L-2-aminooxy-3-phenylpropionic acid (AOPP) Controls Browning and Promotes Adventitious Bud Formation in *Neofinetia falcata* in vitro

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The effect of L-2-aminooxy-3-phenylpropionic acid (AOPP) on bud formation was investigated by determination of total phenolics in explants and medium browning in the micropropagation of the epiphytic orchid *Neofinetia falcata* (Thunb.) H.H. Hu. Explants, upper portion of plantlets, were cultured on a medium containing 0, 0.01, 0.1 or 1.0 mM AOPP. Browning was decreased from 70% to 3% by increasing AOPP concentration up to 1.0 mM. All surviving explants developed buds at 0, 0.01 and 0.1 mM AOPP. The highest mean number of buds (7.2) was produced on medium containing 0.01 mM AOPP. Multiple buds were formed on the explants 8 weeks after planting and multiple shoots had developed after 12 weeks. Total phenolics continuously increased in control explants up to 2.03 mg·gFW⁻¹ after 4 weeks. In contrast, in explants treated with 0.01 mM AOPP, total phenolics ceased to increase for 4 weeks of culture and remained at 1.46 mg·gFW⁻¹, but thereafter increased to 2.24 mg·gFW⁻¹, the same level as the control, after 8 weeks. Medium browning continuously increased in the control, whereas medium containing 0.01 mM AOPP showed little browning after 4 weeks of culture and thereafter increased to the same degree as the control. The present results suggest that 0.01 mM AOPP is the most suitable concentration to control browning and bud formation in *N. falcata*.

Key Words: browning, L-2-aminooxy-3-phenylpropionic acid, *Neofinetia falcata*, orchid, phenol content.

Introduction

*Neofinetia falcata* (Thunb.) H.H. Hu is distributed in temperate and subtropical regions of China, Japan, and Korea (Arditti, 2008). This epiphytic orchid bears flowers with a pleasant fragrance and has a beautiful plant form, which make it a valuable garden plant; however, clonal propagation of *N. falcata* by division produces only two or three genetically identical clones per year, resulting in low regeneration efficiency.

Micropropagation techniques have the potential to enable large-scale production of plants; however, little information on the micropropagation of *N. falcata* has been reported. For example, plant regeneration through a callus induced from aseptic germination of plantlets was reported as a potential propagation method for *N. falcata* (Aoki et al., 1993; Niimi et al., 1995). Shimasaki (1993) induced protocorm-like bodies (PLBs) from young inflorescence explants *in vitro*. We recently reported that the upper portion of the plantlet and leaves of dark preconditioned plants are the most suitable tissue materials for bud formation by *N. falcata* because of browning of the explants and medium (Mitsukuri et al., 2009). However, dark preconditioning takes time and decreases the explant survival rate; therefore, a more simple method is required for tissue culture of *N. falcata*.

Our previous experiments showed that a browning compound is exuded into the medium from the cut surface of orchid explants and decreased the survived explant rate and bud formation by explants (Mitsukuri et al., 2009). In shoot apex cultures of *Cattleya* Lindl., browning of the culture medium was caused by a phenolic compound (Ishii et al., 1979). A phenolic compound also caused browning and necrosis in *in vitro* leaf tissue explants of *Rhynchostylis retusa* BL. (Vij et al., 1984) and *Renanthera imschootiana* Rolfe (Seeni and Latha, 1992); however, Wang et al. (1994) indicated that dark preconditioning of donor plants reduced tissue browning in leaf cultures of *Malus pumila* Mill. In axillary bud cultures of guava (*Psidium guajava* L.),...
dark preconditioning promoted bud development by controlling exudation of the phenolic compound into the culture medium (Meghwal et al., 2001). Sharma and Singh (2002) reported that dark preconditioning of shoot apex cultures of mango (*Mangifera indica* L.) decreased phenol content in explants, controlled exudation of the phenolic compound into the medium, and increased the survived explant rate. Thus, dark preconditioning might reduce the synthesis of phenolic compounds and promote bud formation in *N. falcata* explants.

Cutting of explants also induces browning and death of the tissues owing to activation of phenylpropanoid metabolism and accumulation of phenol compounds in explants of most plant species (Park et al., 2006; Tang and Newton, 2004). In general, phenol compound secretion is related to *in vitro* survived explants and regeneration success (Arezki et al., 2001; Fernandez-Lorenzo et al., 2005).

Phenylalanine ammonia-lyase (PAL) is a key enzyme in the phenylpropanoid biosynthesis pathway. The enzyme catalyzes the deamination of phenylalanine to produce *trans*-cinnamic acid, which is a precursor of most phenolic compounds, and occupies a central position in plant secondary metabolism (Ali et al., 2006).

Amrhein et al. (1977, 1979) reported that L-2-aminoxy-3-phenylpropionic acid (AOPP) is *in vitro* and *in vivo* a potent inhibitor of PAL in many plants. The role of AOPP has also been indicated as a competitive inhibitor in many plants (Brincat et al., 2002; Edahiro and Seki, 2006; Leubner-Metzger and Amrhein, 1994; Mader and Hanke, 1997). In *Betula platyphylla* Sukaczew var. *japonica* H. Har, adventitious bud formation was promoted by AOPP through inhibition of PAL activity and suberization on the callus surface (Fu et al., 1993). Therefore, AOPP may promote adventitious bud formation through control of PAL activity and browning, and thus addition of AOPP to the culture medium may be a more practical method than dark preconditioning.

In the present study, we developed an efficient *in vitro* micropropagation method incorporating AOPP treatment for *N. falcata*. First, the most effective concentration of AOPP for control of browning and bud formation on upper portions of the plantlet was determined. Second, the effect of AOPP on phenolic compound content was analyzed by measuring medium browning and the total phenolics content in explants.

**Materials and Methods**

**Plant material and culture**

Plantlets of *N. falcata* were aseptically grown from seeds on half-strength (1/2) MS basal medium (Murashige and Skoog, 1962) supplemented with 30 g L$^{-1}$ sucrose and 8 g L$^{-1}$ agar. The plantlets were cultured under a 16 h photoperiod with fluorescent light at ca. 70 μmol m$^{-2}$ s$^{-1}$ PPFD (photosynthetic photon flux density) at 25°C. Plantlets with two unfolded leaves were cultured *in vitro* for 5 months until they reached a height of 2.6 mm. These plantlets were then transversely cut at the center of the stem and divided into upper and lower portions. The upper portions were cultured in the conditions described above.

The concentrations of N$^6$-benzyladenine (BA) and α-naphthaleneacetic acid (NAA) in the medium were limited to an effective combination (0.44 μM BA and 5.37 μM NAA) (Mitsukuri et al., 2009). The pH of the medium was adjusted to 5.6, and 50 mL medium was dispensed into 100 mL Erlenmeyer flasks before autoclaving at 121°C for 15 min.

**Application of L-2-aminoxy-3-phenylpropionic acid**

The explants were cultured on basal medium supplemented with AOPP (0, 0.01, 0.1, or 1.0 mM; Wako Pure Chemical Industries, Osaka, Japan), a specific PAL inhibitor in the biosynthesis of phenylpropanoid metabolites. The percentages of browning, survived explants, and survived explants with bud formation, and number of buds per explant were determined at 4 and 8 weeks after planting. Exudation of the compound causing browning in the medium from the cut end of explants was defined as browning. Buds refer to the sum of one terminal bud and adventitious buds. Thirty explants were used for each treatment.

**Phenol content in explants**

Explants cultured on medium containing 0.01 mM AOPP or lacking AOPP (control) were sampled at 0, 4 and 8 weeks after culture, and total phenol contents were measured. Each sample comprised 100 mg fresh tissue from 20–30 explants with three repetitions. The samples were homogenized in a mortar with 80% ethyl alcohol. The suspension liquid was filtered with filter paper. The collected residue was extracted three times with 80% ethyl alcohol and filtered. The filtrate was concentrated by boiling. Folin-Ciocalteu reagent (1.0 mL) was added to the concentrated solution (1.0 mL). After 3 min, 1.0 mL 10% Na$_2$CO$_3$ (sodium carbonate) solution was added and thoroughly mixed. After 60 min, the absorption of the mixture was measured at 530 nm with a Spectronic 20D+ spectrophotometer (Taitech, Saitama, Japan). The phenol content was calculated from the absorbance value based on a standard curve using 0, 25, 50 and 100 mg L$^{-1}$ chlorogenic acid.

**Medium browning**

Media in the culture vessels containing 0.01 mM AOPP and lacking AOPP (control) were sampled at 0, 4 and 8 weeks after culture. Medium browning was measured using a modified protocol (Krishna et al., 2008; Park et al., 2006). Media (5.0 g) sampled from two culture vessels were homogenized in a mortar with 80% ethyl alcohol. The suspension liquid was shaken on a rotary shaker at 100 rpm for 24 h to extract the oxidative phenolic compounds, followed by centrifugation at 4000 rpm for 30 min. Browning level in the medium was measured using a modified protocol (Krishna et al., 2008; Park et al., 2006). Media (5.0 g) sampled from two culture vessels were homogenized in a mortar with 80% ethyl alcohol. The suspension liquid was shaken on a rotary shaker at 100 rpm for 24 h to extract the oxidative phenolic compounds, followed by centrifugation at 4000 rpm for 30 min. Browning level in the medium was measured using a modified protocol (Krishna et al., 2008; Park et al., 2006).
determined from the absorption of the alcoholic extract supernatant at 420 nm with a spectrophotometer.

**Statistical analysis**

Data were analyzed using Tukey’s multiple range tests and Student’s t-test. The correlation between total phenolics content and medium browning was calculated.

**Results**

**Application of L-2-aminooxy-3-phenylpropionic acid**

Medium browning decreased considerably from 37% to 3% with the increase in AOPP concentration up to 1.0 mM (Table 1).

After 4 weeks of culture, although all explants survived on media containing 0, 0.01 and 0.1 mM AOPP, only 37% survived explants were recorded at 1.0 mM AOPP. Adventitious bud formation and terminal bud development were observed in cultured explants. Adventitious buds were formed at 0.01 and 0.1 mM AOPP, but not at 0 and 1.0 mM AOPP. The number of buds per explant was high (1.6) at 0.01 mM compared with 0.1 mM (1.3) and 0 and 1.0 mM AOPP (1.0).

After 8 weeks of culture, browning decreased from 70% to 3% with increasing AOPP concentration up to 1.0 mM. All explants survived at 0 and 0.01 mM AOPP, but no explants survived at 1.0 mM AOPP. The highest (7.2) and lowest (0) number of buds per explant developed at 0.01 and 1.0 mM AOPP, respectively. Multiple buds had formed on the explants after 8 weeks culture and multiple shoots had developed 12 weeks after planting (Fig. 1). Subsequently, these shoots developed into healthy plants with two or three leaves and one or two roots.

**Phenol content in explants**

Total phenolic content continuously increased in control explants from 1.55 mg·gFW\(^{-1}\) up to 2.28 mg·gFW\(^{-1}\) after 8 weeks of culture (Fig. 2). In contrast, total phenolic content in explants treated with 0.01 mM AOPP did not increase from 1.46 mg·gFW\(^{-1}\) until after 4 weeks of culture; however, after 8 weeks of culture, the total phenolic content of AOPP-treated explants had increased to a level similar to that of the control (2.24 mg·gFW\(^{-1}\)).

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**Table 1.** Effects of various concentrations of L-2-aminooxy-3-phenylpropionic acid (AOPP) on browning and bud formation in *N. falcata* after 4 and 8 weeks of culture.

<table>
<thead>
<tr>
<th>Culture period (week)</th>
<th>AOPP (mM)</th>
<th>Browning (%)</th>
<th>Survived explants (%)</th>
<th>Survived explants with buds (%)</th>
<th>Number of buds per survived explant**</th>
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<tbody>
<tr>
<td>4</td>
<td>0</td>
<td>37</td>
<td>100</td>
<td>100</td>
<td>1.0 ± 0 b</td>
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<tr>
<td></td>
<td>0.01</td>
<td>20</td>
<td>100</td>
<td>100</td>
<td>1.6 ± 0.2 a</td>
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<tr>
<td></td>
<td>0.1</td>
<td>13</td>
<td>100</td>
<td>100</td>
<td>1.3 ± 0.1 ab</td>
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<tr>
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<td>100</td>
<td>100</td>
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<tr>
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<td>100</td>
<td>100</td>
<td>7.2 ± 0.7 A</td>
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<tr>
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<td>77</td>
<td>77</td>
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<tr>
<td></td>
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</table>

n = 30

* The rate of browning compound exuded from the explant to medium.

** Mean ± SE As the terminal bud developed in all explants, subtraction of one from the values gives the number of adventitious buds formed.

** For each culture period, different letters indicate a significant difference according to Tukey’s multiple range test (P < 0.05).

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**Fig. 1.** Effects of L-2-aminooxy-3-phenylpropionic acid (AOPP) on adventitious bud formation on upper portion of plantlet in *N. falcata* in vitro. Multiple buds (mb) formed in explants (ex) after 8 weeks of culture (A) and of multiple shoots (sh) developed after 12 weeks of culture (B) on medium containing 0.01 mM AOPP. Scale bars indicate 5.0 mm.

**Fig. 2.** Effect of L-2-aminooxy-3-phenylpropionic acid (AOPP) on total phenolic content in the upper portion of plantlet in *N. falcata* during 8 weeks of culture in vitro. Vertical bars indicate SE (n = 3). NS and ** indicate non-significant and significant at P < 0.001 with Student’s t-test, respectively.
Medium browning

Browning of the medium continuously increased in the control for 8 weeks of culture (Fig. 3). Little browning was observed in the medium containing 0.01 mM AOPP after 4 weeks of culture, but thereafter browning increased markedly. After 8 weeks of culture, the browning level in medium containing 0.01 mM AOPP had increased to the same level as the control (13.0).

Discussion

Tissue browning is attributed to the synthesis of phenolic compounds and decreased bud formation during micropropagation of Orchidaceae (Ishii et al., 1979; Seeni and Latha, 1992; Vij et al., 1984). Browning is controlled by dark preconditioning in Malus pumila (Wang et al., 1994), Psidium guajava (Meghwal et al., 2001), and Mangifera indica (Sharma and Singh, 2002), which increases bud formation. In our previous study, we found that dark preconditioning was an effective treatment for browning control and promotion of bud formation in N. falcata (Mitsukuri et al., 2009).

As a key enzyme in the phenylpropanoid biosynthesis pathway, PAL has a profound impact on the browning and necrosis of plant tissue (Tabiyeh et al., 2006). On the other hand, AOPP controls the production of secondary metabolites from the phenylpropanoid pathway without inhibition of the primary metabolism (Edahiro and Seki, 2006). AOPP regulates PAL activity in phenylpropanoid metabolism in many plants such as apple (Ju et al., 1995), lettuce (Peiser et al., 1998), wheat (Leubner-Metzger and Amrhein, 1994), soybean (Brincat et al., 2002; Mader and Hanke, 1997), and strawberry (Edahiro and Seki, 2006). Fu et al. (1993) reported that AOPP accelerated adventitious bud formation by restraining suberization in Japanese white birch. In the present study, a low concentration of AOPP did not completely inhibit phenol metabolism, but suppressed the increase in phenolic compound content, which had a potential effect.

AOPP controlled browning of the medium and explants, promoted survived explant, and increased adventitious bud formation in N. falcata. We observed that browning of the medium and explant was effectively controlled by 1.0 mM AOPP, but the explant was destroyed; therefore, it can be deduced that a high concentration of AOPP may cause a physiological disorder in the explant by inhibiting the phenylpropanoid pathway. Although secondary metabolism is essential for all living organisms, the production and accumulation of excessive concentrations of phenolic compounds depress survived explant, regeneration, and bud formation. In the present study, the number of adventitious buds increased from 2.4 to 7.2 at 0 and 0.01 mM AOPP, respectively, suggesting that AOPP promotes adventitious bud formation by decreasing phenolic compound content. The shoots formed in the AOPP treatment rooted and grew into healthy plants on 1/2 MS medium. The plants were easily grown in a greenhouse with acclimatization.

In N. falcata, treatment with AOPP controlled phenol compound biosynthesis in the explants and promoted adventitious bud formation, which were similar to the effects of dark preconditioning (Mitsukuri et al., 2009); however, dark preconditioning needs to find the conditioning period of each plant. In our previous study, dark preconditioning reduced the explant survival rate and preconditioned plantlets formed 5.1 buds per survived explant (Mitsukuri et al., 2009). In contrast, in the present study all explants from plantlets grown without dark preconditioning survived and formed 7.2 buds per explant in response to AOPP treatment. Therefore, the decreased efficiency of regeneration resulting from the lower survived explant rate following dark preconditioning suggests that treatment with AOPP is a simpler and more efficient method than dark preconditioning.
Exudation of phenolic compounds and medium browning was observed in leaf cultures of *Doritaenopsis* (Park et al., 2006). In the present study, treatment with AOPP decreased the level of phenolic compounds in the upper portion of explants and medium browning for 4 weeks of culture (Figs. 2 and 3), and the phenol content was strongly correlated with medium browning (Fig. 4). Similar to our findings, Krishna et al. (2008) reported a positive correlation between phenol exudation and necrosis of explants. Thus, AOPP controls phenol content and medium browning for 4 weeks from the start of culture, increasing the plant regeneration potential.

In conclusion, the application of 0.01 mM AOPP controlled phenol production for 4 weeks from the start of culture and promoted adventitious bud formation in *N. falcata*. The addition of AOPP to the medium is a simpler method than dark preconditioning. The effect of AOPP on browning and phenol content means that it has potential for *in vitro* regeneration and bud formation in other plants.

**Literature Cited**


