Research Article

Somatic Embryogenesis Induction from Protocorm-like Bodies and Leaf Segments of *Dendrobium* Sonia 'Earsakul'

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Received February 23, 2014; Accepted December 17, 2014

Abstract

This study aimed to evaluate effects of types and concentrations of plant growth regulators in a half-strength Murashige and Skoog medium on somatic embryogenesis from protocorm-like bodies (PLBs) and leaf segments of *Dendrobium* Sonia 'Earsakul' cultured for 8 weeks. It was found that media containing thidiazuron (TDZ) either at 0.1, 0.3 and 1.0 mg/L alone or in combinations with 0.1 mg/L naphthaleneacetic acid (NAA) or 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) provided no significant differences in percentage of somatic embryogenesis (38.18-49%) from PLBs. Half strength MS supplemented with TDZ either at 1 and 3 mg/L alone or combination with 0.1 mg/L NAA provided higher percentage of somatic embryo formation and number of somatic embryo per explant more than supplemented with 0.1 mg/L NAA alone. Low percentage of somatic embryogenesis was found from leaf segments. Leaf explants with the dorsal surface in contact with a medium provided higher somatic embryogenesis (0-18.75%) than those obtained from the explants with the ventral surface contact with culture medium (0.89-8.04%). Furthermore, basal segments of leaves with ventral surface in contact with a medium showed higher somatic embryogenesis and number of embryos (1.79-16.07% and 0.2-0.85, respectively) than those obtained from apical segments (0-1.78% and 0-0.3, respectively). There was a significant difference in somatic embryogenesis (0-18.75%) of a leaf when a dorsal surface was placed on the culture medium.

***Key Words*:** *Dendrobium*; Plant growth regulators (PGRs); Protocorm-like bodies (PLBs); Somatic embryogenesis

Introduction

In orchid, the genus *Dendrobium* has been very popular and has been exported to the customers around the world. Japan, United States of America (U.S.A.), the People Republic of China (PRC) and the European countries are the major orchid importers from Thailand (Pradhan, 1979; Jones et al., 1998; Kasikorn Research Center, 2006; Information Center, 2007; Mei et al., 2012). The capacity to export this orchid as fresh flowers to the customers overseas occurs as a result of the technological know-how of the Thai orchid farmers and Thai entrepreneurs in both producing the quality orchid flowers and exporting the produces to the foreign customers. The export value of fresh flowers of *Dendrobium* accounts for 86% of all orchid export value (Kaewduangta and Reamkatog, 2011). For this reason, various researches have been conducted in orchid, particularly in the genus *Dendrobium*, to produce the novel varieties which

ISSN 1905-9159

would produce more appealing flower characteristics (Chia et al., 2001; Kunasakdakul and Smitamana, 2003; Khentry et al., 2006; Atichart, 2013; Pinthong et al., 2014).

In *Dendrobium* orchid, micropropagation methods have been subjected to both scientific and technological investigation. The aims of these researches are primarily to invent novel varieties possessing desired characteristics, particularly exotic flower patterns, and subsequently mass produced the plantlets for commercialization. Several orchid plant parts such as protocorm, seed, shoot apical, node and pseudobulb have been investigated as a potential part used for *in vitro* experiment (Liu et al., 1988; Zhang et al., 1993; Alam et al., 2002; Shiau et al., 2005; Khentry et al., 2007; Sunitibala and Kishor, 2009). Callus of *Dendrobium* has also been used in mircropropagation for long term maintenance (Gong et al., 2004; Roy et al., 2007; Khosravi et al., 2008). The protocorm-like bodies (PLBs) have also been regenerated from callus of *Dendrobium* (Roy et al., 2007; Zhao et al., 2008; Mei et al., 2012).

However, unwanted somaclonal variation of plant tissues occurred when the micropropagation received continuous exogenous plant growth regulators (PGRs) in the culture media (Nehra et al., 1992; Lim and Loh, 2003; Bairu et al., 2006). In *Dendrobium*, after PLBs differentiation in the medium supplemented with PGR, the long-term maintenance of PLBs with minimum somaclonal variation is obligatory (Roy et al., 2007). In addition to the somaclonal variation, microbial contamination may also be problematic for long term maintenance of PLBs (Malabadi and Nataraja, 2006; Suzuki et al., 2008; Antony et al., 2010).

Alternative practices for micropropagation of *Dendrobium* without the changes in both genotype and phenotype are thus required. These alternative practices included inducing somatic embryos form leaf explant and establishing thin cross section culture (Nayak et al., 2002; Chung et al., 2005). This study investigated the establishment of somatic embryos formation initiated from axillary bud derived PLBs and leaf explants of young plantlet in *Dendrobium* on medium containing the different types and concentrations of PGRs for re-inducing embryo from different tissues.

Materials and Methods

Plant materials

In experiment 1, the PLBs of *Dendrobium* Sonia 'Earsakul' derived from *in vitro* axillary bud culture was used as initial explants for somatic embryogenic formation. In experiment 2, the age of young plantlets of *Dendrobium* Sonia 'Earsakul' at 6 months of culture (leaf length about 1.0 cm measured from the axillary bud) was cultured *in vitro* to induce PLBs formation and regeneration. Two weeks before the experiments, the PLBs and young plantlet were cultured on Vacin and Went (1949) (VW) solid medium, in which pH was adjusted to 5.5 and with no PRGs added.

Direct somatic embryogenesis

Somatic embryogenesis from PLBs

The PLBs at the stage of shoot apical emergent were transferred onto solidified half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 30 g/L sucrose and different concentrations of thidiazuron (TDZ) either at 0.1, 0.3 and 1.0 mg/L alone or in combinations with 0.1 mg/L naphthaleneacetic acid (NAA) or 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). PLBs were cultured on the media in Petri's dishes with 12 replicates per treatment.

Somatic embryogenesis from leaf segments

A half-strength MS medium supplemented with TDZ either at 1.0 and 3.0 mg/L alone or in combination with 0.1 mg/L NAA was used to culture leaf segments to induce embryogenic callus. A young leaf derived from young plantlet was divided into 2 segments, apical and basal segment (about 0.5 cm long) and cultured on the medium in two orientations, either dorsal or ventral side placing on a medium. All explants were cultured in 120-mL bottles containing 30 mL of culture medium with 14 replicates per treatments.

Culture conditions

Explants were cultured in the cabinet at 26±2°C under a 16-h photoperiod with 1,762 lux illumination in the tissue culture laboratory at the Faculty of Animal Science and Agricultural Technology, Silpakorn University, Phetchaburi Information Technology (IT) Campus.

Data analysis

The data was collected after 8 weeks of culturing both PLBs and leaf explants in the media for inducing somatic embryogenesis. Percentage of the somatic embryogenic formation and number of somatic embryos per culture explant from PLBs and leaf explants were statistically analyzed.

Statistical analysis

In experiment 1, percentage of green leaf, percentage of somatic embryogenesis and number of somatic embryos were collected after 8 weeks of culturing. Data were analysed in a complelety randomized design (CRD) with 7 treatments and 12 replications. In experiment 2, data were analysed in a CRD with 6 treatments and 14 replications for somatic embryogenenic formation on young leaf explants.

Analysis of variance procedures was conducted to evaluate data, in which the F-test was used to test the statistical significance. Duncan's new multiple range test (DMRT) was employed to separate means among treatments ($P<0.05$).

Results

Somatic embryogenesis from PLBs

Somatic embryos could be directly induced from the axillary buds of protocorm-like bodies. No significant difference ($P>0.05$) was observed in all characteristics, including the percentage of green PLBs, the percentage of responded PLBs and the percentage of somatic embryos, after culturing the PLBs on the half-strength MS medium supplemented with TDZ either at 0.1 and 0.3 mg/L alone or in combination with 0.1 mg/L NAA or 1.0 mg/L 2,4-D (Table 1) (Figure 1). However, there was high percentage of green PLBs (ranged between 92.88-98.64%) and the changes in the morphological charcteristics, including enlargement of PLBs and formation of somatic embryo (responsed PLBs) (ranged between 85.26-89.91%) in all treatments (Table 1) (Figure 1). Percentage of somatic embryogenesis, which was induced from PLBs, ranged between 38.18-49% (Table 1). The media did initiate the formation of the somatic embryos from PLBs explants. The media had comparatively less effect in initiating the formation of callus from PLBs (data not shown).

Table 1 Effects of plant growth regulators in ½ MS on somatic embryogenesis from protocorm-like bodies of *Dendrobium* Sonia 'Earsakul' after culturing for 8 weeks

Media	Green PLBs (%)	Responded PLBs (%)	Somatic embryogenesis PLBs (%)
½ MS + 0.1 mg/L TDZ	92.89	86.12	46.41
½ MS + 0.3 mg/L TDZ	92.88	89.82	45.58
½ MS + 1.0 mg/L TDZ	94.72	85.26	49.00
½ MS + 0.1 mg/L TDZ + 0.1 mg/L NAA	94.85	88.24	41.63
½ MS + 0.3 mg/L TDZ + 0.1 mg/L NAA	96.84	88.16	38.18
½ MS + 0.1 mg/L TDZ + 1.0 mg/L 2,4-D	96.41	89.26	45.04
½ MS + 0.3 mg/L TDZ + 1.0 mg/L 2,4-D	98.64	89.91	44.95
F-test	NS	NS	NS

NS, not significant different at the 0.05 level of probability

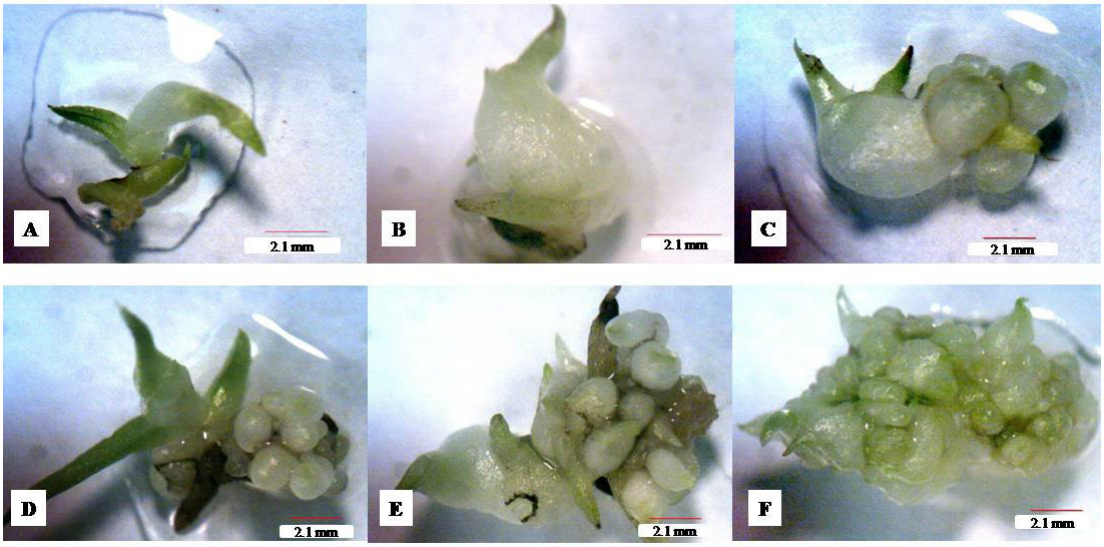

Figure 1 Direct somatic embryo induction from the basal part of protocorm-like bodies of *Dendrobium* Sonia 'Earsakul' after culturing on ½ MS supplemented with 0.3 TDZ for eight weeks; (A) Undifferentiated of protocorm-like bodies; (B) Responded protocorm-like bodies to culture medium but somatic embryos not occurred; (C-F) Somatic embryos occurred in media.

Somatic embryogenesis from leaf segments

The effect of PGRs was illustrated in Table 2-Table 3 and Figure 2. The result showed significant difference on percentage of green leaf segments and the number of somatic embryos per explant from culturing the basal leaf segments when they were placed with ventral surface contacting with the medium (Table 2). The half-strength MS medium supplemented either with 1 and 3 mg/L TDZ alone or in combination between 1 mg/L TDZ with 0.1 mg/L NAA showed the highest green leaf segment (at 93.75%). The lowest green leaf segment (at 73.95%) was observed in the half-strength MS medium supplemented with 0.1 mg/L NAA.

Different supplements in the half-strength MS medium had no effect on the induction of somatic embryogenesis from the leaf segments which were placed with the ventral surface in contact with the medium (at ranged between 0.89-8.04%) (Table 2). For the somatic embryos formation from different leaf segments, there was no statistical significant difference ($P>0.05$) between culturing apical (at 0-1.78%) and basal (1.79-16.07%) leaf segments (Table 2).

The formation of somatic embryos was observed after culturing the leaf segments by placing the ventral surface on the medium (Table 2). The half-strength MS medium supplemented with PGRs affected the number of somatic embryos per explant. The medium supplemented with 1 and 3 mg/L TDZ alone showed highest somatic embryos per explant both on the apical (at 0.11 and 0.30, respectively) and basal leaf segments (at 0.50 and 0.85, respectively) (Table 2). The basal leaf segments (ranged between 0.20-0.85) had the number of somatic embryos per explant more than the apical leaf segments (ranged between 0-0.30) (Table 2).

The effect of PGRs - when the dorsal surface of leaf segments was placed on the medium - was illustrated in Table 3. The result showed significant difference on percentage of green leaf segments and percentage of somatic embryos from culturing the apical leaf segments. The percentage of green leaf segments was affected by PGRs with highly significant difference ($P<0.01$) (Table 3). The half-strength MS medium supplemented either with TDZ alone or in combination with 0.1 mg/L NAA had the greatest effect to the green leaf segments (excepted

Table 2 Effects of plant growth regulators in ½ MS on somatic embryogenesis from leaf segments of *Dendrobium* Sonia 'Earsakul' with ventral surface in contact with medium after culturing for 8 weeks.

Media	Green leaf (%)	Somatic embryogenesis (%) induced from leaf segments			Number of somatic embryo per explant	
		Whole leaf	Apical segments	Basal segments	Apical segments	Basal segments
½ MS	76.92 ab	1.92	0	3.85	0	0.53 a
½ MS + 0.1 mg/L NAA	73.95 b	2.08	0	4.17	0	0.29 a
½ MS + 1.0 mg/L TDZ	93.75 a	5.36	0	10.71	0.11	0.50 a
½ MS + 3.0 mg/L TDZ	93.75 a	4.46	1.78	7.14	0.30	0.85 a
½ MS + 1.0 mg/L TDZ + 0.1 mg/L NAA	93.75 a	0.89	0	1.79	0	0.28 a
½ MS + 3.0 mg/L TDZ + 0.1 mg/L NAA	80.35 a	8.04	0	16.07	0	0.20 b
F-test	**	NS	NS	NS	NS	*

*, significant different at the 0.05 level of probability
**, significant different at the 0.01 level of probability
NS, not significant different at the 0.05 level of probability

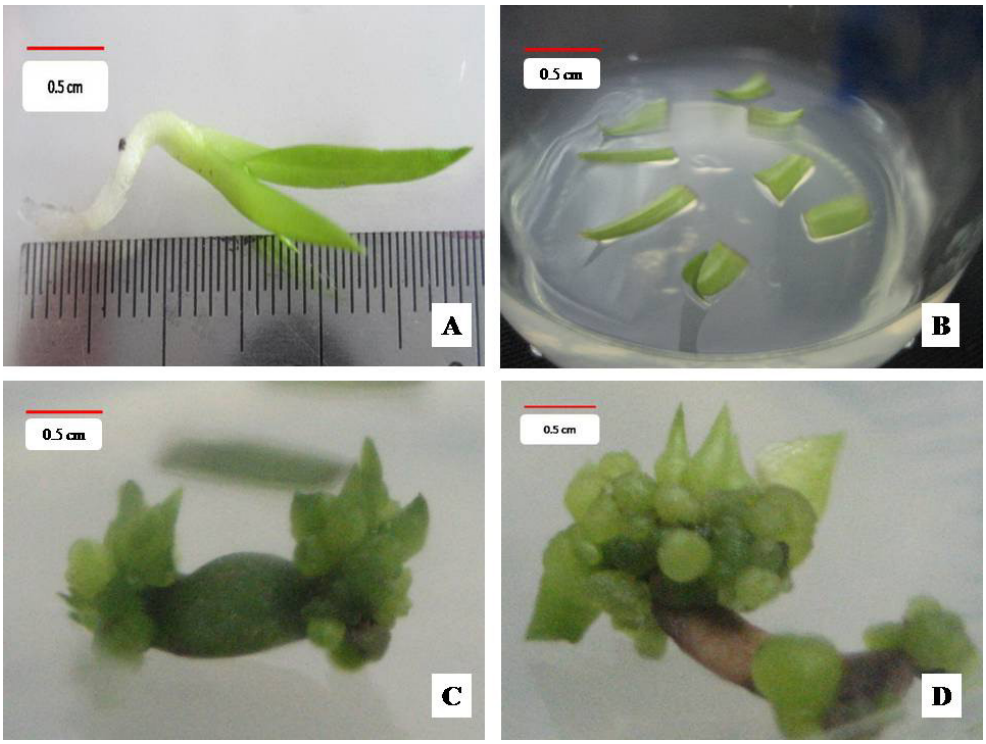

Figure 2 Somatic embryos induction from leaf explants of *Dendrobium* Sonia 'Earsakul' after culturing for eight weeks on media showed as; (A) Young leaf sized 1.0-2.0 cm to be plant material in somatic embryogenesis study; (B) Leaf splicing as apical (left) and basal (right) section and ventral side of leaf contacted with the medium; (C-D) Somatic embryogenesis directly at both cut end of leaf explants (apical and basal leaf segments showed in C and D, respectively).

Table 3 Effects of plant growth regulators in ½ MS on somatic embryogenesis from leaf segments of *Dendrobium* Sonia 'Earsakul' with dorsal surface in contact with medium after culturing for 8 weeks

Media	Green leaf (%)	Somatic embryogenesis (%) induced from leaf segments			Number of somatic embryo per explant	
		Whole leaf	Apical segments	Basal segments	Apical segments	Basal segments
½ MS	35.41 c	0 c	0 b	0	0	0
½ MS + 0.1 mg/L NAA	50.00 bc	0 c	0 b	0	0	0
½ MS + 1.0 mg/L TDZ	86.25 a	15 a	17.50 a	12.50	0.65	0.58
½ MS + 3.0 mg/L TDZ	82.50 a	2.50 bc	0 b	5	0	0.15
½ MS + 1.0 mg/L TDZ + 0.1 mg/L NAA	84.37 a	14.06 ab	12.50 ab	15.62	1.09	0.66
½ MS + 3.0 mg/L TDZ + 0.1 mg/L NAA	59.37 ab	18.75 a	18.75 a	18.75	1.59	0.56
F-test	**	**	*	NS	NS	NS

*, significant different at the 0.05 level of probability
**, significant different at the 0.01 level of probability
NS, not significant different at the 0.05 level of probability

the medium supplemented with 3 mg/L TDZ and 0.1 mg/L NAA) (at 82.50-86.25%). The lowest percentage of green leaf segments was observed either on the half-strength of MS medium (at 35.41%) or that supplemented with 0.1 mg/L NAA (at 50%).

The half-strength MS medium with different supplements had an effect to the somatic embryogenesis when the dorsal surface of leaf segments were placed on the medium (at ranged between 0-18.75%) (Table 3). The half-strength MS medium with different supplements had an effect to the somatic embryogenesis when the apical segments (at 0-18.75%) were tested but they had no effect with the basal segments (at 0-18.75%) (Table 3).

The number of somatic embryos per leaf explant in which the dorsal surface of the segments was placed on the mediums was shown in Table 3. The supplement of PGRs to the half-strength MS medium had no effect to somatic embryos when the dorsal surface of the leaf segments were placed on the medium, regardless of the types of leaf segments used in the tests (Table 3). The number of somatic embryos per leaf explant on the apical leaf segments was between 0-1.59 and that of somatic embryos per leaf explant on the basal leaf segments was between 0-0.66 (Table 3).

Discussion

There was no significant difference in percentage of green PLBs, responded PLBs and somatic embryogenesis after culturing the PLBs on half-strength MS medium supplemented either with different concentrations of TDZ either alone or in combination with 0.1 mg/L NAA or 1.0 mg/L 2,4-D (Table 1). The greenish color of the PLBs which still remained after transferring them to the media indicated that those plant tissues were not affected by culturing them on the medium and the tissue may have gone through cell differentiation.

The orchid explants may enlarge or transform to other plant structures such as PLBs, callus and primordial structure (Temjensangba and Deb, 2005). The supplement of 1-2 mg/L 2,4-D in MS medium promoted callogenesis from culturing apical meristem explants in *Dendrobium* (Anjum et al., 2006). Both 2,4-D and NAA were reported to be the suitable PGRs for callus induction from explants in orchid (Dunlap et al., 1986; Nissen and Sutter, 1990; Mei et al., 2012). Moreover, the TDZ was reported as one of PGRs, when supplemented with auxin, could induce callus formation from explant in orchid (Huan et al., 2004; Chang and Chang, 1998; Chen and Chang, 2000a; Chen and Chang, 2000b). In this study, all of the half-strength MS media contained TDZ (either in TDZ alone or in combination with

NAA or 2,4-D) showed similar effect on percentages of green PLBs, responded PLBs and somatic embryos formation. Thus, the effect of TDZ on these characteristics may supersede the effect of TDZ plus auxin. It was quite rare to observe callus formation in half-strength MS medium supplemented with 1 mg/L 2,4-D and TDZ (either at 0.1 or 0.3 mg/L TDZ) (Table 1). This observation differed from many studies which reported the effect of 2,4-D in promoting callus formation from orchrid explants (Dunlap et al., 1986; Nissen and Sutter, 1990; Anjum et al., 2006). However, the percentage of somatic embryos formation was about half of the percentage of responded PLBs.

The greenish color of the leaf explants which still remained after being transferred the leaf segments to the medium indicated that these explants may undergo morphological transformation (Table 2). Eight weeks after culturing in the induction medium, the number of green leaf explant was high (at 73.95-93.75%), which may indicate that the medium supplemented with PGRs did not cause necrosis in plant tissues.

The age of leaf explant of orchid may affect plant regeneration, in which young leaves were better than older leaves in producing PLBs (Temjensangba and Deb, 2005; Anjum et al., 2006). Temjensangba and Deb (2005) reported that leaf segments produced less PLBs than the whole leaf. However, the thin section of the explants was reported to be 10 times more efficient than the single whole leaf for PLBs induction in *Dendrobium* orchid (Anjum et al., 2006). In this study, young leaf segments (both apical and basal) of *Dendrobium* Sonia 'Earsakul' had the accumulated percentage of somatic embryos more than whole leaf (Table 2). As a result, whole leaf culture was not investigated for somatic embryogenesis.

Efficiency of explant parts of the leaf segments in forming a somatic embryo was varied. The cut ends of the leaf segment had lower number of PLBs than the other parts (Temjensangba and Deb, 2005). The thin section of orchid leaf (both basal ends and the apicals of leaves) possessed potential to proliferated (Anjum et al., 2006). However, in this study, the affect of part of segments (apical or basal segments) on the induction of somatic embryos was dependent on the orientation of leaf explants (whether ventral or dorsal surface of leaf explant in contacted with medium).

The ventral side of the leaf explant in *Oncidium* 'Grower Ramsey' was reported to be a plant part which would give somatic embryos better than the dorsal side when they were placed onto the medium (Chen and Chang, 2001). However, in this study, placing the dorsal surface of leaf explants on a medium gave higher percentage of explant forming somatic embryos than placing the ventral surface on a medium (Table 2 and Table 3).

The dorsal surface of the leaf explants placed on half-strength MS medium either without PGR or supplemented with 0.1 mg/L NAA could not initiate somatic embryogenesis. Half-strength MS medium supplemented only with (at 0.1 or 0.3 mg/L) TDZ promoted somatic embryo formation, regardless of the leaf orientation (Table 2 and Table 3). TDZ was reported to affect somatic embryo induction in leaf explant of orchid (Chen et al., 1999; Chen and Chang, 2000a; Chen and Chang, 2000b; Chen and Chang, 2001). At low concentration, TDZ was reported to have a capacity to increase new plantlet formation in orchid (Ernst, 1994; Hueteman and Preece, 1993; Jitsopakul, 2008).

The apical segment of leaf was reported to be more suitable for use to induce somatic embryo in *Oncidium* 'Grower Ramsey' (Chen and Chang, 2001). However, in this study, the effect of apical or basal segments on somatic embryo induction was not clearly observed whereas orientation of leaf explants plays an important role in somatic embryo induction. The basal leaf segments had higher value of somatic embryos when the ventral surface was placed on the medium (Table 2). This may be because the apical segment of the bending young leaves of *Dendrobium* had less surface area than the basal segment did. However, apical and basal segments of the leaf produced more or less equal number of PLBs when the dorsal surface was placed on the medium (Table 3).

Kuo et al. (2005) reported that the ventral side near the wounded regions had the highest embryogenesis in comparison with other region of the leaf explants in *Phalaenopsis*, whilst the dorsal side of the leaf explants had no observable embryogenesis. In orchids, it was reported that the ventral side of the leaf had higher embryogenesis than the dorsal side (Chen and Chang, 2001; Kuo et al., 2005). In this study, placing the dorsal surface of leaf segments on the medium initiated more somatic embryogenesis than placing the ventral surface of leaf segment on the medium. This is possibly due to the fact that when the dorsal surface of the leaf was placed on the medium, the greater contacting area to the medium may allow better nutrient absorption.

The medium supplemented with either TDZ, NAA or a combination of TDZ and NAA induced somatic embryos on the basal segments of leaf explants when both ventral and dorsal surfaces were placed on medium, except the treatment of the medium supplemented with 0.1 mg/L NAA in which somatic embryo induction was not found when dorsal surface was placed on the medium (Table 3). Either TDZ or the combination of TDZ and NAA could induce apical leaf explants to form somatic embryo when the dorsal surface was placed on the medium (Table 2 and Table 3). The influence of TDZ alone induced apical leaf explants to form somatic embryo when the ventral surface was placed on the medium (Table 2). Thus, TDZ was more effective than NAA in promoting somatic embryo formation.

Conclusion

Both PLBs and leaf explants of *Dendrobium* Sonia 'Earsakul' were induced to form direct somatic embryos on half-strength of MS medium supplemented with PGRs. The medium containing TDZ at either 0.1, 0.3 or 1.0 mg/L alone or in combinations with 0.1 mg/L NAA or 1.0 mg/L 2,4-D showed no significant difference in percentage of somatic embryo formation from PLBs.

As for the effect of explant orientation on somatic embryo formation, placing the dorsal surface of the leaf segment on medium gave a number of somatic embryos higher than placing the ventral surface on the medium. Basal segments of leaves with ventral surface of leaf segment on the medium gave percentage of somatic embryo formation and number of somatic embryo per explants higher than apical leaf segment did. However, apical and basal leaf segments had no differences in percentage of somatic embryo formation and number of somatic embryos when the dorsal surface of leaf segment was placed on the medium. TDZ was more effective than NAA in promoting the formation of somatic embryo. However, the medium containing the PGRs showed the highest percentage of somatic embryo formation and number of somatic embryo per explant, depending on parts of leaf segments used (apical or basal leaf segments) and leaf orientation on the medium (either placing ventral or dorsal surface on the medium).

Acknowledgements

We are grateful to the Silpakorn University Research and Development Institute (SURDI), Silpakorn University, Thailand for funding. We would also like to thank Faculty of Animal Science and Agricultural Technology, Silpakorn University, Phetchaburi IT campus, Cha-am, Phetchaburi, Thailand for providing laboratory facilities.

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