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Multiple Shoot Formation of *Paphiopedilum* 'Delrosi'

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***Abstract*—**Shoots, with three leaves, of *Paphiopedilum* 'Delrosi' were used as explants for multiple shoot induction. Modified Hyponex medium was supplemented with thidiazuron (TDZ), N^6-benzyladenine (BA) or kinetin (Kn) alone and in combinations with 2,4-dichlorophenoxyacetic acid (2,4-D). All explants were cultured for 15 weeks. It was found that TDZ alone at the concentration of 0.45µM or in combination with 4.52µM 2,4-D and 8.88µM BA in combination with 13.56µM 2,4-D promoted multiple shoots. The highest shoot sprouting efficiencies (80.0, 90.0 and 80.0%) and new shoot numbers (1.5, 1.3 and 1.1) were obtained, respectively. Fresh weight, height, numbers of leaf and root of new shoots and initial explants were discussed.

Keywords*—Paphiopedilum*, terrestrial orchids, *in vitro* culture, micropropagation, multiple shoot induction

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I. Introduction

PAPHIOPEDILUM, a member of the family Orchidaceae, is one of economic terrestrial orchids. *Paphiopedilum* 'Delrosi', a hybrid of *P. delenatii* × *P. rothschildianum*, is an attractive pot plant characterized by its white flower overlaid with variable amounts of raspberry color in longitudinal lines with petals and pouch painted in shade of pink [1]. Tissue culture techniques for *Paphiopedilum* have been developed for micropropagation in order to produce plants on demand in a large-scale to supply world market. Many researches on seed germination and appropriate media were reported [2]-[5]. Calli, protocorm-like bodies (PLBs), shoots and plantlets were induced from various types of explants such as seeds and seed-derived protocorms [6], [7], stem nodal explants [8], [9], leaf explants [10], shoots [9], [11]. However, there are a few reports on induction of multiple shoots from single shoots of *Paphiopedilum.*

This study reports the effects of plant growth regulators supplemented in a Hyponex culture medium on multiple shoot induction from seed-derived apical shoots of *Paphiopedilum* 'Delrosi'.

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II. Materials and Methods

A. Plant Material

Three-leaf shoots, approximately 0.354 g. of *Paphiopedilum* 'Delrosi' (*P. delenatii* × *P. rothschildianum*) (Fig. I A) without root, from *in vitro* seedlings obtained from a commercial breeder were used as explants for multiple shoot induction.

B. Media and Culture Conditions

A medium used for multiple shoot induction was Hyponex medium [11] containing 3g/L (6.5N-6P-19K) Hyponex, 100g/L potato homogenate, 2g/L peptone, 2.5g/L sucrose, 1g/L activated charcoal and 2.5g/L gelrite. The medium was supplemented with 0.45 or 2.25µM Thidiazuron (TDZ) or 4.44 or 8.88µM N^6-benzyladenine (BA) or 2.32 or 4.65µM kinetin (Kn) alone or in combinations with 4.52 or 13.56µM 2,4-dichlorophenoxyacetic acid (2,4-D). The pH of all media was adjusted to 5.4 prior to adding gelrite and autoclaving. Explants were cultured, in 240mL glass jars containing 50mL of culture medium, for 15 weeks. All cultured were incubated at 24±1°C and under a 16-h photoperiod at 35–40µmol·m^{-2}·s^{-1} provided by cool-white fluorescent lamps.

C. Statistical Analysis

Percentage of explants induced new shoots, whole fresh weights (FWs) of new shoots and original explants, numbers of new regenerated plantlets were recorded after 15 weeks. Each treatment was replicated 13 times. The completely randomized design (CRD) was used as the experimental design and data were analysed by Duncan's new multiple range test at $P = 0.05$ [12].

III. Results

A. Effects of Plant Growth Regulators Supplemented in Hyponex Medium on Multiple Shoot Formation of *Paphiopedilum 'Delrosi'* Shoots

The high percentage of explants produced multiple shoots was in the range of 80 – 90% obtained from Hyponex medium supplemented with 0.45µM TDZ (80%) or 0.45µM TDZ in combination with 4.52µM 2,4-D (90%) or 8.88µM BA in combination with 13.56µM 2,4-D (80%). New regenerated shoots were 1.5, 1.3 and 1.1 shoots, respectively (Table I; Figs. 1B, 1C, and 1D). Although the highest numbers of new shoots per explant were found from 0.45µM TDZ, the higher whole fresh weights of new regenerated shoots and number of roots per new shoot were obtained from 0.45µM TDZ in

combination with 4.52µM 2,4-D (210.8mg. and 1.3 roots) (Fig 1C) and 8.88µM BA in combination with 13.56µM 2,4-D (268.4mg. and 1.3 roots) (Fig 1D), than whole fresh weights and number of root per new shoot from 0.45µM TDZ (109.9mg and 0.6 root) (Fig. 1B). For other treatments of plant growth regulators, except for 2.32µM Kn in combination with 4.52µM 2,4-D, 10–60% of explants produced new shoots with 0.1–1.5 shoots per explants were obtained. There was no new shoot found from the medium containing 2.32µM Kn in combination with 4.52µM 2,4-D. The medium without plant growth regulator (control) gave 30% of explants produced new shoots with 0.3 new shoot per explants and 15.8mg whole fresh weights of new shoots with 0.2 root per new shoot (Table I; Fig. 1E).

B. Effects of Plant Growth Regulators Supplemented in Hyponex Medium on Growth of Original Shoot Explants of Paphiopedilum 'Delrosi'

Original shoots from all treatments were health with 6.3–7.2 green leaves and 6.2–8.3 regenerated roots (Fig. 1C) compared to control set without plant growth regulator, 6.0 leaves and 5.7 roots) (Fig. 1B, 1C and 1D). However, among the three selected treatments that provided the high regeneration of new shoots, the higher whole FWs (2129.3mg and 2714.7mg.), shoot FWs (1149.9mg and 1553.7mg.) of original shoot explants were found from medium supplemented with 0.45µM TDZ in combination with 4.52µM 2,4-D (Table I; Fig. 1C) and 8.88µM BA in combination with 13.56µM 2,4-D (Table I; Fig. 1D) than whole FW (1942.5mg) and Shoot FW (936.1mg) from medium supplemented with 0.45µM TDZ (Table II; Fig. 1D) .

TABLE I
EFFECTS OF THIDIAZURON (TDZ), 6-BENZYLADENINE (BA) OR KINETIN (KN) ALONE AND IN COMBINATIONS WITH 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D) SUPPLEMENTED IN HYPONEX MEDIUM ON MULTIPLE SHOOT FORMATION OF *PAPHIOPEDILUM* 'DELROSI' SHOOTS AFTER CULTURING FOR 15 WEEKS

Treatment (µM)				% of explant induced new shoot[1]	No. of new shoots[1]	Growth of new shoots[1]		
TDZ	BA	Kn	2,4-D			Whole FW (mg)	No. of leaves/shoot	No. of roots/shoot
-	-	-	-	30.0±15.3 bcd	0.3±0.1 cd	15.8± 10.3 cde	0.8±0.4 bcd	0.2±0.1 b
0.45	-	-	-	80.0±13.3 ab	1.5±0.3 a	109.9± 57.0 abc	2.1±0.4 ab	0.6±0.3 ab
0.45	-	-	4.52	90.0±10.0 a	1.3±0.1 ab	210.8± 57.7 a	2.5±0.4 a	1.3±0.3 a
0.45	-	-	13.56	60.0±16.3 abc	0.9±0.4 abc	60.2± 41.1 abcd	1.5±0.4 abc	0.4±0.3 ab
2.25	-	-	-	50.0±16.7 abcd	0.6±0.2 abcd	11.6± 5.0 bcde	1.0±0.3 abcd	0.1±0.1 b
2.25	-	-	4.52	50.0±16.7 abcd	0.6±0.2 abcd	95.6± 46.6 abcde	1.5±0.5 abcd	0.7±0.3 ab
2.25	-	-	13.56	10.0±10.0 cd	0.1±0.1 cd	14.1± 14.1 de	0.3±0.3 cd	0.2±0.2 b
-	4.44	-	-	60.0±16.3 abc	0.8±0.3 abc	74.0± 35.3 abcd	1.6±0.5 abc	0.5±0.3 ab
-	4.44	-	4.52	10.0±10.0 cd	0.1±0.1 cd	7.5± 7.5 de	0.3±0.3 cd	0.2±0.2 b
-	4.44	-	13.56	30.0±15.3 bcd	0.3±0.2 cd	72.4± 46.1 bcde	1.0±0.5 abcd	1.0±0.6 ab
-	8.88	-	-	40.0±16.3 abcd	1.0±0.5 abcd	103.9± 51.1 abcde	1.1±0.5 abcd	0.4±0.2 ab
-	8.88	-	4.52	50.0±16.7 abcd	0.0±0.4 abcd	182.2± 89.9 abcd	1.5±0.5 abc	0.7±0.4 ab
-	8.88	-	13.56	80.0±13.3 ab	1.1±0.2 ab	268.4± 98.8 ab	2.4±0.5 a	1.3±0.4 a
-	-	2.32	-	40.0±16.3 abcd	0.7±0.3 abcd	69.2± 36.9 bcde	1.2±0.5 abcd	0.6±0.3 ab
-	-	2.32	4.52	0.0± 0.0 d	0.0±0.0 d	0.0± 0.0 e	0.0±0.0 d	0.0±0.0 b
-	-	2.32	13.56	50.0±16.7 abcd	0.6±0.2 abcd	89.9± 59.4 abcde	1.4±0.5 abcd	0.9±0.4 ab
-	-	4.65	-	30.0±15.4 bcd	0.5±0.3 bcd	85.3± 58.3 bcde	0.8±0.4 bcd	0.4±0.3 ab
-	-	4.65	4.52	50.0±16.7 abcd	1.5±0.6 abc	319.8±111.0 abcd	1.9±0.6 abc	1.0±0.4 ab
-	-	4.65	13.56	20.0±13.3 cd	0.3±0.2 cd	36.5± 24.8 cde	0.7±0.4 cd	0.3±0.2 b

[1] Values are mean ± SE (n = 13). Means followed by the same letters within the same column are not significantly different at *P = 0.05* by Duncan's new multiple range test.

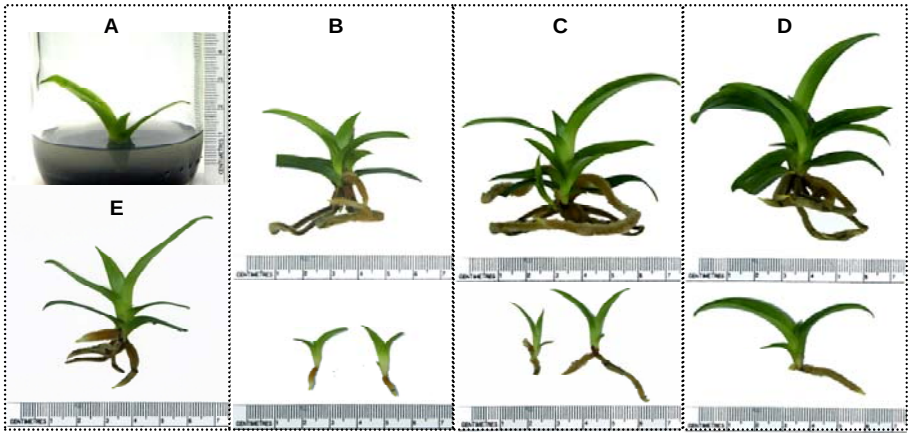


Fig. 1 Effects of plant growth regulators on multiple shoot induction of *Paphiopedilum* 'Delrosi' shoots after culturing for 15 weeks, A = an initial explant, B = 0.45 µM TDZ [the original shoot (above) and new shoots (below)], C = 0.45 µM TDZ and 4.52 µM 2,4-D [the original shoot (above) and new shoots (below)], D = 8.88 µM BA and 13.56 µM 2,4-D [the original shoot (above) and a new shoot (below)], E = control (no plant growth regulator)

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TABLE II
EFFECTS OF THIDIAZURON (TDZ), 6-BENZYLADENINE (BA) OR KINETIN (KN) ALONE AND IN COMBINATIONS WITH 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D) SUPPLEMENTED IN HYPONEX MEDIUM ON GROWTH OF *PAPHIOPEDILUM* 'DELROSI' ORIGINAL SHOOT EXPLANTS AFTER CULTURING FOR 15 WEEKS

Treatment (μM)				Growth of original shoots				
TDZ	BA	Kn	2,4-D	Whole FW (mg)	Shoot FW (mg)	Root FW (mg)	No. of leaves	No. of roots
-	-	-	-	1502.2± 98.4 h	855.2± 43.5 h	647.0±187.5 e	6.0±0.0 d	5.7±0.2 e
0.45	-	-	-	1942.5±141.0 defgh	936.1± 64.2 gh	1006.4±274.5 abcd	6.5±0.2 bcd	6.8±0.2 cd
0.45	-	-	4.52	2129.3±195.1 bcdef	1149.9±101.8 defg	979.4±322.9 abcd	7.2±0.2 ab	7.3±0.4 abcd
0.45	-	-	13.56	2571.1±122.8 ab	1572.9± 74.6 ab	998.2±180.4 abcd	6.7±0.2 abc	7.2±0.2 abcd
2.25	-	-	-	1805.8± 83.7 efgh	1073.0± 37.9 efgh	732.1±241.6 ed	6.5±0.2 bcd	6.7±0.3 cd
2.25	-	-	4.52	2276.4±139.2 abcde	1295.9± 75.0 bcde	980.5±241.0 abcd	6.9±0.2 abc	7.3±0.3 abcd
2.25	-	-	13.56	2726.4±154.3 a	1538.5± 66.5 abc	1187.9±331.4 a	6.5±0.2 bcd	7.9±0.3 ab
-	4.44	-	-	1852.4±202.2 defgh	1034.7± 76.7 efgh	817.7±457.8 cde	6.3±0.2 cd	7.0±0.4 abcd
-	4.44	-	4.52	2030.7±114.2 cdefg	1267.3± 60.0 cdef	763.4±210.1 de	6.7±0.2 abc	6.8±0.4 bcd
-	4.44	-	13.56	2742.9±253.7 a	1647.8±110.7 a	1095.1±474.1 abc	7.0±0.2 abc	7.4±0.5 abcd
-	8.88	-	-	1472.2± 89.4 h	910.0± 68.8 gh	562.2±110.3 e	6.6±0.2 abc	6.7±0.4 cd
-	8.88	-	4.52	2675.0±169.5 a	1520.7± 94.9 abc	1154.3±292.0 ab	6.6±0.4 abc	7.7±0.3 abc
-	8.88	-	13.56	2714.7±195.2 a	1553.7±122.3 ab	1161.0±298.4 ab	6.6±0.2 abc	8.3±0.5 a
-	-	2.32	-	1616.9±122.3 gh	964.1±86.5 gh	652.8±214.1 e	6.3±0.2 cd	6.4±0.3 de
-	-	2.32	4.52	2511.8±157.8 abc	1509.4± 6.1 abc	1002.4±354.8 abcd	6.7±0.2 abc	7.1±0.2 abcd
-	-	2.32	13.56	2609.4±185.7 ab	1500.4±104.8 abc	1109.0±300.7 abc	7.2±0.2 ab	7.9±0.3 a
-	-	4.65	-	1754.4±160.8 fgh	1013.0± 80.9 fgh	741.4±350.8 de	6.7±0.2 abc	6.2±0.4 de
-	-	4.65	4.52	2160.4±180.8 bcdef	1302.5±131.4 bcde	857.9±219.5 bcde	6.3±0.2 cd	7.5±0.3 abc
-	-	4.65	13.56	2324.7±145.9 abcd	1369.3± 93.3 bcd	955.4±206.4 abcd	7.2±0.1 a	7.1±0.2 abcd

[1] Values are mean ± SE (n = 13). Means followed by the same letters within the same column are not significantly different at $P = 0.05$ by Duncan's new multiple range test.

IV. DISCUSSIONS

In general, seeds are still commonly used for micropropagation of *Paphiopedilum* orchids due to multiplication rate from shoot explants is very low. Slow growth and low multiplication rate are the important limiting factor of *in vitro* culture of slipper orchids. For *in vitro* culture media used, a modified half-strength Murashige and Skoog (MS) medium [13] and a modified MS medium were used for *Paphiopedilum* orchids [6], [8]-[10] while a quarter-strength MS medium and Hyponex-peptone medium were used for *Cyperidium* orchids [14], [15].

For plant growth regulations used, Huang et al. reported that a modified MS medium supplemented with 13μM BA in combinations with 1.6μM 1-naphthaleneacetic acid (NAA), 0.15μM adenine sulfate was suitable for promoting shoot and root formation from apical shoots of *Paphiopedilum* hybrids while TDZ inhibited shoot proliferation and rooting [16]. However, Chen et al. [8] presented that 0.45μM TDZ and 4.52μM 2,4-D supplemented in a modified half-strength MS medium enhanced the percentage of explants produced new regenerated shoots from stem nodal explants (66.7 and 80%) of *Paphiopedilum philippenense* hybrids. For numbers of regenerated shoots per explant, the combination of 0.45μM TDZ with 4.52μM 2,4-D and 0.45μM TDZ alone were appropriate for hybrid PH59 and PH60 with 1.6 and 7 new shoots, respectively [8]. The similar results were found from this study. The growth of shoots was very slow and numbers of new regenerated shoots were limited with the low rate of regeneration. The suitable medium, types and concentrations of plant growth regulators varied depending on types of explants, species and hybrids.

V. CONCLUSIONS

The most effective growth regulator for inducing multiple shoots of *Paphiopedilum* 'Delrosi' was TDZ at the low concentration (0.45 μM) supplemented in Hyponex medium.

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