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IN VITRO FLOWERING AND FRUITING IN CULTURE OF *DENDROBIUM OFFICINATE* KIMURA ET MIGO. (ORCHIDACEAE)

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Abstract

Dendrobium officinate Kimura et Migo. is a rare and endangered perennial orchid, which is valued for its attractive flowers and medicinal uses. Its three-to-five-year breeding cycle makes propagation difficult, so we investigated *In vitro* production of flowers and seeds for this species. Calluses were induced from shoot-tip explants of *D. officinate* on Murashige & Skoog (MS) medium with 0.2 mg L^{-1} benzyladenine (BA) and 0.05 mg L^{-1} α-naphthalene acetic acid (NAA). Multiple shoots were regenerated after protocorm-like bodies (PLBs) were transferred onto the same basal medium with 1.0 mg L^{-1} NAA. The plantlets, 2-4 cm in height, maintained *In vitro* were induced to flower, and the highest rates of inflorescence (83.2%) and normal flowers (73.6%) were produced on the MS medium with 15% (v/v) coconut water and 0.1 mg L^{-1} thidiazuron (TDZ) within 9 weeks. Histological analysis showed the origin of the floral primordial and normal morphologies of the pollen and female organs. The normal flowers were artificially pollinated and capsules developed. Viable seeds were produced and most of them germinated on the MS medium. This reproduction system could be used to study the molecular basis of flowering or to improve plant breeding programs.

Key words: Capsule formation; *Dendrobium officinate*; Histological analysis; *In vitro* flowering.

Introduction

Dendrobium is one of the largest genera in the family Orchidaceae. There are more than 1100 *Dendrobium* species worldwide, and they are primarily distributed from Southeast Asia to New Guinea and Australia (Puchooa, 2004). *Dendrobium officinate* Kimura et Migo. is a rare and endangered perennial orchid in South and Southeast Asia; it is valued for its attractive flowers and medicinal uses (Zhao *et al*., 2007; Shiau *et al*., 2005). The natural habitat of *D. officinate* has been largely destroyed, and populations of *D. officinate* are declining due to overexploitation and low rates of natural propagation. The entire breeding cycle of *D. officinate* lasts three to five years, due to the long juvenile phase of this orchid. Therefore, efforts to increase the population stands of this species are critical (Yin & Hong, 2009).

The precise mechanism of flowering is still unclear despite it being of significance both physiologically and as a main regulator of fruit or seed yield in horticulture and agriculture (Munir *et al*., 2010). Artificial pollination and breeding could increase *D. officinate* propagation, since a single capsule of *D. officinate* may contain 1,500 to 3,000,000 seeds (Tsi, 1999). *In vitro* flowering could shorten the juvenile phase, making rapid breeding of this rare orchid possible (Zhang, 2007). Induction of precocious flowering *In vitro* has been shown in several *Dendrobium* orchids (Wang *et al*., 2009; Hee *et al*., 2007; Sim *et al*., 2007). With viable seed production, *In vitro* flowering in culture could play an important role in orchid breeding. However, production of functional orchid seeds in culture following *In vitro* flowering and pollinating has only been reported in a *Dendrobium* hybrid (Hee *et al*., 2007).

In this study, we established an entire regeneration system for *D. officinate*, describing the processes of protocorm-like body (PLB) formation, plantlet development, flowering, self-pollinated capsule formation, and seed germination. These findings are important for the study of physiological and molecular mechanisms of flower induction, providing the potential to advance the breeding programs for *D. officinate*.

Materials and Methods

Plant materials, culture media, and culture conditions: Shoot tips obtained from greenhouse-grown *D. officinate* plants were used as explants; tips were surface sterilized in 70% ethanol for 20s, placed in an aqueous solution of 0.1% $HgCl_2$ for 2 min, and thoroughly rinsed with sterile water. Explants were cultured on MS basal medium (Murashige & Skoog, 1962), supplemented with 3% (w/v) sucrose, 0.03% (w/v) activated charcoal, 0.8% agar, 0.5 mg L^{-1} benzyladenine (BA), and 0.1 mg L^{-1} α-naphthalene acetic acid (NAA). The media were adjusted to pH 5.8 before autoclaving at 121°C for 20 min. Callus masses (5-8 mm diameter) induced from explants were subcultured on the same medium and then transferred to MS medium without any plant growth regulators (PGR) for PLB regeneration. Callus-derived PLBs that were about 3-4mm in diameter with macroscopic shoot-apexes were transferred onto MS medium 1.0 mg L-1 NAA for the development of shoots. The cultures were maintained at 25 ± 2°C with a 10 h photoperiod provided by white fluorescent lights at 42 μmol $m^{-2}s^{-1}$.

***In vitro* flowering, pollination, and capsule development in culture:** Plantlets about 2-4 cm in height with two or three leaves were induced to flower on the MS medium with 15% (v/v) coconut water and different PGRs. The effect of different concentrations (0.05-2.0 mg L^{-1}) of BA and thidiazuron (TDZ) was studied on *In vitro* flowering. The *In vitro*–developed normal flowers were self-pollinated using sterile forceps. Upon pollination, the plantlets were observed for seedpod formation. The capsules were harvested upon maturation and cut open. Seeds from these *In vitro* capsules were germinated on 1/2 MS medium.

Pollen germination *In vitro*: Pollen released from the anthers of flowers in full bloom was placed on an agar (8 g L^{-1}) medium containing 10% sucrose, 100 mg L^{-1} H_3BO_3, 300 mg L^{-1} $Ca(NO_3)_2$ H_2O, 200 mg L^{-1} $MgSO_4$ $7H_2O$, and 200 mg L^{-1} KNO_3 for germination studies (Brewbaker & Kwack 1963).

Histological analysis: The materials used for histological observations were fixed in FAA (formalin/acetic acid/50% ethanol, 1:1:18 *v*/*v*/*v*), dehydrated in an ethanol series, and embedded in paraffin wax. The embedded materials were cut into sections 8 µm thick and stained with 1% safranin-O and 0.5% fast green. Sections were observed and photographed using a light microscope (Olympus CX41).

Statistical analysis: Each experiment was repeated three times, and the means and standard errors were calculated. The statistical significance between the mean values was assessed using Duncan's multiple-range test, where a probability of $p<0.05$ was considered significant.

Results

PLB formation, shoot development, and root induction: A light yellowish and fragile callus mass emerged from the swollen and disorganized shoot tip three weeks after inoculation. The calli continued to proliferate after being transferred to the fresh medium. Following transfer to the PGR-free medium, the calli continued to proliferate and gradually turned green. A large amount of PLBs were produced from the surface of the calli. A large proportion of these PLBs readily germinated into well-developed shoots on the same medium or after being transferred to a fresh PGR-free medium. Healthy shoots with two to three leaves and one to two roots were observed 3-5 weeks after newly formed PLBs with shoots were transferred to MS medium containing 1.0 mg L^{-1} NAA.

Inflorescence induction and flowering *In vitro*: The 2-4 cm plantlets were transferred to the flower-inducing culture. On the appropriate medium, inflorescence stalks were observed in the induced axillary shoots or they developed from the shoot apex of the plantlets within 4-8 weeks (Fig. 1c). Some inflorescence stalks produced normal flower buds, which eventually developed into normal flowers (Fig. 1d). Other buds developed into abnormal flowers, which were morphologically distorted (Fig. 1e). Histological analysis of the inflorescence apex revealed that the apex contained several floret primordia (Fig. 2a). The longitudinal sectioning of a normal floral bud showed the development of a column with normal stigma and pollen (Fig. 2b).

Both BA and TDZ affected the *In vitro* flowering of *D. officinate* (Table 1). Explants incubated in MS medium without BA or treated only with 0.05-0.1 mg L^{-1} BA did not flower. However, flower formation was observed at a BA concentration of 0.5-3.0 mg L^{-1}. Inflorescence induction increased with an increasing BA concentration up to 2.0 mg L^{-1} (64.6%), and then decreased when BA reached 3.0 mg L^{-1}. The percentage of normal flower experienced a similar trend, reaching at 52.7% before decrease to 44.6%. Both proportion of inflorescence and normal flower depended upon the TDZ concentrations in the MS medium as well. Increasing the concentration of TDZ from 0.05 to 0.1 mg L^{-1} resulted in a gradual rise in frequency of inflorescence from 65.4% to 83.2%. In addition, there was a parallel increase in mormal flower induction. The highest rates of inflorescence (83.2%) with the maximum normal flowers (73.6%) was recorded in the MS medium containing 0.1 mg L^{-1} TDZ. It is clear that TDZ promoted inflorescence development and normal flowering better than BA at low concentrations (0.05-0.5 mg L^{-1}), but normal-flower induction was reduced at high concentrations (1.0-2.0 mg L^{-1}). Plantlets grown in PGR-free medium did not produce inflorescences.

Table 1. Effect of BA and TDZ on the flowering development of *D. officinate* with coconut water (15%) added to the medium.

Cytokinin	Conc.(mg L^{-1})	% Culture with inflorescence stalk	% Culture with normal flower
control	0	0 g	0 h
BA	0.05	0 g	0 h
	0.1	0 g	0 h
	0.5	16.3 ± 0.4 f	5.1 ± 0.4 g
	1.0	55.4 ± 1.3 c	43.2 ± 0.9 d
	2.0	64.6 ± 1.2 b	52.7 ± 0.6 b
	3.0	54.5 ± 1.3 c	44.6 ± 0.9 cd
TDZ	0.05	65.4 ± 2.4 b	40.2 ± 2.1 d
	0.1	83.2 ± 3.5a	73.6 ± 2.5 a
	0.5	64.1 ± 1.1 b	47.5 ± 2.1 c
	1.0	35.3 ± 1.7 d	29.1 ± 1.4 e
	2.0	34.6 ± 1.0 de	23.4 ± 0.5 f
	3.0	31.9 ± 1.3 e	24.8 ± 0.7 f

Data were recorded after 9 weeks of flower-inducing culture. Each treatment consisted of four replicates with 10 explants in each culture. Values represent mean ± S.E. Means followed by the same letter in a column are not significantly different at the $p<0.05$ level of significance

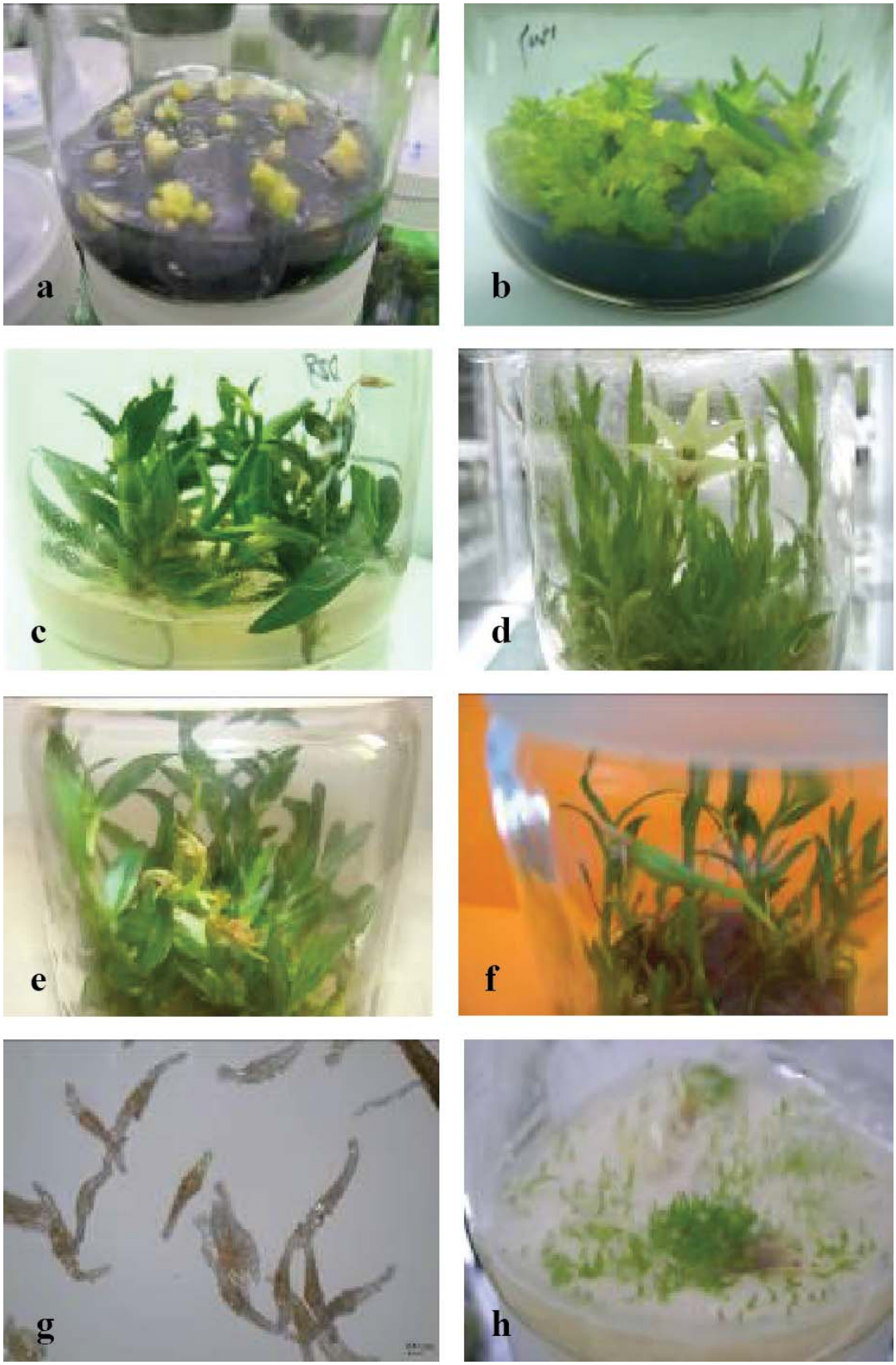

Fig. 1. PLB proliferation, *In vitro* floral morphology, and fruit formation in the culture of *D. officinate*. (a) Callus induced from shoot tips. (b) PLB proliferation and differentiation. (c) Inflorescence with floral bud. (d) Normal flower. (e) Abnormal flower. (f) Capsule 60 d after artificial pollination. (g) Seeds. (h) Seed germination.

Table 2. Characteristics of *In vitro* and *In vivo* flowering for *D. officinate*.

	Inflorescence stalk		No. per stalk	
	Stalk no. per plantlet	Length (cm)	Flower buds	Normal flower
In vivo	4.2 ± 1.1 a	23.7 ± 2.6 a	12.2 ± 1.2 a	10.6 ± 1.4 a
In vitro	1.2 ± 0.3 b	3.5 ± 1.0 b	4.3 ± 0.3 b	2.3 ± 0.2 b

The *In vitro* seedlings were grown in culture with 0.1 mg L^{-1} TDZ. Values are the mean of three experiments, and each experiment consisted of 10 plantlets. Values represent mean ± S.E. Means followed by the same letter in a column are not significantly different at the $p<0.05$ level of significance. Assessment of flower development was made for individual flower buds at bloom.

Table 3. Characteristics of *In vitro* and *In vivo* capsules for *D. officinate*.

Days after pollination		15 d	30 d	60 d	90 d	120 d
In vivo	Length (mm)	15.66 ± 0.45 c	24.44 ± 0.2 b	38.26 ± 0.3 a	39.32 ± 0.4 a	39.51 ± 0.2 a
	Diameter (mm)	3.42 ± 0.19 c	5.56 ±0.27 b	8.19 ± 0.43 a	8.37 ± 0.32 a	8.43 ± 0.38 a
In vitro	Length (mm)	13.32 ± 0.27 c	23.26 ± 0.1 b	36.88 ± 0.31 a	38.02 ± 0.22 a	38.03 ± 0.27 a
	Diameter (mm)	3.19 ± 0.32 c	5.02 ± 0.11 b	7.71 ± 0.13 a	7.81 ± 0.09 a	7.88 ± 0.14 a

Each result was based on three independent experiments. Values represent mean ± S.E. Means followed by the same letter in a row are not significantly different at the $p<0.05$ level of significance

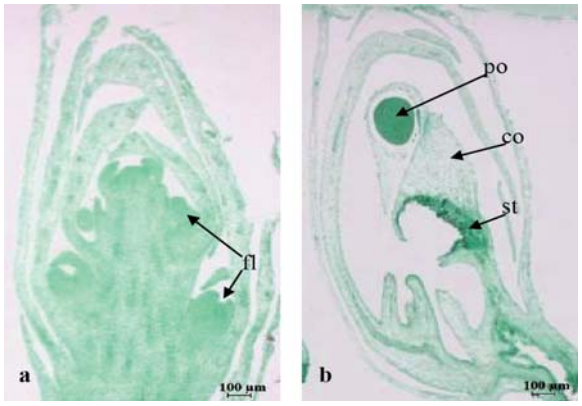

Fig. 2. Histological analysis of inflorescence and floral buds. (a) Apex of young inflorescence with floral primordial (fl). (b) Longitudinal section of a floral bud column (co), showing pollen (po) and stigma (st).

Plantlets flowering *In vitro*: Each flowering plantlet *In vitro* produced one to two inflorescence stalks, with an average of four to five flower buds per inflorescence stalk (Table 2). Inflorescence stalks produced *In vitro* were only 15% as long as greenhouse-grown plants, and the number of stalk *In vitro* was more than three times less than that *In vivo*. In addition, there were two or three normal flowers per inflorescence stalk in culture with 0.1 mg L^{-1} TDZ, while the greenhouse-grown seedlings possessed more than 10 flowers per stalk. The size of the flowers *In vitro* was smaller than the plant grown *In vivo*. Nevertheless, the shapes of the *In vitro* flowers were normal, and the flowers had all floral organs including three sepals, three petals, and a column with anthers and stigma. The pollen from *In vitro*–developed flowers was functional, and some triads were observed (Fig. 3a). Seeds germinated on an agar medium after 48 h culture (Fig. 3b), which demonstrated that the pollen grains were viable.

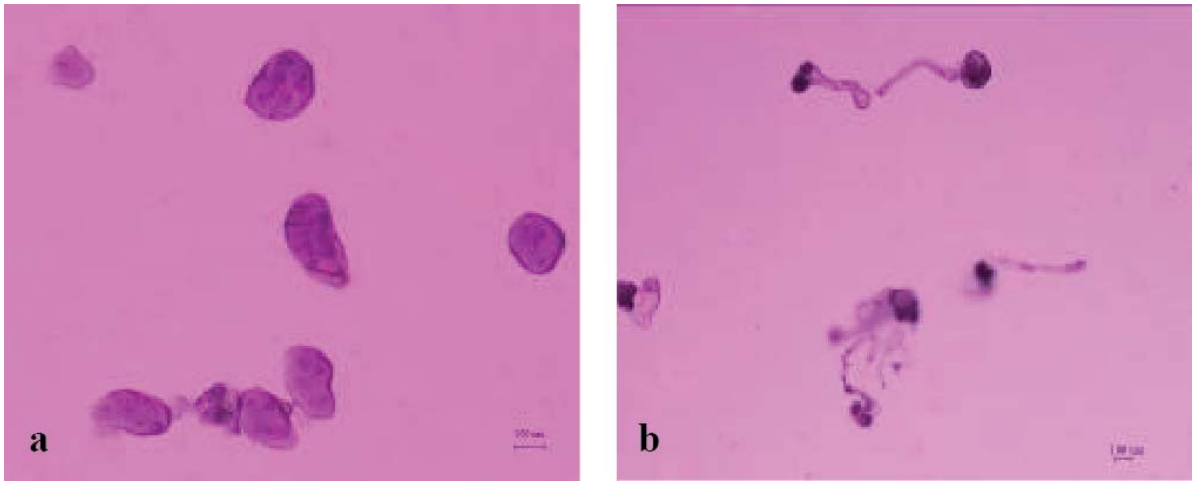

Fig. 3. Sporads and *In vitro* pollen germination. (a) Sporads derived from an *In vitro*–developed flower. tri refers to triad. (b) Germination of pollen derived from an *In vitro*–developed flower. pt refers to pollen tube.

Capsule development and seed production: *In vitro* artificial pollination could lead to successful capsule development. The capsules developed quickly after pollination, the length and diameter of them saw a gradual growth over the prior 60 days, reaching at 36.88 and 7.71mm respectively (Fig. 1f). After that, the figures remained at a stable level. The size and shape of the fruit *In vitro* were similar to those grown *In vivo*. The mature capsules with fertile seeds formed after 120 d of culture, when the capsules had turned yellowish. These capsules were harvested upon maturation, and seeds were sown on 1/2 MS medium (Fig. 1g). After 60 days, 86.6% of the seeds germinated into PLBs or green shoots and grew into seedlings (Fig. 1h) (Table 3).

Discussion

The results presented here describe an efficient protocol for multiple-plantlet regeneration from shoot-tip explants of *D. officinate*, followed by flowering and fruiting of regenerated plantlets *In vitro*. *In vitro* flowering of *D. officinate* has previously been documented when seeds were cultured on MS medium (Wang *et al*., 1997), but reports on fruiting and seed set were lacking. The present study offered another regenerative system for *In vitro* flowering, which allowed the formation of multiple shoots, flowering plantlets, and fertile seed production. This reproductive system might play an important role in orchid breeding because shoot-tip explants can be easily obtained.

The cytokinin requirement for the growth and development of flower buds has been reported in several plants (Heylen & Vendrig, 1988; Huang *et al*., 1999; Zhang, 2007), and BA was found to be effective for early floral induction in orchids (Chang & Chang, 2003; Wang *et al*., 1997; Sim *et al*., 2007). However, TDZ has been considered to be more potent than most of the commonly used cytokinins (Sajid & Aftab, 2009; Naz *et al*., 2009). The importance of TDZ for *In vitro* flower induction has been reported in *D. nobile* and *Cymbidium ensifolium,* where TDZ had a stronger flower-inducing effect than BA (Chang & Chang, 2003; Wang *et al*., 2009). The results in this study agreed with those findings. However, abnormal flowers occurred in the culture with higher TDZ concentrations (Table 2), so the cytokinins may influence a delicate regulative system in floral development. Some genes controlling shoot apical meristem activity may be related to floral regulation (Lindsay *et al*., 2006).

The cultures with optimal BA and TDZ concentrations produced normal flowers; these flowers resembled the flowers of field-grown plants and had complete structures. However, the *In vitro* plantlets produced fewer and smaller inflorescences than the field-grown plants (Table 2). This could be due to the smaller size of the *In vitro* plantlets, since reproductive output may be affected by plant size (Sletvold, 2002).

The morphology of pollen and female organs in the *In vitro* flowers was normal. The morphological and anatomical examination showed that a normal column with pollen and stigma formed in the *In vitro*-developed flowers (Fig. 2b), and the pollen could germinate on the culturing medium (Fig. 3b). Furthermore, the success of *In vitro* pollination and viable seed formation demonstrated that the gametes produced from *In vitro* flowers were functional. In addition, the technology of seed production in culture could have tremendous applications in the breeding of *D. officinate* (Hee *et al*., 2007).

Conclusion

In summary, *D. officinate* flowered and produced viable seeds in culture in this study. Such a reproduction system is ideally suited for the study of the molecular basis and hormonal regulation of flowering and development, while also having practical implications for effective plant breeding.

Acknowledgements

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