Effect of Aluminum Chloride on the Organogenesis of Two Types of Cymbidium In Vitro

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We examined the effects of aluminum chloride (AlCl3) on the organogenesis of Cymbidium Sweet Waffle ‘Tarte’ from inoculated protocorm-like bodies (PLBs) and of Cymbidium kanran ‘Murotonishiki’ from rhizomes to determine the appropriate concentration of AlCl3 for organogenesis. The explants were cultured in modified MS media supplemented with various concentrations of AlCl3 and maintained at 25±1°C and a 24 h light period for 6 weeks for C. Sweet Waffle ‘Tarte’ and 10 weeks for C. kanran ‘Murotonishiki.’ In C. Sweet Waffle ‘Tarte’ 1.0 mg L−1 AlCl3 significantly increased PLB formation, shoot formation from PLBs and root formation from shoot. In C. kanran ‘Murotonishiki,’ 1.0 mg L−1 AlCl3 induced shoot from rhizome and developed roots from shoot. On the other hand, high concentration of AlCl3 (10 mg L−1) resulted the formation of protocorm-like shoots in both Cymbidiums. We concluded that the concentration of 1.0 mg L−1 AlCl3 is optimum for the root and shoot formation of the Cymbidium orchids.

Keywords: orchidaceae, protocorm-like bodies, protocorm-like shoots, regeneration, rhizome

INTRODUCTION

The primary morphogenic pathway leading to the whole plant regeneration involves shoot organogenesis followed by root organogenesis in vitro (Malepszy, 2009). Apart from plant growth regulators, many treatments have been applied to improve the efficiency of regeneration of explant. One of them is incubation of cultures for a certain time under stress condition (low and high temperature, drought, salinity, metal). These types of stress have been found to have a positive effect on regeneration of plants (Pujalón et al., 2008). Aluminum (Al) is the 3rd most abundant element in the Earth’s crust (after oxygen and silicon), accounting for roughly 7% by mass. In soil, Al ions can be toxic to plants, but in combination with other minerals, it increases plant growth by enhancing phosphorus availability and activating the genes associated with abiotic stress (Noor et al., 2019). The effect of Al on plant growth, both toxic and beneficial, depends on the concentration and varies with species, physiological age, and growth conditions (Bojórquez-Quintal et al., 2017). Aluminum chloride (AlCl3) can produce metallic stress condition when added to culture media (Gallego et al., 2002). It enhanced shoot regeneration in date palm (Al-Mayahi, 2019), and increased micro-tuber and tuberous root production in Gloriosa superba L. (Subaramani et al., 2019). However, whether it can be used in orchid in vitro culture has not been reported yet. Cymbidium species are highly valued in the flower market due to its attractive foliage, flower color and pleasant aroma. So, a high quality plantlet is always on demand. Based on morphological and ecological characters, the genus Cymbidium can be differentiated into two types, protocorm-forming and rhizome-forming (Shimasaki and Uemoto, 1987). The protocorm and protocorm-like body (PLB) forming type of Cymbidium are epiphytic, mostly common in tropical or subtropical regions and the rhizome-forming type includes terrestrial or saprophyte, which is widely distributed in oriental regions. The organogenetic pathways of PLB-forming and rhizome-forming types of Cymbidium are different (Ogura-Tsujita et al., 2007). The PLBs of PLB-forming Cymbidium are developed from apical meristem culture in vitro and developed shoots and roots within short period. In case of rhizome-forming types, rhizomes are developed directly from apical meristem culture in vitro and started forming more branches. However, shoot formation of a rhizome-forming type of Cymbidium is difficult compare with of PLB-forming type under an in vitro condition because rhizome has long dormancy period than PLBs (Shimasaki and Uemoto, 1987). In the present study we investigated the metallic stress effects of AlCl3 on in vitro cultures of two types of Cymbidium to identify its optimum concentration for regeneration of PLBs or rhizomes from inoculated PLB or rhizome, respectively, and formation of shoots and roots.

MATERIALS AND METHODS

Plant materials
Cymbidium Sweet Waffle ‘Tarte’ and Cymbidium kan-
run ‘Murotonishiki’ were used as representatives of PLB-forming type and rhizome-forming type Cymbidium, respectively. Cymbidium Sweet Waffle ‘Tarte’ was provided by Mr. Masamori Ujike, Bio-U Company, Kagawa, Japan. Clonal protocorm-like bodies (PLBs) of Cymbidium Sweet Waffle ‘Tarte’ and rhizome cultures of Cymbidium kanran ‘Murotonishiki’ were obtained by shoot meristem cultures and proliferated in modified Murashige and Skoog (Shimasaki and Uemoto, 1990) medium by transferring new medium every two months. After obtaining a desirable number of explants, approximately 3 mm diameter PLBs (average fresh weight, 30 mg) of C. Sweet Waffle ‘Tarte’ and approximately 5 mm long apical segments of rhizomes of C. kanran ‘Murotonishiki’ (average fresh weight, 20 mg) were used as explants.

**Culture medium**

Modified MS medium (Shimasaki and Uemoto, 1990) supplemented with 412.5 mg L\(^{-1}\) NH\(_4\)NO\(_3\), 950 mg L\(^{-1}\) KNO\(_3\), 20 g L\(^{-1}\) sucrose, 2.2 g L\(^{-1}\) Phytagel (Sigma-Aldrich Co., USA) and 1 mM 4-morpholinooethanesulfonic acid sodium salt (MES-Na) (Sigma-Aldrich Co., USA) was used as basal media. AlCl\(_3\) (Fujifilm Wako Pure Chemical Co., Japan) at various concentrations (0, 0.01, 0.1, 1 and 10 mg L\(^{-1}\)) was added to the basal media before autoclaving. Culture bottles (UM culture jar, 250 mL; AsOne, Japan) with plastic caps were used, with each bottle receiving 30 mL of medium. The media were adjusted to pH 5.5–5.8 and sterilized by autoclaving at 121°C for 15 min. Five explants were put in each culture vessel and three culture vessels were used for one replication. There were five replications. Accordingly, 15 explants were used for each replication. All the cultures were established and grown under white LED (LT L20KY 9W 1532) at a low photon flux density (PFD) of 25 µmol m\(^{-2}\) s\(^{-1}\) and maintained at 25±1°C and a 24 h day\(^{-1}\) light period for 6 weeks for Cymbidium Sweet Waffle ‘Tarte’ and 10 weeks for Cymbidium kanran ‘Murotonishiki.’

**Statistical analysis**

Statistical evaluation was done by using one-way analysis of variance (ANOVA) and then analyzed with Tukey’s honestly significant difference test (Tukey’s HSD) at \(P \leq 0.05\) using KaleidaGraph-4.5.0 (Synergy Soft-ware, USA) to test for significant differences among the sample means.

### RESULTS

**Effect of AlCl\(_3\) on the organogenesis of Cymbidium Sweet Waffle ‘Tarte’**

Significant variation was observed in the organogenesis of inoculated PLBs of C. Sweet Waffle ‘Tarte’ with elicitation by AlCl\(_3\), which increased PLB formation, fresh weight and the regeneration of the shoots and roots per explant (Table 1). After 6 weeks of culture, AlCl\(_3\) significantly increased the number of PLBs. Vegetative shoots differentiation was observed by adding 0.1-1.0 mg L\(^{-1}\) AlCl\(_3\). Adding of 1.0 mg L\(^{-1}\) AlCl\(_3\) to the culture media enhanced the differentiation of the shoots from the apex meristem of the PLBs (Fig. 1B) and the shoots formed roots (Fig. 1C). The concentration of 1.0 mg L\(^{-1}\) AlCl\(_3\) also enhanced the formation of roots from shoots. Bigger PLBs (>2 mm in diameter) did not form except with 1.0 mg L\(^{-1}\) AlCl\(_3\). Fresh weight also increased with 1.0 mg L\(^{-1}\) AlCl\(_3\); 10 mg L\(^{-1}\) AlCl\(_3\) induced protocorm-like shoots from the apex segment of the cultured PLBs (Fig. 1D). Protocorm-like shoots are flattened with shorter and thicker shoots than normal shoots. These protocorm-like shoots did not show any further development. Therefore, 1.0 mg L\(^{-1}\) AlCl\(_3\) was found to be optimum for the organogenesis of C. Sweet Waffle ‘Tarte.’

**Effect of AlCl\(_3\) on the organogenesis of Cymbidium kanran ‘Murotonishiki’**

The effects of AlCl\(_3\) on the differentiation of inoculated rhizomes of C. kanran ‘Murotonishiki’ after 10 weeks of culture is shown in Table 2. The rhizomes grew and developed rhizome branches and shoot or protocorm-like shoots (Fig 2B-D). Rhizome branch had several nodes and apexes, which showed the physiology of gravitropism in vitro. Adding 1.0 mg L\(^{-1}\) AlCl\(_3\) to the culture media significantly increased the number of new rhizome branches per explant. Differentiation of shoots and roots from inoculated rhizomes was only found by using 1.0 mg L\(^{-1}\) AlCl\(_3\). The concentration of 0.01–0.1 mg L\(^{-1}\) AlCl\(_3\) did not induce any shoots but 1.0 mg L\(^{-1}\) AlCl\(_3\) resulted in

### Table 1. Effect of AlCl\(_3\) on the organogenesis of Cymbidium Sweet Waffle ‘Tarte’ after 6 weeks of culture.

| AlCl\(_3\) (mg L\(^{-1}\)) | PLBs | Shoots | Protocorm-like shoots | Roots | PLBs (>2 mm) | Bigger PLBs | Shoots | Roots | Protocorm-like shoots | Fresh weight (mg) |
|--------------------------|------|--------|-----------------------|-------|--------------|-------------|--------|-------|-----------------------|-----------------|
| 0                        | 6.0±0.2c | 0        | 0                     | 0     | 53           | 0           | 0      | 0     | 0                     | 158.7±0.5d       |
| 0.01                     | 7.7±0.1c | 0        | 0                     | 0     | 67           | 0           | 0      | 0     | 0                     | 190.5±1.1d       |
| 0.1                      | 10.5±0.1b | 0.7±0.2b | 0                     | 0.5±0.02b | 87     | 0           | 47     | 40    | 0                     | 253.3±0.8b       |
| 1.0                      | 14.5±0.1a | 1.4±0.01a | 0                     | 0.9±0.04a | 100   | 40          | 60     | 47    | 0                     | 323.3±1.0a       |
| 10                       | 3.0±0.2a  | 0.3±0.03a | 1.8±0.07a             | 0     | 40           | 0           | 6      | 0     | 67                    | 205.0±1.3c       |

Values represent mean±SE (n = 15). Means in the same column followed by different letters show significant differences by Tukey’s test (\(P \leq 0.05\)).

\(\text{Average number} = \text{Average number of new PLBs or shoots or roots/culture}\)

\(\%\) Formation rate (%) = (Number of cultures forming new PLB(s) or bigger size PLBs (>2 mm in diameter) or shoots or roots or protocorm-like shoots/total number of cultures)×100

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differentiation of shoots from inoculated rhizome and roots from shoot. So, AlCl₃ in the culture media increased the
differentiation of shoots from the terminal bud of the rhi-
zome, and later these shoots formed roots (Fig. 2C). It also
increased the length of the longest rhizome branch and
fresh weight. However, adding 10 mg L⁻¹ AlCl₃ resulted in
protocorm-like shoots (Fig. 2D) from the apex segment of
the cultured rhizomes. Protocorm-like shoots, triggered
from the rhizome, resemble shoots but were very short and
thick, and fail to show any further development. Therefore,
1.0 mg L⁻¹ of AlCl₃ was found to be optimum for the
organogenesis of *C. kanran* 'Murotonishiki.'

**DISCUSSION**

The abundance of aluminum (Al) in acid soil is
responsible to suppress plant growth (Kopittke et al., 2016)
but low concentration of Al may stimulate the growth of
native or acid-loving plants (Osaki et al., 1997). Al has
been reported to induce several oxidative stress genes in
*Arabidopsis thaliana* (Richards et al., 1998). Low levels of
Al can involve in the defense mechanism and enhance
resistance to stress (Hamel et al., 1998; Kaur et al., 2016).
When plants are exposed to abiotic stress, oxidative

| AlCl₃ (mg L⁻¹) | Average number of branches | Length of longest rhizome (mm) | Rate of formation (%) | Fresh weight (mg) |
|--------------|---------------------------|-------------------------------|----------------------|-------------------|
|              | Rhizomes | Shoots | Protocorm-like shoots | Roots | Rhizomes | Shoots | Roots | Protocorm-like shoots |
| 0            | 2.9±0.1c | 0      | 0                  | 0     | 2.7±0.1c | 47     | 0    | 0     | 0     | 49.0±0.1e |
| 0.01         | 3.1±0.1c | 0      | 0                  | 0     | 3.3±0.1bc | 60     | 0    | 0     | 0     | 77.7±0.5c |
| 0.1          | 3.9±0.1b | 0      | 0                  | 0     | 4.4±0.1b | 73     | 0    | 0     | 0     | 118.3±0.6b |
| 1.0          | 5.2±0.1a | 1.2±0.02a | 0          | 0.7±0.01a | 6.6±0.2a | 87     | 33   | 13    | 0     | 165.0±0.4a |
| 10           | 1.5±0.1d | 0.2±0.01b | 2.2±0.1a    | 0     | 2.3±0.1c | 13     | 6    | 0     | 80    | 64.3±0.3d |

Values represent means±SE (n = 15). Means in the same column followed by different letters show significant differences by Tukey’s test (P ≤ 0.05).

Average number of branches = Number of branches/one rhizome culture

Rate of formation (%) = (Number of cultures forming new rhizomes or shoots or roots or protocorm-like shoots/total number of cultures)×100

Fig. 1  Morphology of different stages during plant organogenesis of *Cymbidium* Sweet Waffle ‘Tarte.’ (A) PLB as explant, (B) proliferation of PLBs and differentiation of shoots from PLBs, (C) new plantlet from PLB, (D) Flattened and short protocorm-like shoot formation from PLBs. PLB: protocorm-like body, S: shoot, R: root, PLS: protocorm-like shoot

Table 2  Effect of AlCl₃ on the organogenesis of *Cymbidium kanran* ‘Murotonishiki’ after 10 weeks of culture.
stresses occur due to production of relative oxygen species (ROS). Plants have unique defense mechanism to remove ROS radicals which helps to ease the stress condition. Application of Al can up-regulates many oxidative stress responsive genes which can protect cell from oxidative damage (Cançado et al., 2005; Maron et al., 2008). AlCl3 can increase the production of bioactive compounds by modifying plant secondary metabolism (Ramirez-Estrada et al., 2016). It also improve the plant defense mechanism by increasing activities of various antioxidant enzymes i.e. superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and prolin (Zafar et al., 2017; Al-Mayahi, 2019). In this study we found that low concentration of AlCl3 (1.0 mg L\(^{-1}\)) was sufficient for the organogenesis of both Cymbidium orchids. Protocorm-like shoots were observed at the high concentration of aluminum (10 mg L\(^{-1}\)) which did not show any further developmental stages. Shimasaki and Uemoto (1990) also did not find any further development of rhizomes after formation of protocorm-like shoots in Cymbidium kanran. Zafar et al. (2017) found that in vitro growth of calluses in Rauvolfia serpentina is enhanced by low doses of AlCl3 (up to 0.15 mM). Subramani et al. (2019) found that AlCl3, at 125-150 μM results in significant increases in the micro-tuber and tuberous root production in Gloriosa superba L. but a higher concentration decreases production. Al-Mayahi (2019) found that a low concentration of AlCl3 induces vegetative growth in date palm (Phoenix dactylifera L. cv. Urm-Aldehin) in an in vitro culture by absorbing nutrients in the shoots, but a high concentration hinders the growth by disturbing cell division in the meristematic zone. Therefore, the effective dose of AlCl3 varies depending on the plant species. Thus, to identify the appropriate amount of AlCl3 for a particular species is very important. In our experiment, small amount of AlCl3 (1.0 mg L\(^{-1}\)) may induce metallic stress condition which may reduce the dormant period of the rhizome of C. kanran ‘Murotonishiki’ and enhance the organogenesis of both Cymbidium orchids. In contrast, a high concentration of AlCl3 (≥ 10 mg L\(^{-1}\)) acted as growth retardant by producing protocorm-like shoots in both Cymbidium orchids. This phenomenon indicated the metallic stress effects of Al on the cultures of Cymbidium. Although the exact mechanism of AlCl3 cannot be explained by our study but it revealed that AlCl3 can play a significantly role in micropagation of orchid.

In orchid in vitro cultures, plant growth regulators (e.g. auxin, cytokinin etc.) are added to culture media for regeneration of roots and shoots (Shimasaki and Uemoto, 1990, Hamada et al., 2010). The ratio of auxin and cytokinin play a vital role to regenerate shoots from rhizome branch and roots from shoot in oriental Cymbidium (Kokubo et al., 1980; Shimasaki and Uemoto, 1991). However, high concentration of BA (6-Benzylaminopurine) in culture medium increase mutation rates in regenerated plants (Arditti and Ernst, 1993; Huetteman and Preece, 1993). Furthermore, auxin and cytokinin affect the growth periods during plant life cycle. Thus, application of a plant growth regulator in Cymbidium in vitro is very complicated. In our study, we demonstrated that a low concentration AlCl3, significantly enhanced the PLB formation, regeneration of shoots and roots in PLB forming-type Cymbidium, and induced shoots and roots in rhizome-forming type Cymbidium. This result suggests that AlCl3 may act as a plant growth regulator especially for the regeneration of shoot from rhizome branch and development of roots from shoot in oriental Cymbidium in vitro. Organogenesis is the initial step for regeneration of explants in vitro. It regenerates plant by organ formation through dedifferentiation of differentiated cells and reorganization of cell division to create particular organ primordia and meristems after the establishment of the vascular connection between the explant and the newly regenerating organ (Bidabadi and
Jain, 2020). In this study, adding AlCl$_3$ to the basal medium significantly influenced the organogenesis of both Cymbidium species so it can play a vital role in orchid tissue culture.

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