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Full Length Research Paper

In vitro propagation of orchid (*Dendrobium nobile*) *var.* Emma white

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Axillary buds of orchid *Dendrobium nobile* var. Emma white were proliferated by using phytotechnology medium (O753) supplemented with 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mgl^{-1} of benzylaminopurine (BAP) and kinetin (Kin) as well as coconut water (CW) at the rate of 50, 100, 150, 200, 250 and 300 ml. Maximum number of shoots (4.33), as well as fresh and dry weights (752.5 and 52.99 mg) were obtained at 2 mgl^{-1} BAP, while 1.5 mgl^{-1} of Kin exhibited the highest shoot length (4.18 cm). Higher concentrations of BAP, Kin (3.0 mgl^{-1}) and CW (300 ml) resulted in yellowing, necrotic shoots and poor growth. Root induction was carried out by using two auxins namely indolbutyric acid (IBA) and naphthalene acetic acid (NAA) at different concentrations (0.5, 1, 1.5, 2.0, 2.5 and 3.0 mgl^{-1}) on the modified MS medium. IBA at a level of 2 mgl^{-1} increased the rooting percentage (97.5%) number of roots (4.70) and root length (3.47 cm) more efficiently than NAA. Higher concentrations of IBA and NAA (3.0 mg l^{-1}) showed poor results of rooting.

Key words: Orchids, *in vitro* propagation, coconut water, *Dendrobium nobile*.

INTRODUCTION

Orchids are commercially grown worldwide as cut flower and potted plants with 8% share in floriculture trade. It is considered outstanding in the ornamentals due to its diverse colors, shapes, forms and long lasting blooms (Toukuhara and Mii, 2001). Among orchids, *Dendrobium* occupies a foremost position with marvelous varieties like Emma white, which is admirable for purity of blooms and prolonged shelf life (Vendrame, 2008). It is propagated by seed, division of clumps or rhizomes, cuttings, separation of offshoots and keikis produced from the stem or pseudobulbs. The major constraints in its conventional propagation are slow in clonal multiplication and in provision of insufficient clones within a short time frame (Martin and Madassary, 2006). Hence, *in vitro* propagation is used as an alternative for rapid mass multiplication of the valuable varieties, in that it has progressed well during last decades and preferably has been used in recent years in developed countries. *In vitro* propagation of *Dendrobium nobile* is successfully carried out through the use of appropriate plant medium and growth regulators (Aktar et al., 2008).

Tissue culture techniques are ultimately an effective solution for the mass multiplication of *D. nobile* in a short time span (Malabadi et al., 2005). Nayak et al. (2002) used zeatin riboside (ZR), N^6- benzyladenine (BA) and kinetin (Kin) to induce shoot proliferation. Similarly, Malabadi et al. (2005) presented an idea for mass multiplication of *D. nobile* by using different concentrations (11 to 15 µM g/l) of triacontanol. Aktar et al. (2008) supplemented ½ MS and KC medium with banana pulp to compare their effects on the growth of *D. nobile*. However, *in vitro* propagation of orchid is highly dependant on the choice of genotypes (Islam et al., 2003). Establishment of a reliable cloning methodology for this orchid is important in terms of enabling the rapid propagation and production of a large number of high quality plants. Therefore, the present study was designed to establish the protocol for *in vitro* shoot proliferation and rooting of fascinating orchid var. Emma white. The development of protocols for extensive and clonal multiplication of highly priced varieties of Dendrobium will serve as a foundation for commercial scale propagation.

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Abbreviations: BAP, Benzylaminopurine; **Kin,** kinetin; **CW**, coconut water; **IBA**, indolbutyric acid; **NAA,** naphthalene acetic acid.

Table 1. Effect of different durations of NaOCl application on infection and survival percentage of cultured axillary buds after three weeks.

NaOCl solution (% v/v)	Time of sterilization (minute)	Necrosis (%)*	Infection (%)*			Survival (%)*
			Bacterial	Fungal	Total	
10	6	0	47.5	40	87.5	12.5
	8	5	42.5	30	72.5	22.5
	10	12.5	35	32.5	67.5	20.5

40, explants/treatment

It will lead to its production in Pakistan, and a substantial reduction in its import and saving foreign exchange as well.

MATERIALS AND METHODS

Lateral shoots of orchid *Dendrobium nobile* var. Emma white, about 8 cm long, arising from the base of the stem of healthy plants, maintained in the Glass House were isolated and placed under running tap water for half an hour to remove dust and other foreign particles. After washing, 1 to 1.5 cm long cuttings containing axillary buds were prepared from these lateral shoots by removal of the surrounding leaves and sheath. Disinfection of axillary buds was carried out by using 10% (v/v) sodium hypochlorite (NaOCl) solution (active chlorine 6 to 14%) for 6, 8 and 10 min with continuous agitation followed by 4 to 5 thorough washings with distilled autoclaved water. Shoot segments having axillary buds were given a final size by trimming up to 1 cm with subsequent inoculation in test tubes (1 plant per test tube), containing phytotechnology medium (O753) under aseptic conditions. Observations were taken after three weeks for necrosis, infection (fungal and bacterial) and survival percentage. For shoot proliferation, axillary buds were transferred to culture jars (5 plants per jar) containing shoot proliferation media, via phytotechnology medium (O753) supplemented with varying concentrations of BAP, Kin and CW (Table 2). The pH of the media was adjusted to 5.5 before autoclaving at 121°C for 15 min. Data were recorded after six weeks using the following parameters:

Number of shoots per explant

Number of shoots per explant was observed through visual observation.

Shoot length (cm)

The data of shoot length (cm) were taken by a measuring scale.

Fresh and dry weights of shoots (mg)

Fresh weight was taken by using electrical weighing balance followed by keeping the shoots at a temperature of 60°C for 48 h apiece dry weight. For rooting, proliferated shoots (2 cm in length) with 2 to 3 expanded leaves were detached from the shoot clumps. These shoots were transferred to the modified MS medium (Murashige and Skoog, 1962; Arditti, 2008), supplemented with different concentrations of IBA and NAA (Table 3). The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 15 min. The experiment was complete randomize design with four replications per treatment and ten explants per replication. Data were recorded after six weeks regarding rooting percentage, number of roots and length of roots (cm). All cultures were incubated at 25 ± 1°C under 16/8 h of photoperiod (2,000 lux) with white fluorescent tubes (Philips TL 40 W/54).

Statistical analysis

The difference among treatment means was compared by least significance difference test (LSD) at 5% probability level (Steel et al., 1997).

RESULTS

Disinfestations of explant

Maximum survival percentage of cultures (22.5%) with limited necrosis (5%) and infection (72.5 %) was observed when sterilization of axillary buds was done for 8 min with 10% (v/v) NaOCl when compared to that for 6 and 10 min (Table 1). The comparison of 8 and 10 min NaOCl treatment shows that the former was relatively better in terms of survival percentage and reduced necrosis.

Shoot number and shoot length (cm)

Effect of different concentrations of BAP, Kin and CW varied significantly with the number and length of shoots per explant (Table 4). BAP yielded maximum shoot number (4.33) but reduced length of shoots (3.52 cm) at 2 mgl^{-1} (T_4). Among different concentrations of CW, higher number (3.42) and length of shoots (4.03 cm) was produced at 100 ml l^{-1} (T_{14}), whereas Kin provided minimum shoot number (2.45), but the length of the resulted shoot was higher (4.18 cm) than that of CW and BAP at 1.5 mg l^{-1} (Figure 1). Increase in the concentration of BAP, Kin and CW have a promoting effect for both number and length of shoots up to a certain level, while the results obtained at control (leaf number, 1.65 cm; leaf length, 1.68 cm) and higher concentrations (T_6, T_{12} and T_{18}) were found to inhibit the formation of shoots.

Table 2. Variable concentrations of BAP, Kin and CW used for shoot proliferation of *D. nobile* var. Emma white.

Treatment	Concentration (mg l^{-1})
T_0	(0.0)
BAP	
T_1	(0.5)
T_2	(1.0)
T_3	(1.5)
T_4	(2.0)
T_5	(2.5)
T_6	(3.0)
Kin	
T_7	(0.5)
T_8	(1.0)
T_9	(1.5)
T_{10}	(2.0)
T_{11}	(2.5)
T_{12}	(3.0)
CW (ml l^{-1})	
T_{13}	50
T_{14}	100
T_{15}	150
T_{16}	200
T_{17}	250
T_{18}	300

$LSD_{5\ \%}$: means followed by the same letter are not significantly different at $p < 0.05$. **BAP,** Benzylaminopurine; **Kin,** kinetin; **CW,** coconut water.

Table 3. Variable concentrations of IBA and NAA used for rooting of *D. nobile* var. Emma white.

Treatment	Concentration (mg l^{-1})
T_0	(0.0)
IBA	
T_1	(0.5)
T_2	(1.0)
T_3	(1.5)
T_4	(2.0)
T_5	(2.5)
T_6	(3.0)
NAA	
T_7	(0.5)
T_8	(1.0)
T_9	(1.5)
T_{10}	(2.0)
T_{11}	(2.5)
T_{12}	(3.0)

$LSD_{5\ \%}$: means followed by the same letter are not significantly different at $p < 0.05$. **IBA,** indolbutyric acid; **NAA,** naphthalene acetic acid.

Shoot fresh and dry weight (mg)

Highest fresh weight (752.5 mg) and dry weight (52.99 mg) was recorded on the medium supplemented with 2 mg l^{-1} BAP (T_4), followed by 660.9 and 46.47 mg fresh and dry weight, respectively at 1.5 mg l^{-1} BAP (T_3) (Table 4). Comparatively, higher fresh weight (566.3 mg) obtained on T_{14} containing 100 ml l^{-1} of CW was followed by 562.7 mg fresh, as well as 38.7 mg dry weight, on the medium fortified with Kin (T_9). Contrarily, higher concentration of both cytokinins and CW showed an inhibiting effect. Control (T_0) did not revealed any effective response both on the fresh (314.5 mg) and dry weight (28.9 mg) of proliferated shoots as compared to optimum concentra-tions of BAP, CW and Kin.

Rooting percentage (%)

IBA gave a better rooting percentage (97.5%) at 2 mg l^{-1}(T_4), followed by 85.0% at 1.5 mg l^{-1} NAA (T_9) (Table 5).

The minimum rooting percentage was recorded in the control as compared to other treatments containing different levels of auxins. Gradual increase in rooting percentage was observed with an increase in auxin concentration, but higher levels showed a declining trend than optimum levels.

Root number and root length (cm)

Comparatively, the highest number (4.70) and length (3.47 cm) of roots was observed at 2 mg l^{-1} IBA (T_4) (Table 5), while the highest number (3.30) and length (2.25 cm) of roots in NAA was found at 1.5 mg l^{-1} (T_9) (Figures 2a and b). Poor results (1.40) were produced at control (T_0) than certain specific concentrations of auxins with less number as well as length of roots (Figure 2c). Minimum as well as maximum concentrations of both auxins produced inferior results than the optimal level.

DISCUSSION

Disinfestations of explant

NaOCl is widely applied to orchid explants and seed for disinfestations because of its oxidizing nature to kill the microorganisms. It is useful in reliable plant sterilization as it is inexpensive and an easily available chemical

Table 4: Effect of different concentrations of BAP, Kin and CW on number of shoots, length (cm) and fresh as well as dry weights (mg) of orchid *Dendrobium nobile var*. Emma White.

Treatment (mg l^{-1})	Number of shoots per Explant	Shoot length of Proliferated (cm)	Fresh Weight Shoots (mg)	Dry Weight Shoots (mg)
T_0 (0.0)	1.65 kl	1.68 k	314.5 r	28.5 n
BAP				
T_1 (0.5)	2.78 d	2.87 i	513.8 l	41.90 d
T_2 (1.0)	3.03 c	3.08 h	523.5 k	43.22 c
T_3 (1.5)	3.50 b	3.45 g	660.9 b	46.47 b
T_4 (2.0)	4.33 a	3.52 f	752.5 a	52.99 a
T_5 (2.5)	2.50 e	2.84 i	450.9 n	35.0 j
T_6 (3.0)	1.90 hi	2.73 j	422.1 o	31.23 l
Kin				
T_7 (0.5)	1.75 jk	3.83 c	546.8 g	35.88 ij
T_8 (1.0)	1.98 h	4.03 b	554.8 f	37.35 gh
T_9 (1.5)	2.45 e	4.19 a	562.7 d	38.17 fg
T_{10} (2.0)	1.80 ij	3.66 e	540.3 h	36.00 ij
T_{11}(2.5)	1.55 lm	3.48 fg	534.8 i	35.25 j
T_{12} (3.0)	1.45 m	3.07 h	526.0 j	35.00 j
CW(ml l^{-1})				
T_{13} (50)	3.10 c	3.89 c	556.5 e	39.08 ef
T_{14} (100)	3.42 b	4.03 b	566.3 c	39.88 e
T_{15} (150)	2.68 d	3.73 d	489.5 m	36.35 hi
T_{16} (200)	2.55 e	3.68 de	415.8 p	32.75 k
T_{17} (250)	2.30 f	3.64 e	371.3 q	30.33 lm
T_{18} (300)	2.13 g	2.81 i	305.3 s	29.40 mn
LSD $_{5\%}$	0.109	0.063	0.622	1.076

Means followed by the same letter are not significantly different at $p < 0.05$

Table 5: Effect of different concentrations of IBA and NAA on rooting percentage (%), root number and length of orchid *Dendrobium nobile var*. Emma White.

Treatments (mg l^{-1})	Rooting percentage (%)	Number of Roots	Length of Roots (cm)
T_0 (0.0)	35 i	1.40 i	1.07 j
IBA			
T_1 (0.5)	75.0 cd	2.52 e	1.92 fg
T_2 (1.0)	82.5 bc	3.10 cd	2.54 c
T_3 (1.5)	95.0 b	3.72 b	2.96 b
T_4 (2.0)	97.5 a	4.70 a	3.47 a
T_5 (2.5)	72.5 de	2.90 d	2.24 de
T_6 (3.0)	65.0 ef	2.32 ef	1.59 h
NAA			
T_7 (0.5)	52.5 gh	2.02 gh	1.85 g
T_8 (1.0)	65.0 ef	2.35 ef	2.10 de
T_9 (1.5)	85.0 c	3.30 c	2.25 d
T_{10} (2.0)	57.5 fg	2.25 fg	2.07 ef
T_{11} (2.5)	52.5 gh	1.90 h	1.65 h
T_{12} (3.0)	47.5 h	1.55 i	1.42 i
LSD $_{5\%}$	7.680	0.230	0.163

Means followed by the same letter are not significantly different at $p < 0.05$

Figure 1. (a) Highest length (4.18 cm) at 1.5 mg l^{-1} Kin (T_9); (b) reduced length (3.52 cm) at 2.0 mg l^{-1} BAP (T_4) and (c) poor results (1.68 cm) at control (T_0).

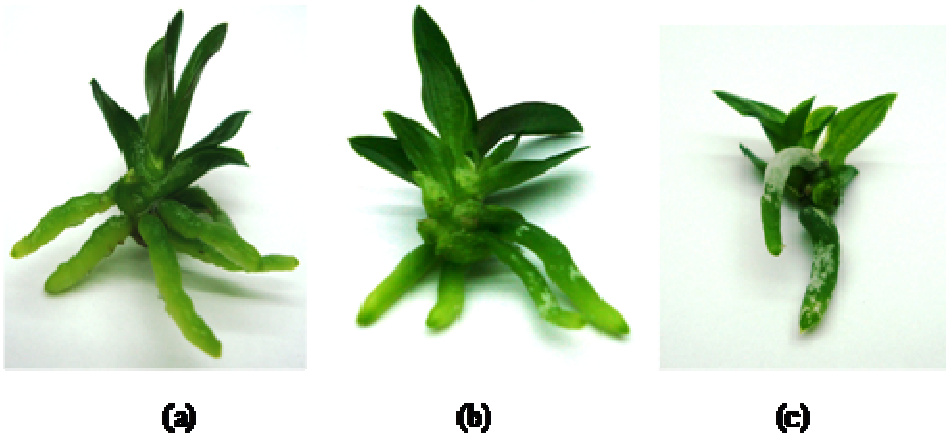

Figure 2. Number of roots per explant. (a) 4.70 at 2 mg l^{-1} IBA (T_4); (b) 3.30 at 1.5 mgl^{-1} NAA (T_9) and (c) 1.40 at control (T_0).

(Canli and Kazaz, 2009). Moreover, contaminations are not controlled properly if NaOCl is applied for a shorter time period. Bacterial and fungal infections can be eliminated accurately by proper immersion time of explants in the sterilization agent (Yildiz and Er, 2002). Furthermore, Canli and Kazaz (2009) reported that the time and concentration of NaOCl are equally important to produce sterile plants in order to eliminate contaminants from the plant surface.

Shoot number and shoot length (cm)

The type and concentration of growth regulators are an initial consideration for micropropagation of orchid species (Genkov and Ivanova, 1995). Addition of cytokinin to the medium increase the number of shoots which demonstrates the significance of exogenous cytokinin to enhance the multiple shoots. Among the BAP, CW and Kin used for shoot proliferation, BAP influences shoot proliferation by stimulating quick cell divisions to induce large number of multiple shoots (Yakimova et al., 2000; Roy and Banerjee, 2002; Ronzhina, 2003). Results are also supported by the findings of Roy and Banerjee (2002) who reported that it enhances the shoot multiplication more actively than Kin. BAP provided smaller lengths of proliferated shoots in contrast to shoots number. Being a strong cytokinin, it depresses shoot length by an increase in number of axillary buds (Hameed et al., 2006). Yakimova et al. (2000) demon-

strated that the culture plants producing large number of shoot buds exhibit minimum shoot length because all the nutrients are utilized for the formation of lateral shoots.

The treatments (T_{15}) containing CW gave high number of shoots after BAP (T_4). Similar to the function of plant growth regulators, growth additives are also used in the culture medium to enhance the frequency and number of new forming shoot buds (Akhtar et al., 2008). CW is cost-effectively employed for the micropropagation of imperative species of orchids due to its endless benefits (Peixe et al., 2007). It is a natural growth promoter which contains higher levels of zeatin, zeatin riboside, 1,3-diphenylurea (contains cytokinin-like activity), auxins, nitrogenous compounds, inorganic elements, organic acids, sugars and their alcohols, peptides, vitamins, amino acids and many other unknown components in its composition (Tokuhara and Mii, 2001; Nasib et al., 2008). George et al. (2008) demonstrated that physiologically, active substances present in CW promote the cell divisions which further enhance shoot multiplication. Amino acids increase the number of shoots by inducing cell divisions, and more than ten natural N^6- substitute adenine compounds, along with zeatin present in the CW, may be involved in cell division tended towards multiple shoot formation (Mohr et al., 1995).

CW (T_{15}) contributed the second highest results for shoot length after Kin (T_9). It acts as a complex multi-functional growth promoter which influences the growth parameters like shoot number and length due to presence of organic and inorganic elements in it (Nasib et al., 2008). Akhtar et al. (2008) also reported that it collectively stimulates shoot bud formation and plantlet growth, whereas Malik et al. (2000) observed that Kin cannot perform well for induction of multiple shoots because of nutrient diversion towards shoot apex. However, Roy and Banerjee (2002) found a production of less number of shoots in carnation on the media containing Kin.

In the present study, Kin (T_9) showed the best elongated shoots. As such, the results coincide with the findings of Sirchi et al. (2008) that Kin actively stimulates the nutrient mobilization from source to sink areas due to which it act as a triggering element to increase the length of microshoots. Higher concentrations of BAP, CW and Kin than the specified limit of concentration caused a reduction in their number and in their length of shoots. Roy and Banerjee (2003) reported that application of exogenous cytokinins at supra optimal levels cause inhibitory impacts on shoot length and number.

Shoot fresh and dry weight (mg)

Fresh and dry weight, due to biomass accumulation of cytokinin, increase number of leaves, shoots and shoot length by stimulating cell division and elongation through nutrient mobilization (Peres et al., 2001). In the present study, Kn (T_9) did not contribute obvious results in increasing fresh and dry weights of proliferated shoots than BAP (T_4) and CW (T_{16}). According to Vulysteker et al. (1997), fresh weight is dependant on the shoot number and it might decrease due to reduced number of shoots. Bennet et al., (1994) demonstrated that less fresh weight might be due to the reason that Kin shows less effectiveness than BAP in order to trigger the enzymes responsible for enhancement of vegetative growth. Supra-optimal concentrations of BAP, Kin and CW illustrated the deleterious effects of and reduced the fresh as well as dry weights of proliferated shoots. Higher concentrations of cytokinins are responsible for elevating the ethylene production causing senescence in plant tissues, and it subsequently gives smaller fresh weights (George et al., 2008).

Rooting percentage (%)

Auxins are considered as the efficient plant growth regulators which accelerate the processes of root induction and development by differentiation of vascular bundles. These auxins may not have direct effect on the development of shoots, but may be effective mostly through induction of roots (Husen and Pal, 2007). They promote rooting in the plants through changes in the biochemical systems of the plants (Henrique et al., 2006). Among various auxins, IBA is known to stimulate rooting more efficiently due to its weak toxicity and greater stability for induction of roots (Han et al., 2009). Liu et al. (2002) described that IBA is physiologically a more active auxin than NAA and IAA in promoting the root initiation as it acts as a precursor for endogenous IAA.

Root number and root length (cm)

Auxin application to microshoots is said to intensify the number of adventitious roots by increasing the level of endogenous contents of enzymes. They are considered to have an increased effect on cell division, elongation and differentiation (Husen and Pal, 2007). Han et al. (2009) revealed that auxins induces the sprouting of shoot buds which then stimulates the growth substances present in the roots for their growth and elongation. Auxin application induces the complex processes of lateral root formation through repetitive cell divisions (Liu et al., 2002). George et al. (2008) reported their significance that they are considered fundamental for the establishment and maintenance of polarity of the plants and its organs (vascular system). NAA produced less number of roots than IBA probably due to the reason that NAA accumulates and cannot be rapidly catabolized particularly at higher levels than that of IBA (Vuylsteker et al., 1997). In contrast to these results, Rout (2006) quoted that NAA promotes the number of roots by enhancing cell division

in the root primordial.

Higher concentrations of IBA and NAA were found to have a reduced number and length of roots than the optimum level. Ozel et al. (2006) reported that higher levels of IBA, applied to plants, inhibit the formation of shoot buds and this might further stop the production of roots as the auxin in the root primordial is shifted from the shoot apex. IBA yielded the good results of root number because it is very effective to increase endogenous auxin contents and show higher stability against catabolism and inactivation by conjugation with growth inhibitors (George et al., 2008).

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