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dichlorophenol from China National Pharmaceutical Industry Corp. Ltd., Shanghai, China; β-mercaptoethanol from Tianrui Chemical Co. Ltd., Shanghai, China. Phosphate and borate buffers were made fresh.

Plant Material and Culture Media

The axillary buds of tree peony from uniform, clonally propagated in vitro stock mother plants, originally derived from 5-year-old ex vitro plants, were used. Three tree peony cultivars ('Feng Dan Bai', 'Wu Long Peng Sheng' and 'Tai Ping Hong') were gathered from Luoyang City in February, 2010.

Axillary buds were placed on MS medium supplemented with 0.3 mg/l of NAA, 0.3 mg/l of 6-BA and 3% of sucrose and solidified by 7.5 g/l of agar power in 100-ml flasks (three axillary buds per flask) after sterilisation of the culture at 24±1°C. The flasks were placed under a 12-h photoperiod at 36 μmol $m^{-2}s^{-1}$. After 35 days, standardised shoots formed (4-5 leaves, ~5 cm in height, and no roots), and these were used as explants for all subsequent experiments.

Assay Methods

Adventitious shoots (one per flask) were transferred to WPM supplemented with 4 mg/l of IBA and 3% of sucrose, and solidified by 2.0 g/l of Phytagel for culture under the same conditions as for shoot induction. No antioxidant compounds were added to the medium. The enzyme activities and total phenolic content of whole plantlets were determined after culturing for 0, 1, 2, 3, 4, 5, 7, 9, 12, 15 and 20 days. Ten plantlets were cut into small pieces and mixed, and 0.5-g aliquots were used in enzyme assays. Each treatment contained 10 replicates and was repeated three times.

All media used were adjusted to pH 5.8 (measured with a pHS-3B meter, Hongyi Instrument Co., Shanghai, China) with 1 M NaOH before autoclaving at 121°C for 15 min at 121 psi.

POD activity

The plantlets (0.5 g) from different rooting culture periods were ground immediately on ice by adding 5 ml of phosphate buffer (pH 7.0). The crude enzyme extract was obtained after centrifugation at 12085×g for 15 min at 4°C. POD activity was measured following the Guaiacol method [19]. Briefly, 0.3 ml of crude enzyme, 2.0 ml of phosphate buffer (pH 7.0), 0.5 ml of 0.2% aqueous guaiacol and 0.5 ml of 0.15% H_2O_2 were mixed. POD absorbance values were recorded immediately at 470 nm using a UV-3200 spectrophotometer (Mei Puda Instrument Co., Shanghai, China). One unit of POD activity is equivalent to an increase in 0.01 times the amount of enzyme for 1 g of fresh weight/min.

PPO activity

The plantlets (0.5 g) from different rooting culture periods were ground immediately on ice by adding 5 ml of phosphate buffer (pH 6.0). The crude enzyme extract was obtained after centrifugation at 12085×g for 15 min at 4°C. PPO activity was measured according to Zhu et al. [20]. Briefly, 0.1 ml crude enzyme, 3.9 ml phosphate buffer (pH 6.0) and 1 ml 0.l M aqueous catechol

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were mixed in a 30°C water bath for 10 min. Then 2 ml of 20% trichloroacetic acid was added quickly to stop the reaction. PPO absorbance values were recorded immediately at 525 nm. One unit of PPO activity is equivalent to an increase in 0.01 times the amount of enzyme for 1 g of fresh weight/min.

IAAO activity

The activity of IAAO, extracted by the same method as that for PPO, was measured according to Zhang [21]. Briefly, 1 ml crude enzyme, 2 ml 1 mM aqueous $MnCl_2$, 1 ml 1 mM aqueous 2,4-dichlorophenol, 2 ml 1 mM aqueous IAA and 5 ml phosphate buffer (pH 6) were mixed in a 30°C water bath for 30 min. Then 2 ml of this mixture and 4 ml of reaction solution (1.0 ml 0.5M $FeCl_3$ + 50 ml 35% perchloric acid) were mixed in the dark in a 30°C water bath for 30 min. IAAO absorbance values were recorded at 530 nm. One unit of IAAO activity was expressed in terms of μg of IAA degraded/g fresh weight/h.

PAL activity

The plantlets (0.5 g) from different rooting culture periods were ground immediately on ice with addition of 5 ml pre-cooled 0.1 M borate buffer (pH 8.8, containing 5 mM β-mercaptoethanol and 0.25 g PVP-30). The supernatant was used for the determination after centrifugation at 12085×g for 15 min at 4°C. PAL activity was measured by the method of Li [22] with minor modifications. Briefly, 1 ml crude enzyme, 1 ml 0.02 M L-phenylalanine in borate buffer (pH 8.8) and 2 ml distilled water were mixed in a 30°C water bath for 30 min. PAL absorbance values were measured at 290 nm using water as control. One unit of PAL activity is equivalent to an increase in 0.01 times the amount of enzyme for 1 g of fresh weight/h.

Total phenolic content

Polyphenols were extracted by a methanol and water extraction method [23]. Plantlets (0.5 g) from different rooting culture periods were placed in a 55°C water bath for 30 min after grinding in 5 ml of 40% methanol. A crude extract of polyphenols was obtained after centrifugation at 3000×g for 10 min. Polyphenols content was determined by the Folin-Ciocalteu method [24]. Briefly, 1.0 ml of crude polyphenol extract, 1.0 ml of distilled water, 0.5 ml of Folin-Ciocalteu's reagent and 0.5 ml of 7.5% Na_2CO_3 were mixed for the reaction to take place at room temperature for 1 h. Absorbance values were then recorded at 765 nm using water as control. Gallic acid was used as standard. The total phenolic content was expressed as μg/g fresh weight.

Morphological parameters

Rooting percentage, average number of roots/plant, and root length (i.e. total length of all roots/total root number) of the three cultivars were determined after 60 days of rooting since root tips would emerge, on average, within 30-40 days while all roots appeared within 40-50 days of culture. The rooting index (RI) [25] was calculated as: RI = average number of roots/plant × root length × % rooting (where % rooting = (number of plants that formed roots / total number of plants)

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× 100). RI is a composite indicator for the measurement of root conditions that can fully explain the degree of difficulty of in vitro rooting.

Experimental design and statistical analyses

In all experiments, each treatment had 10 samples per treatment (tissue culture and biochemical experiments) and was repeated in triplicate. Means were separated by one-way analysis of variance and significant differences were assessed using Duncan's multiple range test at $P = 0.05$ using DPS software version 3.01.

RESULTS AND DISCUSSION

Rooting

The rooting percentage and RI were significantly different ($P < 0.05$) among the three tree peony cultivars. The rooting percentage of 'Feng Dan Bai' was highest (51.72%) with RI of 5.2, while rooting percentages of 'Wu Long Peng Sheng' and 'Tai Ping Hong' were lowest, i.e. 13.8 and 14.29% respectively (RI = 1.94 and 1.61 respectively; Table 1). From the results it can be concluded that 'Feng Dan Bai' is an easy-to-root cultivar while 'Wu Long Peng Sheng' and 'Tai Ping Hong' are difficult-to-root cultivars.

Table 1. Rooting in three tree peony cultivars (n = 30)

Cultivar	Root number	Root length (cm)	Rooting percentage	Rooting index (RI)
Feng Dan Bai	2.8 ± 0.2 a	3.59 ± 0.6 b	51.72 ± 3.23 a	5.2 ± 0.71 a
Wu Long Peng Sheng	2.0 ± 1 a	7.03 ± 2.16 a	13.8 ± 5.48 b	1.94 ± 1.14 b
Tai Ping Hong	2.25 ± 0.58 a	5.02 ± 1.5 ab	14.29 ± 5.2 b	1.61 ± 0.7 b

Note: Each value is the mean ± SD of triplicate. Different letters within a column indicate significant difference at $P < 0.05$ according to DMRT.

POD Activity

POD activity changed during the in vitro rooting of the three tree peony cultivars (Figure 1). The change in POD activity of 'Feng Dan Bai' showed a jagged trend, peaking twice on the 3rd and 9th days. The activity was significantly different ($P < 0.05$) from that of 'Tai Ping Hong' and 'Wu Long Peng Sheng' in the early days of rooting culture. The activity of 'Tai Ping Hong' and 'Wu Long Peng Sheng' was not significantly different, although a similar jagged trend was observed, albeit with a lower amplitude.

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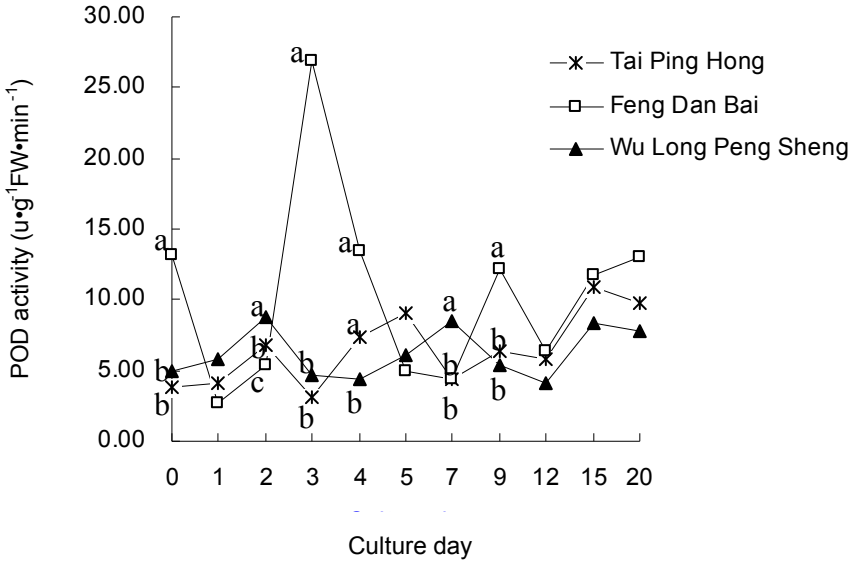

Figure 1. Trends in POD activity change in plantlets of different cultivars during the rooting process. Different letters in each column indicate significant difference between the three cultivars on the same day at $P < 0.05$ using DMRT. Non-significant differences on some days are not indicated by any letters. Each treatment had 10 samples and was repeated in triplicate.

Some studies have shown that there is a close relationship between the activity of POD, PPO and IAAO and the occurrence and growth of adventitious roots in plants and that the changes in these enzymes differ at different periods of rooting [26]. Other studies indicated that the increase in POD activity was a signal of rooting ability in the periods of root induction and expression [7, 27], implying that POD activity would reach two peaks in the induction and expression periods. Pacheco et al. [28] also observed two peaks in the vitro rooting of *Eucalyptus globulus* cuttings, the first on the first day of rooting culture, which might have been related to root induction, and the second peak on the 10th day when the root had broken through the epidermis. In our study, the POD activity of easy-to-root 'Feng Dan Bai' cultivar had two peaks on the 3rd and 9th day, corresponding to possible root primordium induction and expression. However, POD activity of 'Tai Ping Hong' and 'Wu Long Peng Sheng' did not have the same behaviour as that of 'Feng Dan Bai'. Molassiotis et al. [29] observed that the rooting stems of rootstock GF-677 (*Prunus amygdalus* × *P. persica*) showed a maximum soluble POD activity on the 9th day and peaked in ionically-bound POD to cell wall POD activity on the 6th and 12th days on the rooting medium; a similar behaviour was not observed in non-rooting stems. In the case of the KIBA (potassium salt indole-3-butyric acid) treatment of *A. unedo* genotype D, POD activity showed one peak on day 10, but in the control treatment there was no peak in activity [30].

PPO Activity

PPO activity changed during the in vitro rooting of the three tree peony cultivars (Figure 2). The activity showed a jagged trend with peaks and troughs similar for all three cultivars. The activity in 'Feng Dan Bai' peaked on the 4th day and was significantly higher than in 'Tai Ping Hong' and

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'Wu Long Peng Sheng'. The activity was also significantly different ($P < 0.05$) among the three tree peony cultivars on the 7th and 15th day.

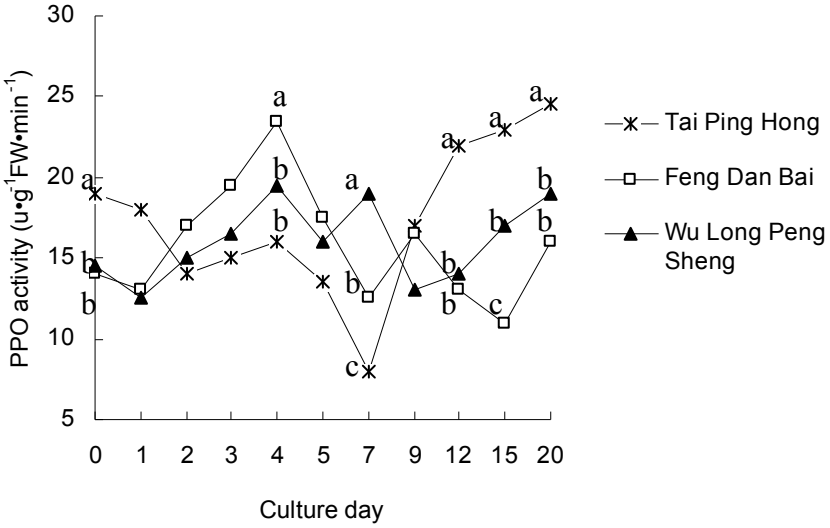

Figure 2. Trends in PPO activity change in plantlets of different cultivars during the rooting process. Different letters in each column indicate significant difference between the three cultivars on the same day at $P < 0.05$ using DMRT. Non-significant differences on some days are not indicated by any letters. Each treatment had 10 samples and was repeated in triplicate.

Bhattacharya [31] proved that PPO can catalyse the metabolism of auxin, promoting the generation and development of adventitious roots. Moreover, PPO can also catalyse phenolic compounds and IAA to form 'IAA-phenol complexes', which would be a type of rooting cofactor that can promote the occurrence and development of adventitious roots [27]. In this experiment on tree peony, after 3-4 days of culture, the PPO activity of all three cultivars increased, the increase being significantly greater, however, in easy-to-root 'Feng Dan Bai' than in difficult-to-root 'Tai Ping Hong' and 'Wu Long Peng Sheng', which may be more advantageous to the formation of 'IAA-phenol complexes' that promote the induction of root primordia. During days 12-15 of rooting, the PPO activity of 'Tai Ping Hong' and 'Wu Long Peng Sheng' continued to rise, while that of 'Feng Dan Bai' began to drop and became significantly lower than that of the other two cultivars on the 15th day. This reduction in PPO activity may have participated in or been responsible for the formation of root primordia. Molnar and Lacroix [32] found that when *Hydrangea macrophylla* formed adventitious roots from stem tissue, PPO activity increased dramatically when the root tip emerged. Habaguchi [33] observed the same result in carrot callus culture: when root tips emerged from callus, PPO activity increased sharply. In this study on tree peony, after rooting for 20 days, the PPO activity of all three cultivars increased, which may be related to the emergence of root tips.

IAAO Activity

The changes in IAAO activity during rooting of the three tree peony cultivars are shown in Figure 3. Similarly to POD and PPO, IAAO activity was jagged for all three cultivars and was higher in 'Tai Ping Hong' and 'Wu Long Peng Sheng' than in 'Feng Dan Bai' at all times, particularly on

the 2nd, 3rd, 7th and 9th days. The activity was similar in 'Tai Ping Hong' and 'Wu Long Peng Sheng' on the 2nd, 3rd, 4th, 5th, 9th and 12th days. It was significantly different ($P < 0.05$) among the three tree peony cultivars on the 7th day.

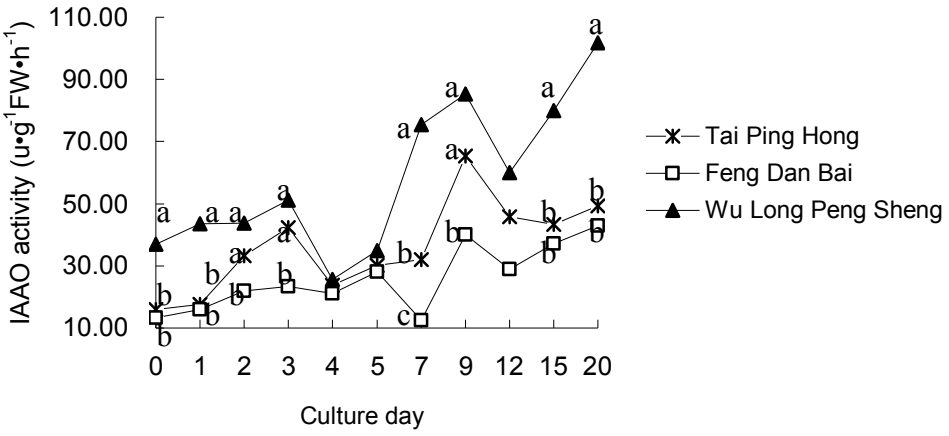

Figure 3. Trends in IAAO activity change in plantlets of different cultivars during the rooting process. Different letters in each column indicate significant difference between the three cultivars on the same day at P<0.05 using DMRT. Non-significant differences on some days are not indicated by any letters. Each treatment had 10 samples and was repeated in triplicate.

IAAO can degrade IAA and modify its level in plants, thereby affecting the rooting of plantlets, and it is theoretically likely that there is a high IAAO activity (in roots, leaves and stems) in difficult-to-root cultivars, which can strongly degrade IAA [34-35]. In this case, more IAA is destroyed and consequently little IAA would be transported downwards to the roots, resulting in few or no roots being induced. Conversely, an easy-to-root cultivar would have a low IAAO activity and hence a lower ability to degrade IAA that facilitates rooting [36]. In this study, IAAO activity of 'Feng Dan Bai' was consistently lower than that of 'Tai Ping Hong' and 'Wu Long Peng Sheng'.

Consistent with the results of our study, Hu et al. [26] found that the IAAO activity in *Corylus avellana* cuttings was higher in the control (0% of rooting) than in the plants treated with IBA (60% of rooting). The IAAO activity of *Camellia sinensis* (L.) Kuntze was also higher in the control (0% rooting) cuttings as compared to IBA-treated cuttings (92.6% rooting) [9]. IAAO activity in *Vigna radiata* L. cv. 105 remained higher in controls than in cuttings treated with PUT and IBA [37].

PAL Activity

The PAL activity followed a pattern similar to that of POD, PPO and IAAO during the in vitro rooting of the three tree peony cultivars (Figure 4). The activity is similar among the three tree peony cultivars on the 0, 1st, 2nd, 4th, 5th and 12th days, although there were significant differences on the 3rd day. PAL activity of 'Feng Dan Bai' was also significantly lower than that of 'Tai Ping Hong' and 'Wu Long Peng Sheng' on the 7th and 9th days.

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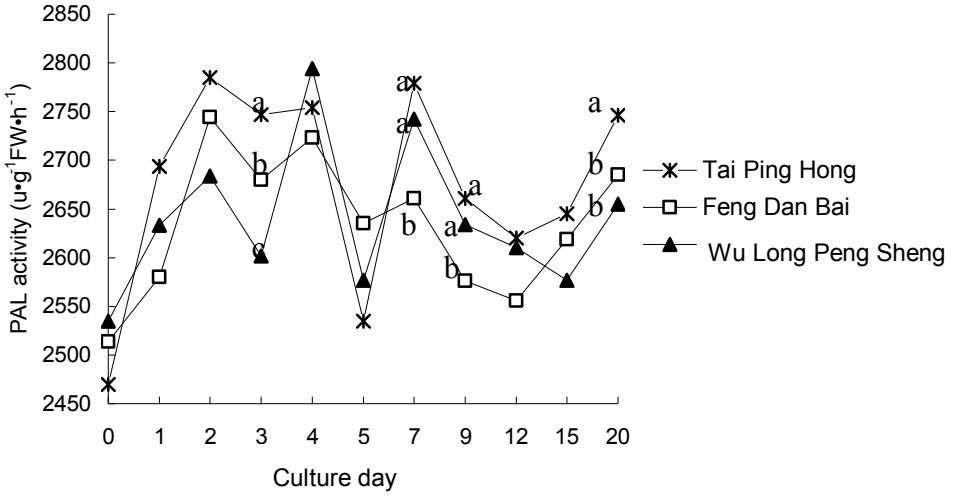

Figure 4. Trends in PAL activity change in plantlets of different cultivars during the rooting process. Different letters in each column indicate significant difference between the three cultivars on the same day at $P < 0.05$ using DMRT. Non-significant differences on some days are not indicated by any letters. Each treatment had 10 samples and was repeated in triplicate.

PAL is a key enzyme in the synthesis of phenolic acids and its activity is affected by external and internal factors in cells [38]. Highly positive correlations were observed between phenolic content and PAL activity or free phenylalanine content in buds and scales in *Lilium davidii* var. *unicolor* bulbs [39]. The total phenolic content of three varieties of *Phalaenopsis* was positively correlated with their PAL activity during tissue culture [40]. Smith-Becker et al. [41] observed that the constitutive activity of PAL in stems and petioles of cucumber was approximately 20-fold higher than in leaves. However, no difference in PAL activity was detected between control and plants inoculated with *Pseudomonas syringae* pv. *syringae* in the leaf directly above the inoculated leaf. In this study, there were changes in PAL activity during the rooting process, implying that PAL participated in the rooting process of tree peony plantlets. However, there was no significant difference in the activity among the three tree peony cultivars, and there was no correlation with the total phenolic content, which seemed to indicate that PAL did not play a leading role in the rooting of tree peony plantlets. Other factors which may be at play would have to be further studied.

Total Phenolic Content

Figure 5 shows changes in the total phenolic content during the in vitro rooting of the three tree peony cultivars. The jagged patterns of 'Tai Ping Hong' and 'Wu Long Peng Sheng' were similar to each other, but different from that of 'Feng Dan Bai'. Except for the 1st and 12th days of rooting, the phenolic content of 'Feng Dan Bai' was significantly higher than that of 'Tai Ping Hong' and 'Wu Long Peng Sheng' during day 2-7 and day 15-20 of rooting.

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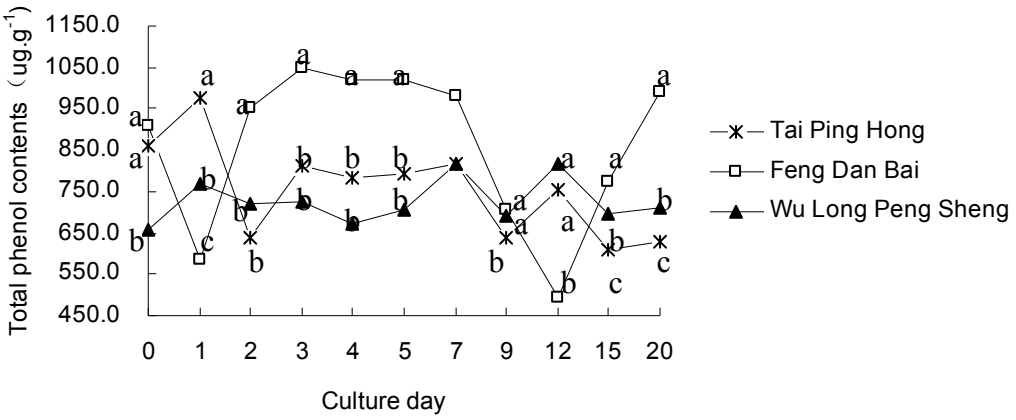

Figure 5. Trends in total phenolic content change in plantlets of different cultivars during the rooting process. Different letters in each column indicate significant difference between the three cultivars on the same day at $P<0.05$ using DMRT. Non-significant differences on some days are not indicated by any letters. Each treatment had 10 samples and was repeated in triplicate.

The function of phenolic compounds in rooting is well established. Hartman et al. [16] proposed one view: differences in the content of phenols indicate differences between easy-to-root and difficult-to-root cuttings, the former having a higher content of phenols than the latter. The content of some flavonoids in *Eucalyptus* seedlings was positively correlated with rooting ability [42]. In a *Rhizobium* isolate of *Vigna mungo* (mung bean), the root nodules contained a higher amount of IAA and phenolic acids than non-nodulated roots [43]. Another study showed that exogenous phenolic compounds and IAA, when used in conjunction, could synergistically promote rooting. Pentahydroxyflavone plus IBA, for example, significantly increased the rooting rate of walnut [44]. In this experiment, the total phenolic content of easy-to-root 'Feng Dan Bai' was also generally higher than that of difficult-to-root 'Wu Long Peng Sheng' and 'Tai Ping Hong', which is in line with the above observations and implies an active role of phenolic compounds in the promotion of rooting.

CONCLUSIONS

Enzyme-related activities and total phenol content, which were related to the rooting of tree peony, changed in the first 20 days (early phase of in vitro rooting) for all three tree peony cultivars. These changes differed over the rooting period and there were significant differences between easy-to-root 'Feng Dan Bai' and difficult-to-root 'Wu Long Peng Sheng' and 'Tai Ping Hong' cultivars as determined initially by RI values. POD activity and total phenolic content were higher in easy-to-root 'Feng Dan Bai' cultivar than in difficult-to-root 'Tai Ping Hong' and 'Wu Long Peng Sheng' cultivars on the whole, while IAAO activity was lower in 'Feng Dan Bai' than in 'Tai Ping Hong' and 'Wu Long Peng Sheng' throughout the entire experimental period. However, no clear conclusions could be drawn for PPO and PAL activities in relation to the rooting ability of the three tree peony cultivars. The changes in the activity of the POD and IAAO enzymes and phenol content can thus be used as a marker and predictor of rooting ability in tree peony during the early days of in vitro rooting as depicted in Figure 6.

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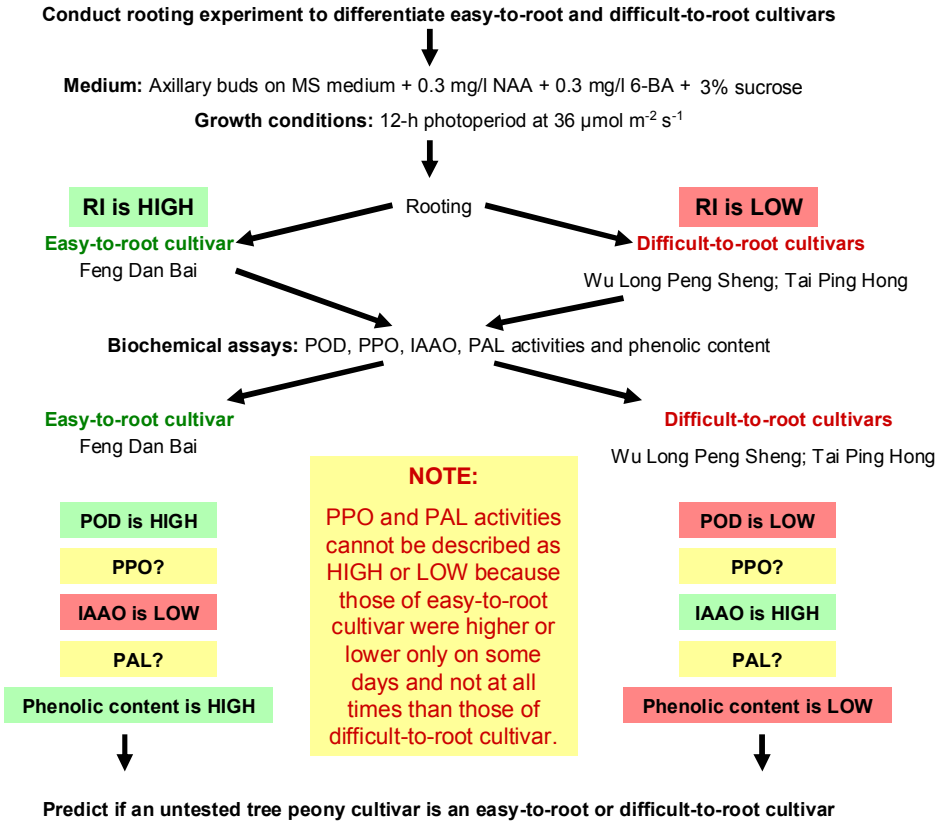

Figure 6. Proposed scheme for classifying a tree peony cultivar into easy-to-root or difficult-to-root cultivar based on RI value and other biochemical parameters. Note that RI, POD and IAAO represent 'stable' parameters while PPO and PAL are 'unstable' parameters for predicting the nature of in vitro rooting of a cultivar.

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