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RESEARCH ARTICLE

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Biochemical changes in barberries during adventitious root formation: the role of indole-3-butyric acid and hydrogen peroxide

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Abstract

Peroxidase, polyphenol oxidase (PPO), phenolic compounds and total sugars (TS) were investigated during root formation in cuttings of *Berberis vulgaris* var. asperma (*BVA*) and *Berberis thunbergii* var. atropurpurea (*BTA*) treated with indole-3-butyric acid (IBA) and IBA + H_2O_2. Rooting was observed on *BTA* cuttings but not on *BVA* cuttings. The *BTA* cuttings treated with IBA and IBA + H_2O_2 showed higher rooting percentages, number of roots, and root length over the control. Those treated with IBA + H_2O_2 recorded the lowest peroxidase activity after planting. *BTA* cuttings treated with IBA + H_2O_2 showed the highest peroxidase activity at 50 d after planting; *BVA* cuttings under different treatments showed no significant difference for peroxidase activity at planting time or up to 80 d after planting. PPO activity for the *BTA* cuttings in the control treatment was lower than for other treatments during root formation. The cuttings in the IBA and IBA+H_2O_2 treatments showed increased PPO activity from 0 to 50 d after planting and a slight decrease in PPO activity from 60 to 80 d after planting. PPO activity for the *BVA* cuttings was significantly lower than for *BTA* during root formation. The *BTA* cuttings treated with IBA and IBA + H_2O_2 showed the highest phenolic compound content during root formation. The *BVA* cuttings displayed higher TS than *BTA* during the initial stage of root formation. A comparison of the anatomical structure of easy-to-root and difficult-to-root cuttings indicated that physical inhibitors did not affect the rooting capacity of *BVA*.

Additional key words: *Berberis vulgaris* var. asperma; *Berberis thunbergii* var. atropurpurea; peroxidase; polyphenol oxidase; phenolic compounds.

Introduction

More than 40 cultivars of Japanese barberry (*Berberis thunbergii* DC) are utilized as ornamental shrubs in landscaping because they are hardy, drought tolerant, easy to grow and generally attractive. They vary in leaf colour (green, purple, yellow or variegated) and plant habitat, and are easily propagated from seeds and cuttings (Lubell *et al*., 2008). *Berberis thunbergii* var. atropurpurea (*BTA*) has adapted to the drought conditions of Khorasan province in Iran. It is a deciduous shrub with purple leaves and yellow aggregate blooms (Lubell *et al*., 2008). *Berberis vulgaris* var. asperma (*BVA*) is native to Iran and Khorasan province and is known for its drought and salt tolerance.

BVA is commonly known in Iran as seedless barberry; its fruit is consumed fresh and is used to prepare syrups, jams, beverages, jellies, flavoring, and coloring agents. *BVA* cuttings are generally difficult to root and they are usually propagated by suckers. The development of seedless barberry orchards is limited because of the low rooting ability of its cuttings (Balandari & Kafi, 2001). The salinity of water and soil make large areas in eastern Iran (latitude 32.5-34.5° N) unsuitable for most agricultural crops. In the last 20 years, *BVA* has become a major crop in the area, with about 6000

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Abbreviations used: *BTA* (*Berberis thunbergii* var. atropurpurea); *BVA* (*Berberis vulgaris* var. asperma); IBA (indole-3-butyric acid); PPO (polyphenol oxidase); TS (total sugars).

ha under cultivation and an annual production of 941-4500 t (Balandari & Kafi, 2001; Tehranifar, 2003).

Adventitious rooting consists of three successive, but interdependent, physiological phases (induction, initiation and expression) with different requirements (Hartmann *et al*., 1989). The induction phase comprises molecular and biochemical events with no visible change. The initiation phase is characterized by cell division and root primordia organization. The expression phase consists of the intra-stem growth of root-primordia and the emergence of roots (Ford *et al*., 2001; Sebastiani *et al*., 2002). The ability to form adventitious roots is affected by genotype, stock plants, types of cuttings, collection date, and endogenous factors related to cutting (Kibber *et al*., 2004; Tworkoski & Takeda, 2007). Numerous studies on adventitious root formation have shown that changes in phenolic compounds, sugar and protein content significantly affect root formation (Kaur *et al*., 2002; Sivaci & Yalcim, 2006; Kevresan *et al*., 2007; Satish *et al*., 2008). Carbohydrates are the direct products of photosynthesis; they serve as an energy source and provide the carbon necessary for production of new tissue (Sivaci & Yalcim, 2006).

Previous studies have noted changes in enzyme activity, such as polyphenol oxidase (PPO) and peroxidase, during rooting (Gaspar *et al*., 1992; Gunes, 2000; Ludwig-Muller, 2003; Yilmaz *et al*., 2003). PPO is involved in the oxidation of phenol and contributes energy for cell division and cell differentiation in organogenesis of explants in tissue cultures (Satisha *et al*., 2008). Peroxidase is a marker of the inductive and initiative phases of root formation in plants (Yilmaz *et al*., 2003; Kevresan *et al*., 2007).

Currently, indole-3-butyric acid (IBA) is the most widely used auxin to stimulate rooting in cuttings. It has a strong ability to promote root initiation, low toxicity and good stability in comparison to naphthalene acetic acid and indole-3-acetic acid (Strzelecka, 2007). It has been reported that hydrogen peroxide (H_2O_2) also effectively increases the activity of enzymes such as superoxide dismutase and catalase and is also related to morphogenesis. Li *et al*. (2009a) reported that exogenous hydrogen peroxide promotes the formation and development of adventitious roots in mung beans (*Vigna radiate*) and demonstrated that H_2O_2 may function as a signal molecule in the auxin-induced formation of adventitious roots.

The present study investigated biochemical and anatomical changes during rooting of *Berberis thunbergii* var. atropurpurea (easy-to-root) and *Berberis vulgaris* var. asperma (hard-to-root) barberry species. It also examined the influence of IBA and H_2O_2 on the formation and development of adventitious roots for these two species.

Material and methods

Plants

The barberries species selected were *Berberis thunbergii* var. atropurpurea (easy-to-root) and *Berberis vulgaris* var. asperma (difficult-to-root) cultivars. Hardwood cuttings 6-9 mm in thickness and 180-200 mm in length with four buds were prepared from the basal parts of 1-year-old shoots collected from 8-year-old barberry shrubs. The shrubs were maintained in the experimental orchard of Ferdowsi University in the city of Mashhad. The cuttings were treated with cold water (tap water) for 24 h to leach out rooting inhibitors.

Cutting treatment

IBA solution (3 g L^{-1}) was freshly prepared by dissolving IBA powder (Sigma Chemical Co.) in an ethanol/water/glycerol (2.5:6.5:1 v/v/v) solution. Hydrogen peroxide (3.5%, w/v) solution was prepared by diluting 35% (w/v) H_2O_2 stock solution (Merck) in distilled water.

Hardwood cuttings of the two species were treated with IBA and H_2O_2 solutions by wetting 2.5 cm of their basal ends. The cuttings were planted in a nursery bed with bottom heat in a sand medium without nutrients for 80 d. The following rooting treatments were used: (i) control, dipped in distilled water for 90 s; (ii) dipped in 3 g L^{-1} IBA for 60 s; and (iii) dipped in 3.5% (w/v) H_2O_2 30 s + dipped in 3 g L^{-1} IBA 60 s.

Chemical composition

Fresh stem tissue was prepared for the enzyme assays according to the method proposed by Rout (2006). Protein concentrations were determined as recommended by Bradford (1976) and bovine albumin (BSA) was used as the standard. Peroxidase enzyme was determined spectrophotometrically at 470 nm after 10 min incubation at 30 ± 0.5 °C using 3,3-dimethyl-

glutaric acid (3,3-DGA)-NaOH and guaiacol (Beffa *et al.,* 1990). One unit of peroxidase activity corresponds to ΔA 470 of 1.0 for 1 mg of protein in 10 min.

Polyphenol-oxidase enzyme assay was carried out using pyrogallol as the substrate (Kar & Mishra, 1976). The activity was determined as mg^{-1} protein min^{-1}. Each value was the mean of five replicates. Total phenol was extracted and estimated using Folin-Ciocalteu reagent (Jayaprakasha *et al*., 2001) and expressed as mg g^{-1} of fresh weight. Total sugar (TS) was estimated using anthrone agent according to the method described by Irigoyen *et al*. (1992) and expressed as mg g^{-1} of fresh weight.

Anatomical studies

Basal sections of cuttings were fixed in formalin/methanol/acetic acid (1:18:1 v/v/v) for 2 wk. Section were then mounted by hand on slides, stained with saffranin for 30 min and fast green for 3 min and rinsed in distilled water. The samples were photographed using a camera (U-TVO-5XC-2, Olympus Optical Co., Tokyo) attached to an optical microscope (BX41TF, Olympus Optical Co., Tokyo) under the control of a computer.

Statistical analysis

The hardwood cuttings were sampled at 0, 10, 20, 30, 40, 50, 60, 70, and 80 d after planting. Cuttings of each species and treatment were used for biochemical analysis; 30 cuttings were used for root scoring. The treatments were arranged in a completely randomized design with four replications of 30 cuttings each. Analysis of the data was conducted using SAS software (version 9.1) and the mean values and standard error (SE) were calculated for all tests.

Results

Rooting percentage, number of roots and root length

The rooting percentage differed between species and was observed only on cuttings of *BTA* (Fig. 1). The highest rooting percentage (73.33%), number of roots and root lengths were observed on cuttings treated with IBA + H_2O_2. The control cuttings of *B. thumbergii* var. atropurpurea showed the lowest rooting percentage (43.33%), number of roots and root length (Table 1).

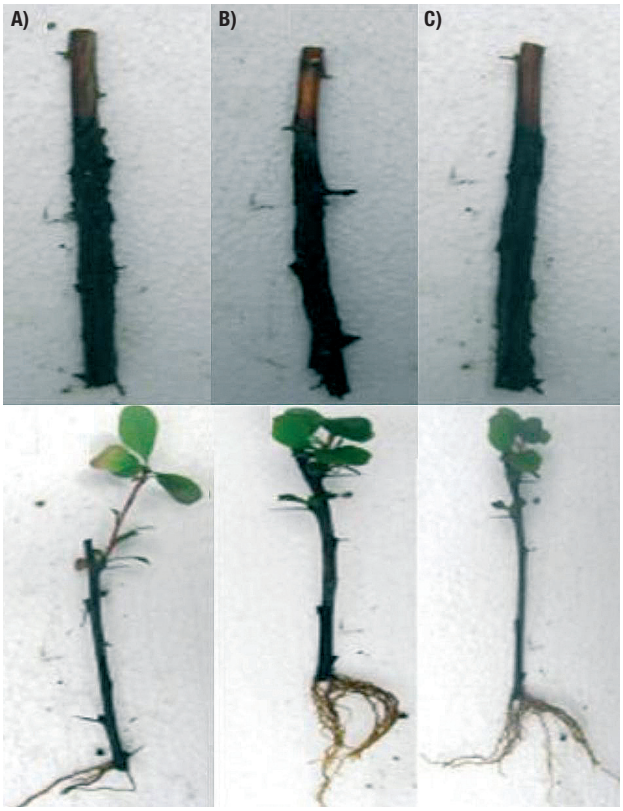

Figure 1. Root formation in the cuttings of *Berberis vulgaris* var. asperma (up) and *Berberis thunbergii* var. atropurpurea (down). A: control, B: treated with IBA, C: treated with IBA + H_2O_2.

Peroxidase activity

BTA

The cuttings treated with IBA + H_2O_2 showed the lowest peroxidase activity after planting. The peroxidase activity of the cuttings decreased slightly up to 40 d after planting and the lowest peroxidase activity was observed in cuttings treated with IBA + H_2O_2. A sharp increase was observed in peroxidase activity in each treatment at 40 d after planting (Fig. 2A). The cuttings treated with IBA + H_2O_2 showed the highest peroxidase activity at 50 d after planting. The highest peroxidase activity in the IBA and control treatments were observed at 60 d after planting. Peroxidase activity was highest in the

Table 1. Root formation in the cuttings of *Berberis thunbergii* var. atropurpurea (*BTA*) and *Berberis vulgaris* var. asperma (*BVA*) with different treatments

Genotype	Treatment	Rooting (%)	Roots (No.)	Length of root (average, in mm)
BTA	Control	43.33±5.8c	6±1.04c	34.67±4.04b
	IBA	63.00±3.2b	10±2.31b	57.00±6.50a
	IBA + H_2O_2	73.33±4.5a	14±1.50a	59.33±5.68a
BVA	Control	0	0	0
	IBA	0	0	0
	IBA + H_2O_2	0	0	0

Means with the same letters in each column are not significantly different by Duncan's multiple range test ($p \leqslant 0.05$) ± SE.

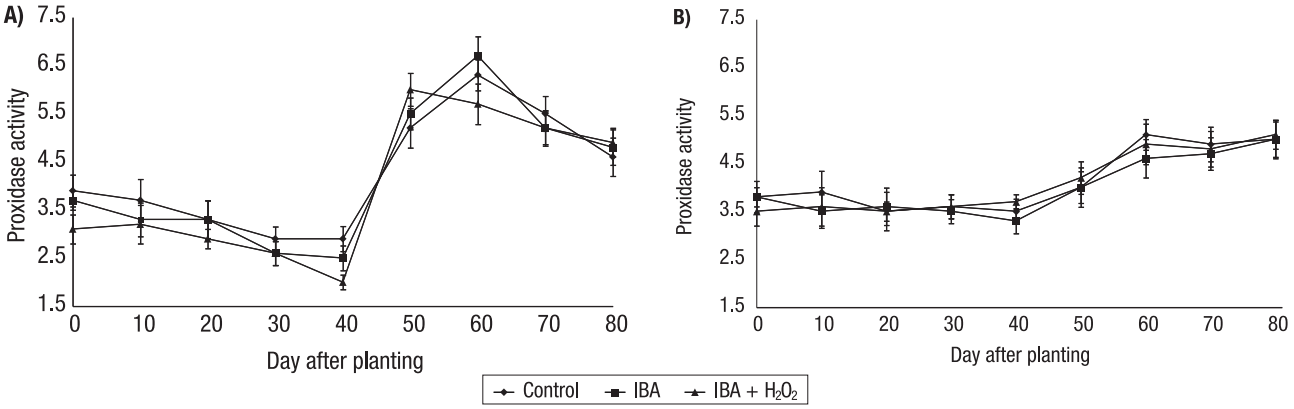

Figure 2. Peroxidase activity in the cuttings of *Berberis thunbergii* var. atropurpurea (A) and *Berberis vulgaris* var. asperma (B) with different treatments during root formation. One unit of peroxidase activity is equivalent to a ΔA470 of 1.0 for 1 mg of protein in 10 min. Values are means ± SE.

IBA treatment. Peroxidase activity decreased rapidly for all treatments from 60 to 80 d after planting.

BVA

There were no significant differences between treatments for peroxidase activity at planting time. All treatments showed constant levels of peroxidase activity up to 40 d after planting and increasing activity from 40 to 80 d after planting (Fig. 2B).

Polyphenol oxidase activity

BTA

Polyphenol oxidase activity of the control cuttings was lower than for the other treatments during root formation. It gradually increased for all treatments from 0 to 60 d after planting and then decreased slightly from 60 to 80 d after planting. The IBA and IBA + H_2O_2 treatments showed an increase in PPO activity from 0 to 50 d after planting; the highest PPO activity was observed for the IBA + H_2O_2 treatment. In both treatments, PPO activity decreased slightly by 80 d after planting (Fig. 3A).

BVA

Polyphenol oxidase activity of the *BVA* cuttings was significantly lower than for *BTA* during root formation. The cuttings treated with IBA, and IBA + H_2O_2 and the control showed no significant differences in PPO activity from 0 to 80 d after planting. Polyphenol oxidase activity of some

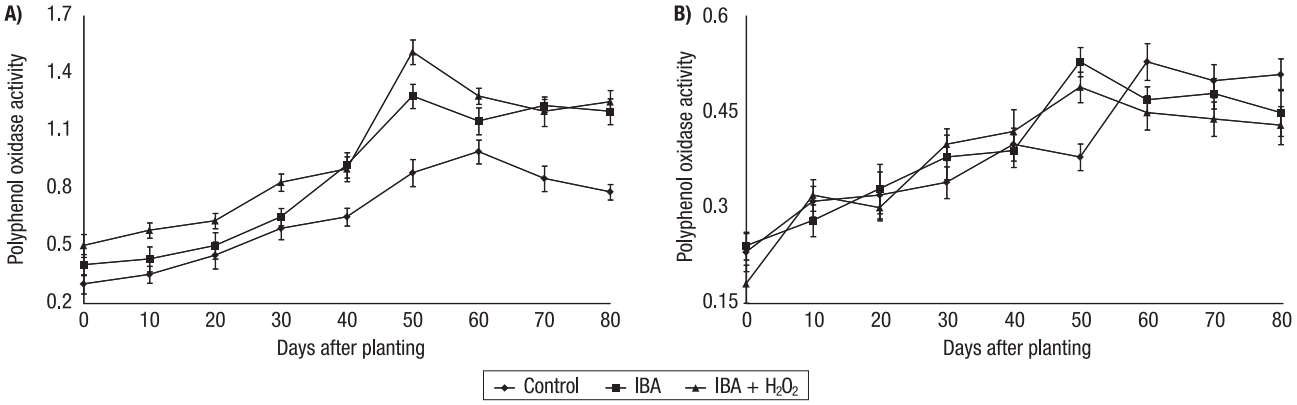

Figure 3. Polyphenol oxidase activity in cuttings of *Berberis thunbergii* var. atropurpurea (A) and *Berberis vulgaris* var. asperma (B) with different treatments during root formation. The activity was determined in terms of enzyme activity mg^{-1} protein min^{-1}. Values are means ± SE.

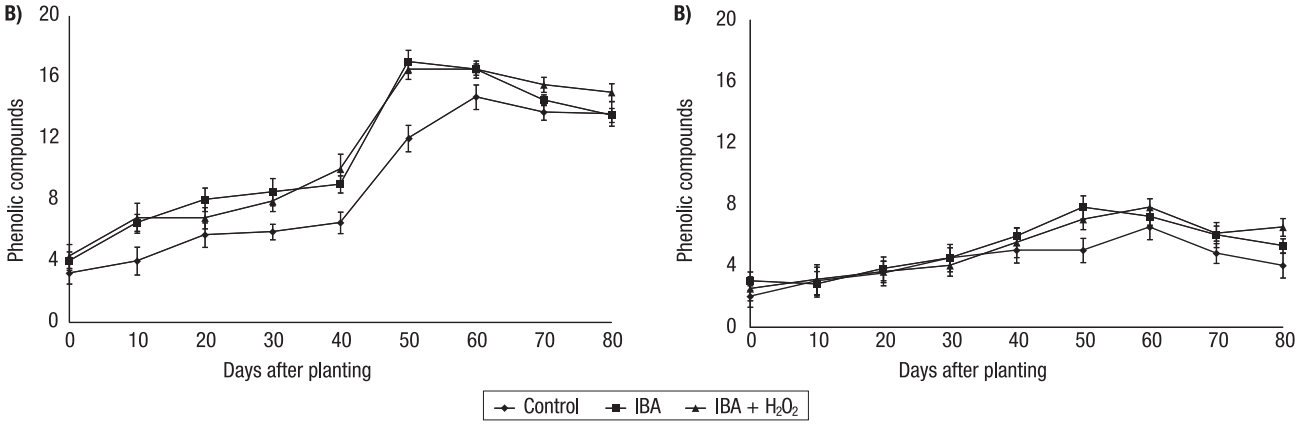

Figure 4. Phenolic compounds in cuttings of *Berberis thunbergii* var. atropurpurea (A) and *Berberis vulgaris* var. asperma (B) with different treatments during root formation. The phenol content was expressed as mg g^{-1} fresh weight. Values are means ± SE.

treatments increased gradually, but not significantly, during root formation (Fig. 3B).

Phenolic compounds

BTA

The IBA and IBA + H_2O_2 treatments showed higher phenolic compound concentrations over the control during root formation. Phenolic compounds of all treatments gradually increased up to 40 d after planting. It was observed that phenolic compound concentrations in the IBA and IBA + H_2O_2 treatments increased rapidly from 40 to 50 d after planting and then decreased slightly, while for the control treatment, it increased rapidly from 40 to 60 d after planting and then gradually decreased from 60 to 80 d after planting (Fig. 4A).

BVA

The phenolic compound content in *BVA* was significantly lower than for *BTA* during the initial stage of root formation. The cuttings treated with IBA and IBA + H_2O_2 and the control showed no significant differences in phenolic compounds between 0 to 80 d after planting. The phenolic compounds increased gradually, but not significantly, from 0 to 50 d after planting and then decreased slightly from 60 to 80 d after planting (Fig. 4B).

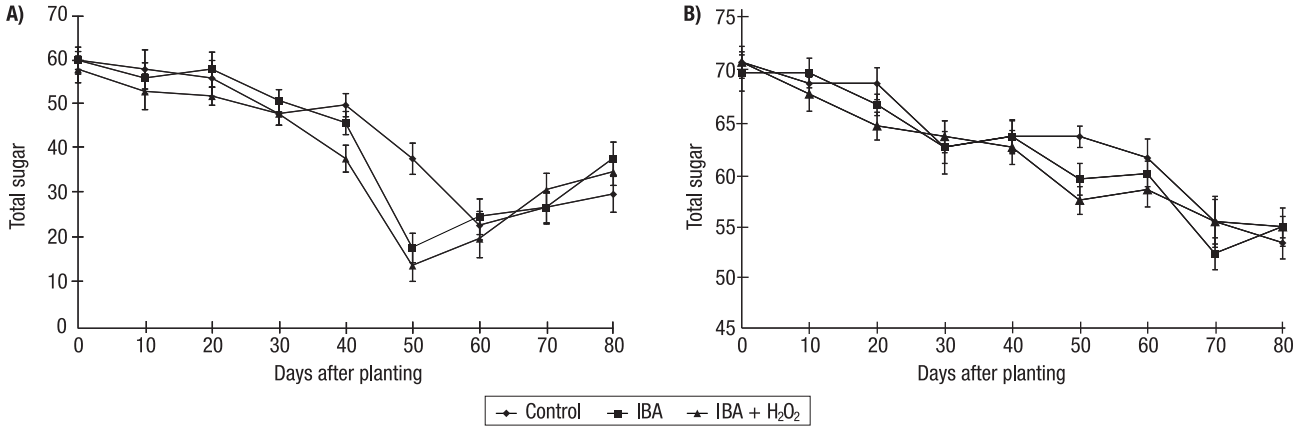

Figure 5. Total sugars in cuttings of *Berberis thunbergii* var. atropurpurea (A) and *Berberis vulgaris* var. asperma (B) with different treatments during root formation. Total sugars were expressed as mg g^{-1} fresh weight. Values are means ± SE.

Total sugars

BTA

The cuttings treated with IBA and IBA + H_2O_2 and the control showed no significant differences in TS during the initial stage of root formation. IBA and IBA + H_2O_2 treatments showed a slight decrease in TS from 0 to 40 d after planting and a strong decrease from 40 to 50 d after planting; from 60 to 80 d, TS increased. The control showed a gradual decrease in TS from 0 to 50 d after planting. The rate of TS depletion increased from 50 to 60 d after planting and continued to increase gradually from 60 to 80 d after planting (Fig. 5A).

BVA

The *Berberis vulgaris* var. asperma showed higher TS than the *Berberis thunbergii* var. atropurpurea during the initial stage of root formation. The cuttings treated with IBA and IBA + H_2O_2 and the control showed no significant differences in TS from 0 to 60 d after planting; TS decreased gradually from 60 to 80 d after planting (Fig. 5B).

Anatomical studies

Transverse sections of the *BVA* and *BTA* cuttings were compared before transfer to the rooting medium (Fig. 6). The cortex tissue of *BVA* contained more cell layers than did that of *BTA*. The dye density of the cell wall indicated lignification of the cell wall. This indicates that the cell wall of the cortex layer of *BVA* was thicker than that of *BTA*. The sclerenchymatic tissue of *BVA* consisted of more cell layers than that of *BTA* sclerenchyma, while the cell wall of the sclerenchymatic tissue of *BVA* was thinner than that of *BTA*. Other *BVA* and *BTA* tissues showed no significant differences.

Discussion

The ability to form adventitious roots has been associated with the physiologic and ontogenetic age of plant material, genotype and the characteristics of the cuttings, such as growth conditions of the stock plant (Kibber *et al*., 2004). There was a significant difference in rooting ability between *BVA* and *BTA* (Table 1, Fig. 1). The rooting ability of *BTA* was strongly affected by IBA and IBA + H_2O_2, while the cuttings of *BVA* showed no response to the treatments. Previous studies have shown that IBA promotes root formation (Kaur *et al*., 2002; Ludwig-Muller, 2003; Rout, 2006). Li *et al*. (2009b) reported that the application of exogenous H_2O_2 promoted the formation and growth of adventitious roots in mung bean seedlings and that its effects were dose and time dependent. H_2O_2 treatment of olive cuttings at the beginning of rooting promoted early rooting, increased the root percentage and number of roots, but the application of H_2O_2 alone and with IBA promoted rooting ability in both easy- and difficult-to-root cuttings (Sebastiani & Tognetti, 2004).

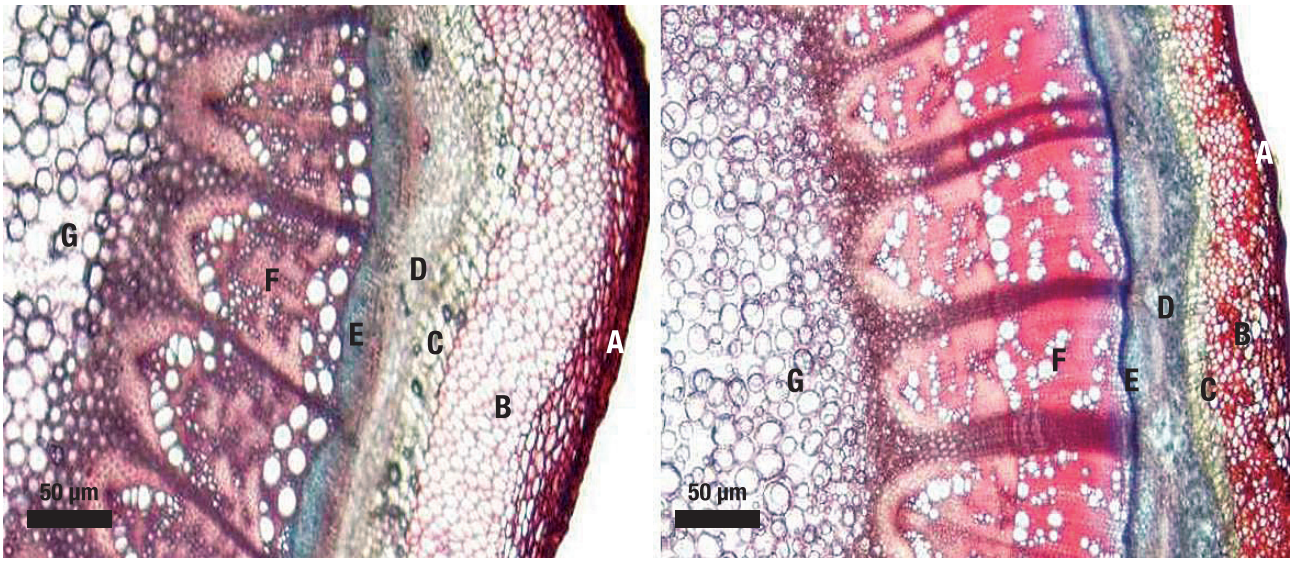

Figure 6. Transverse section of cuttings of *Berberis vulgaris* var. asperma (left) and *Berberis thunbergii* var. atropurpurea (right). A, cortex cells; B, sclerenchymatic tissue; C, parenchymatic tissue; D, phloem vascular; E, vascular cambium; F, xylem vascular; G, pith.

Plant peroxidases are known to affect auxin metabolism and lignification in the cell wall in the presence of phenol (Rout, 2006). Peroxidase activity increased during cell division and primordium formation. Peroxidase helps form the cofactors necessary for root initiation (Gunes, 2000). Rout (2006) showed that IBA-treated cuttings of *Camellia sinensis* L. recorded higher peroxidase activity than the control and that increased peroxidase activity at the early stages of rooting was a good marker of later rooting success. Seedlings of mung beans treated with either IBA or H_2O_2 showed decreased peroxidase activity during the root induction phase and increased activity during the initiation and expression phases. These treatments increased root formation (Li *et al*., 2009b).

Changes in peroxidase activity in *BTA* delineate the root formation phases. It appears that the induction phase of root formation occurred from 0 to 40 d after planting and the initiation and expression phases from 40 to 70 d after planting. Cuttings treated with IBA and IBA + H_2O_2 showed decreased peroxidase activity during the induction phase and increased peroxidase activity during the initiation and expression phases of root formation. Cuttings of *BVA* showed no obvious changes in peroxidase activity (Fig. 2).

Polyphenol oxidase is a key element of cell division, differentiation and primordium development (Huystee & Cairns, 1982). Rout (2006) reported that PPO activity increased in *Camellia sinensis* L. cuttings during the induction and initiation phases and then decreased during the expression phase. IBA treatment increased PPO activity over the control during root formation. It has been shown that cuttings of grape rootstock with low rooting ability showed decreased PPO activity during the induction and initiation phases and increased PPO during the expression phase (Satish *et al*., 2008). Yilaz *et al*. (2003) reported that there was a relationship between PPO activity and root formation in grape cuttings with different rooting abilities. Difficult-to-root cuttings showed lower PPO activity than easy-to-root cuttings during root formation.

In the present study, PPO activity of *BVA* was significantly lower than *BTA* during the root formation period. IBA and IBA + H_2O_2 treatments increased PPO activity in cuttings of *BTA*. PPO activity increased from 0 to 50 d after planting (induction and initiation phase) and then decreased from 50 to 80 d after planting (expression phase) (Fig. 3).

A negative correlation has been observed for phenolic compounds and seed germination (White, 1994) and a positive correlation has been observed between phenolics and totipotency (Tomas & Ravindra, 1999). Phenolic compounds have inhibitory or stimulatory effects on plant growth depending upon the species (Ozyuigit, 2008).

Phenolic compounds of *BVA* were significantly lower than for *BTA* during root formation and showed no response to the stages of root formation (Fig. 4B).

For *BTA*, phenolic compounds increased during the induction and initiation phases and decreased during the expression phase. IBA and IBA + H_2O_2 treatments increased the phenolic compounds during root formation of *BTA* (Fig. 4A). Satish *et al.* (2008) have shown that phenolic compounds play a key role in the rooting of cuttings and that those with higher phenolic compounds in the initial stage of rooting formed roots rapidly.

Although cuttings of *Berberis vulgaris* var. asperma showed higher TS concentration than those of *Berberis thunbergii* var. atropurpurea during the initial stage of root formation, no stimulation of root formation was observed. The *BTA* treatment showed depletion of TS during the expression phase of rooting, which could be related to carbohydrates being a source of energy. Comparison of the anatomical structure of easy-to-root (*BVA*) and difficult-to-root (*BTA*) cuttings showed that physical inhibitors did not affect rooting of *BVA*. This indicates that the low rooting ability of *BVA* is related to its biochemical properties.

Acknowledgments

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