Journal of Genetic Engineering and Biotechnology (2015) 13, 227–233

HOSTED BY

ELSEVIER

Academy of Scientific Research & Technology and National Research Center, Egypt

Journal of Genetic Engineering and Biotechnology

www.elsevier.com/locate/jgeb

ARTICLE

In vitro regeneration from protocorms in *Dendrobium aqueum* Lindley – An imperiled orchid

Selvaraju Parthibhan [a,1], **Mandali Venkateswara Rao** [a,1], **Thiruppathi Senthil Kumar** [b,*]

[a] *Department of Plant Science, Bharathidasan University, Tiruchirappalli 620 024, Tamil Nadu, India*
[b] *Department of Industry University Collaboration, Bharathidasan University, Tiruchirappalli 620024, Tamil Nadu, India*

Received 27 October 2014; revised 3 June 2015; accepted 4 July 2015
Available online 21 July 2015

KEYWORDS

Dendrobium aqueum;
Protocorms;
Plant growth regulators;
Natural additives;
Hardening

Abstract An efficient *in vitro* plant regeneration protocol from protocorms of *Dendrobium aqueum* was developed. The uniformly developed protocorms (*in vitro* origin) having shoot initials were cultured on half macro strength MS medium (1/2 MS) supplemented with cytokinins (BA, 2iP, KIN and TDZ) at 1, 3, 5, 7, 10 mg l^{-1}, natural additives (BP and CW) at 1%, 3%, 5%, 7%, 10% and auxins (IBA, NAA, 2,4-D) at 1, 3, 5, 7, 10 mg l^{-1} to study their efficacy on complete plant development. A maximum of 9.4 shoots per explant were generated on 3 mg l^{-1} of NAA followed by 3% of BP (7.0 shoots). Shoot elongation (1.52 cm) was achieved on 1/2 MS medium fortified with NAA 7 mg l^{-1} followed by TDZ 7 mg l^{-1} (1.37 cm). Shoots cultured on 1/2 MS medium supplemented with IBA 5 mg l^{-1} produced an average of 8.75 roots per shoot, however the lengthiest roots (1.48 cm) were noted in NAA 7 mg l^{-1}. Healthy rooted plantlets successfully acclimatized in *ex vitro* condition. The role of complete plantlet production by natural additives could be useful for conservation and cost effective commercial production of orchids.

1. Introduction

The genus *Dendrobium* is known for its commercial value as cut flowers in most countries and as traditional medicine of China and India. India has traded floriculture products including orchids worth Rs. 455.90 crores in the year 2013–14 to USA, Netherlands, Germany, UK, Japan, Canada, Japan and other countries (Agricultural & processed food products export development authority, India – APEDA 2013). Several ethno medicinal properties (*viz*. anti-diabetic, anti-pyretic, immune regulatory, anti-cancer, cure to skin disease, anxiety, panic and stomach ache) and important phyto chemicals (*viz*. benzyl derivatives, phenanthrene derivatives, alkaloids, flavonoids, pigments, sesquiterpenoids, anti-tumour, anti-mutagenic and anti-pyretic) have been recorded and reviewed in many *Dendrobium* species [9] and [44].

Abbreviations: MS, Murashige and Skoog (1962); BP, banana pulp; CW, coconut water; PGR, plant growth regulators.

* Corresponding author. Tel.: +91 9442156480.
E-mail addresses: thibhan@gmail.com (S. Parthibhan), mvrao_456@yahoo.co.in (M.V. Rao), senthil2551964@yahoo.co.in (T. Senthil Kumar).
[1] Tel.: +91 9442147460, +91 9788522895.

Peer review under responsibility of National Research Center, Egypt.

http://dx.doi.org/10.1016/j.jgeb.2015.07.001

Dendrobium aqueum Lindley is an epiphytic orchid endemic to Eastern Ghats of south India [26]. It is a sympodial orchid with clavate stems, leaves are ovate to lanceolate in shape and acuminate. The flowers are white to faintly yellowish in colour and the fruits are persistent. Continuous pressure of forest degradation, deforestation, shifting cultivation, tree cutting, lopping, biological invasion and indiscriminate exploitation are of the major threats to Eastern Ghats [39]. Therefore, orchid populations have been under threat in their habitat in addition to their inherent lower germination rate due to the absence of nutritive endosperm with uneven climate change. Incidentally *D. aqueum* is one of the victims placed under near threatened category of IUCN [12]. *In vitro* propagation of this species has been studied through asymbiotic seed germination of immature and mature pod seeds [50,36] and [40]. However, mass multiplication can be achieved through micropropagation from *ex vitro* and/or *in vitro* explants which would be very useful to restoration of valuable and RED listed orchids in nature. Efficient protocols for mass propagation through micropropagation have been developed to conserve many orchid species including *Dendrobium*, *Vanilla*, *Cypripedium*, *Coelogyne*, *Geodorum*, *Ipsea*, *Anoectochilus* etc., through different explants [44]. Regeneration of shoot buds or protocorms like bodies (PLBs) and plantlet development in *in vitro* necessitate an exogenous supply of auxins and/or cytokinins for many orchid species [1].

Moreover, individual treatments of cytokinins, auxins and natural supplements have been recorded to be the most important factors to promote and improve the plant development from PLBs [34,32,24,22]. Hence, in order to obtain an efficient regeneration system with high frequency, *in vitro* seed derived protocorms with leafy shoot initials were experimented on cytokinins, natural additives and auxins either individually or in combination.

2. Materials and methods

2.1. Plant material

Protocorms with leafy shoot initials raised from *in vitro* green pods seeds of *D. aqueum* cultured on half macro strength Murashige and Skoog (1/2 MS) basal medium [30], were used as the explants [36]. As the protocorm explants were obtained through *in vitro* cultures, no sterilization was required.

2.2. Effect of plant growth regulators and additives on shoot growth

Protocorms were inoculated on 1/2 MS medium supplemented discretely with 1.0, 3.0, 5.0, 7.0 and 10.0 mg l^{-1} of cytokinins [6-benzylaminopurine (BA), N6-(2-isopentyl) adenine (2iP), kinetin (KIN) and Thiadiazuron (TDZ)], 1.0, 3.0, 5.0, 7.0 and 10.0% (w/v and v/v) of natural additives [banana pulp (BP) and coconut water (CW)] and 1.0, 3.0, 5.0, 7.0 and 10.0 mg l^{-1} of auxins [Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), a-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and picloram (PIC)] for shoot growth. Natural additives, fresh bananas dialectally termed 'Poovan' and green tender coconuts were procured from Indian local market. Skin peeled bananas sliced according to the required weight and completely crushed using pestle and mortar on media preparation.

Further to determine the synergistic effect of the best responded PGRs in multiple shoot formation, explants were cultured on 1/2 MS medium containing 3.0 mg l^{-1} NAA in combination with 1.0, 3.0, 5.0, 7.0 and 10.0 mg l^{-1} of TDZ and 2iP.

2.3. Culture condition

The basal 1/2 MS medium was fortified with 2% sucrose, 100 mg l^{-1} myo-inositol and was solidified with 0.7% (w/v) agar. The pH was adjusted to 5.7 ± 0.2 either with 0.1 N NaOH or HCl prior to autoclaving at 120 °C and 105 kPa for 15 min. All cultures were aseptically maintained at 25 ± 2 °C under a 16/8 h (light/dark) photoperiod with a light intensity of 40 μmol m^{-2} s^{-1} by white fluorescent light.

2.4. Histological observation

Explants with PGR induced multiple shoot initials were fixed in FAA solution (50% ethanol: 40% formalin: acetic acid, 90:5:5 v/v/v) and directly taken to hand sectioning [13]. Dehydration in ethanol series was neglected due to severe level of tissue shrinkage. The fixed tissues were manually sectioned longitudinally using several fine blades and immediately stained with 1% safranin. The handmade fine, clear sections were then observed under a triangular compound microscope (Kyowa, Tokyo) and photographed using Full HD Cybershot Sony camera.

2.5. Experimental design and data statistical analysis

Protocorm explants were cultured in 100 ml culture bottles with five explants, each treatment comprised of three bottles and the experiment was repeated twice. Regular observations of cultures were made once a week. Total number of protocorms responding with healthy shoots was recorded after 5 weeks and the total number and length of shoots as well as roots were recorded after 15 weeks of culture. The percentage of the explants regenerating a shoot and the average shoot number and length were calculated by the following formulas:

$$\text{Percentage of response} = \frac{\text{Total number of explants with a shoot}}{\text{Total number of explants cultured}} \times 100$$

$$\text{Average number or length of shoot/root} = \frac{\text{Total number of } \frac{\text{shoots}}{\text{roots}} \text{ or sum of length of } \frac{\text{shoots}}{\text{roots}}}{\text{Total number of explants cultured}}$$

The average number, length of shoots and roots were analysed statistically using one-way analysis of variance (ANOVA), and the mean ± standard error (S.E.) of triplicates were represented and compared using Duncan's multiple range test at 5% level of significance using SPSS-PASW statistic program software version 18.0.0.

2.6. Acclimatization

Well rooted plantlets with four to five fully expanded leaves were hardened on 1/2 MS basal medium without sucrose for

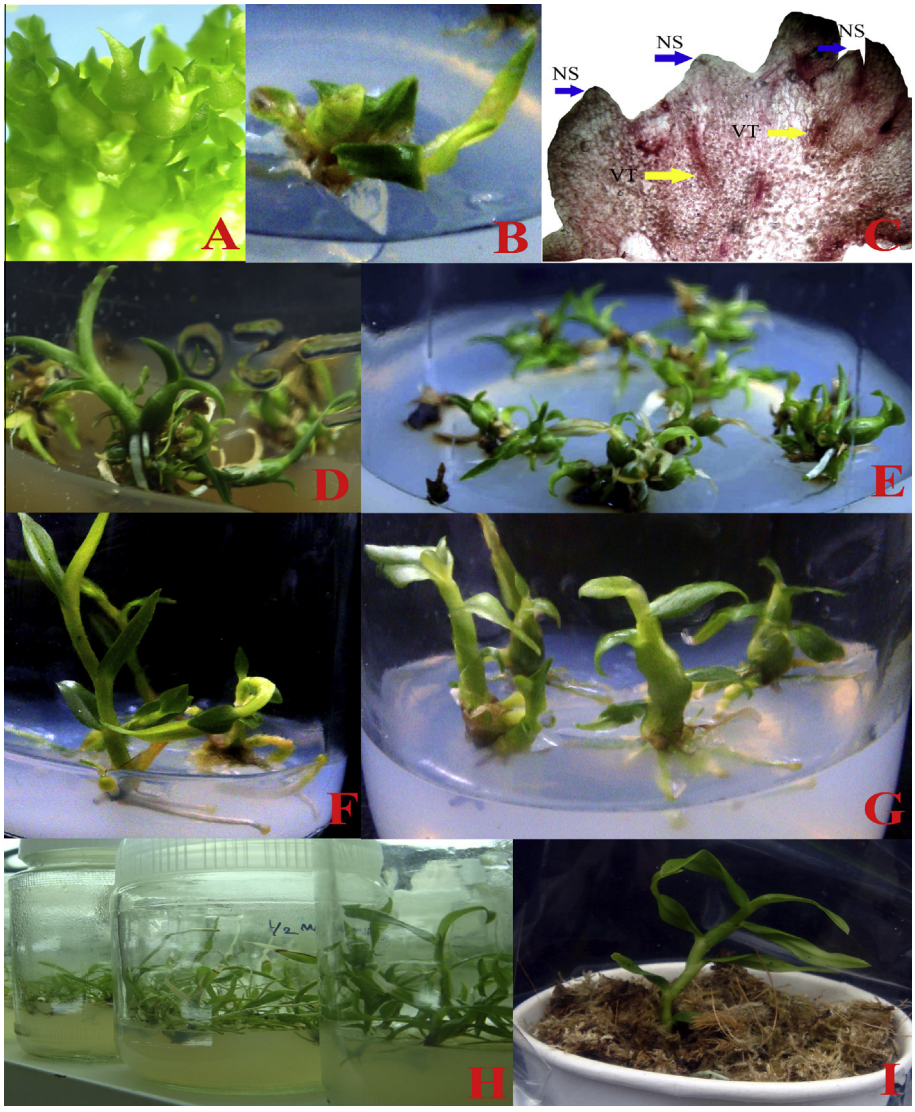

Figure 1 Micropropagation of *Dendrobium aqueum* Lindley through protocorm explants. (A) Protocorm explants with leafy shoot initials growing on 1/2 MS medium (15 weeks old), (B) multiple shoot initiation on 1/2 MS medium containing NAA 1.0 mg l^{-1} after 5 weeks, (C) longitudinal section through multiple shoot induced protocorms showing new shoots (NS) and vascular traces (VT), (D) multiple shoots and root formation on 1/2 MS medium containing banana pulp (BP) 3% after 15 weeks of culture, (E) multiple shoots on 1/2 MS medium containing coconut water (CW) 3.0 mg l^{-1} after 15 weeks, (F) elongated shoots on 1/2 MS medium containing TDZ 7.0 mg l^{-1} after 15 weeks, (G) multiple root formation on 1/2 MS medium containing IBA 5 mg l^{-1} after 15 weeks, (H) well acclimatized, rooted plantlets growing on 1/2 MS medium without sucrose after (after 3 weeks of acclimatization), (I) hardened plantlet of *D. aqueum* in pot (after 6 weeks of hardening) containing charcoal and brick (1:1) covered with mosses.

about 5 weeks then transferred to small pots containing 1:1 (w/w) mixture of brick pieces and charcoal. Moss was used to lay over the potting mixture to maintain the moisture and polyethylene bags were used to cover the pots to maintain high humidity. Plantlets were moistened using diluted 1/2 MS macro nutrients alone and kept at 25 ± 1 °C in artificial light provided by cool white fluorescent tubes for 4–6 weeks. Well acclimatized plantlets were then transferred to green house.

3. Results and discussion

As it was mentioned by Chen and Chen [4], *in vitro* studies on the genus *Dendrobium* can be categorized into, shoot-bud proliferation, regeneration from callus and direct somatic embryogenesis. The present study exerts one of the efforts for shoot bud proliferation from protocorms with novel results for conservation and idea for cost effective production.

3.1. Effect of cytokinins on shoot and root development

The protocorm explants composed of highly meristematic cells having leafy shoot initials connected with vascular tissues. Due to the effect of PGRs, multiple number of new shoots were developed with the extending vascular connection from the explant. Handmade histological sections from explants, showed clear multiple shoot formation with individual

Table 1 Effect of cytokinins on shoot development and rooting in protocorm explants of *Dendrobium aqueum* after 15 weeks of culture.

Cytokinins	Conc. (mg l^{-1})	Freq. of resp. (%)	No. of shoots per explant	Avg. shoot length (cm)	No. of roots per explant	Avg. root length (cm)
BA	1	60	0.80 ± 0.37^{b}	0.45 ± 0.06^{d}	1.50 ± 0.29^{cd}	0.30 ± 0.08^{cdef}
	3	60	0.80 ± 0.37^{b}	0.65 ± 0.12^{bcd}	0.00 ± 0.00^{d}	0.00 ± 0.00^{f}
	5	80	1.20 ± 0.37^{b}	0.65 ± 0.14^{bcd}	0.00 ± 0.00^{d}	0.00 ± 0.00^{f}
	7	80	0.80 ± 0.20^{b}	0.80 ± 0.26^{bcd}	0.00 ± 0.00^{d}	0.00 ± 0.00^{f}
	10	80	1.40 ± 0.40^{b}	0.47 ± 0.02^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{f}
KIN	1	60	1.00 ± 0.44^{b}	0.60 ± 0.20 ^{cd}	3.25 ± 1.31^{bc}	0.58 ± 0.17^{bc}
	3	60	1.60 ± 0.68^{b}	0.65 ± 0.02^{bcd}	5.25 ± 0.95^{a}	0.70 ± 0.24^{ab}
	5	60	1.80 ± 0.73^{b}	0.92 ± 0.19^{bc}	4.75 ± 0.75^{ab}	0.98 ± 0.19^{a}
	7	80	1.40 ± 0.60^{b}	0.60 ± 0.04 ^{cd}	6.25 ± 1.80^{a}	0.40 ± 0.07^{bcde}
	10	80	1.00 ± 0.32^{b}	0.67 ± 0.10^{bcd}	1.75 ± 0.75^{cd}	0.23 ± 0.09^{cdef}
2-IP	1	60	1.00 ± 0.45^{b}	0.57 ± 0.04 ^{cd}	0.00 ± 0.00^{d}	0.00 ± 0.00^{f}
	3	60	1.60 ± 0.75^{b}	0.75 ± 0.05^{bcd}	0.50 ± 0.50^{d}	0.20 ± 0.20^{def}
	5	60	5.00 ± 1.6^{a}	0.52 ± 0.04^{d}	1.75 ± 0.25^{cd}	0.45 ± 0.22^{bcd}
	7	60	1.40 ± 0.7^{b}	0.57 ± 0.02 ^{cd}	0.00 ± 0.00^{d}	0.00 ± 0.00^{f}
	10	80	1.80 ± 0.49^{b}	0.62 ± 0.12^{bcd}	0.00 ± 0.00^{d}	0.00 ± 0.00^{f}
TDZ	1	80	2.00 ± 0.55^{b}	0.77 ± 0.15^{bcd}	0.00 ± 0.00^{d}	0.00 ± 0.00^{f}
	3	100	2.00 ± 0.32^{b}	1.00 ± 0.00^{b}	0.25 ± 0.25^{d}	0.03 ± 0.03^{ef}
	5	100	2.40 ± 0.51^{b}	0.95 ± 0.02^{bc}	1.50 ± 0.29^{cd}	0.28 ± 0.11^{cdef}
	7	80	1.20 ± 0.37^{b}	1.37 ± 0.09^{a}	0.75 ± 0.25^{d}	0.13 ± 0.05^{def}
	10	80	2.20 ± 0.73^{b}	0.80 ± 0.08^{bcd}	0.75 ± 0.48^{d}	0.15 ± 0.10^{def}

The values followed by different letters within columns are significantly different from each other at 5% level. Data represent mean ± S.E.

vascular traces (Fig. 1C). Each new shoot initials continued to grow separately and develop leafy shoots, which were later developed into one to two individual roots.

Protocorms cultured on 1/2 MS basal medium produced a maximum of 1.5 shoots per explant (Fig. 1A), whereas incorporation of different growth regulating factors significantly increased the multiple shoot and root development amongst and within cytokinins, organic supplements and auxins. Explants on all treatments induced direct shoots without any callus formation after 3–5 weeks on culture media (Fig. 1B). Amongst the four cytokinins tested, 2iP at 5 mg l^{-1} induced a maximum number of 5.00 shoots with 1.75 roots per explant. Similarly in *Dactylorhiza incarnate* ssp. *incarnata*, the highest shoot and root growth rate was observed on the medium containing 2iP [35]. Bektas et al., [3] have also reported, both BA and 2iP highly favored shoot formation of protocorms in *Orchis coriophora*.

TDZ responded a maximum response of shoot induction (100%) with higher number of 2.40 shoots/protocorms at 5.0 mg l^{-1}, whereas elongated shoots formed at 7 mg l^{-1} compared to other cytokinins (Table 1), which was the second lengthened shoots (1.37 cm) of all treatments (Fig. 1F). Even though, 2iP responded to an average of 5.00 shoots/protocorm, the shoot induction frequency was low (60%) and the developed shoots showed stunted growth. Several reports on orchids especially in *Dendrobium* have agreed that TDZ can strongly stimulate multiple shoots formation and elongation [33,16,25,51,23]. The possible reasons defined that the TDZ is a phenyl urea derivative, more active stimulator of shoot formation due to high cytokinin activity and more persistence nature in plant tissues, by which it induces elongation and rooting of regenerated shoots [10,27,38].

The role of BAP in plantlet production from PLBs of *Dendrobium formosum*, reported very effective in terms of height and highest number of shoot and root formation [32], however in *Dendrobium nobile* [34], *Dendrobium huoshanense* [22] and *Doritis pulcherrima* [29] BAP was found inhibitory to the conversion of protocorms into plantlets and root formation. Similar inhibitory response with very lesser multiple shoots and stunted growth was observed on medium containing BA in *D. aqueum*.

An effective conversion of PLBs into shoots was recorded in *D. huoshanense* at 4 mg l^{-1} of KIN [22] whereas in our study, protocorms cultured on every concentration of KIN produced multiple roots equally to IBA (Table 3). A maximum number of 6.25 and 5.25 roots per explant were observed on 7 mg l^{-1} and 3 mg l^{-1} of KIN along with an average length of 0.98 cm roots on KIN 5 mg l^{-1}. These results suggest that KIN has some critical role on root development and no reports so far on orchids but similar root growth on KIN alone was reported in *Dactylorhiza majalis* [35] and in a fishtail fern [2].

3.2. Effect of natural additives on shoot and root development

A number of complex organic additives like peptone, beef extract, casein hydrolysate and natural additives like tomato juice, potato extract, coconut water (CW), banana extract (BE) etc. are commonly added to plant tissue culture media including orchids [8,28]. Of the two natural additives used in our experiments (BP and CW), medium supplemented with 3% BP induced maximum (80%) shoot formation with an average of 7.00 shoots per protocorm explant which was comparatively higher than the cytokinins tested (Table 2). Moreover increase in the concentration of BP to 7%, highest

Table 2 Effect of natural additives on shoot and root development of *D. aqueum* after 15 weeks of culture.

Natural additives	Conc. (%)	Freq. of response (%)	No. of shoots per explant	Avg. shoot length (cm)	No. of roots per explant	Avg. root length (cm)
Banana pulp (g/l)	1	60	5.60 ± 2.77^{ab}	0.47 ± 0.04^{ab}	2.75 ± 0.8^{b}	0.33 ± 0.0^{de}
	3	80	7.00 ± 2.17^{a}	0.62 ± 0.02^{ab}	2.50 ± 0.5^{b}	0.53 ± 0.1^{cde}
	5	80	3.20 ± 0.86^{abc}	0.50 ± 0.04^{ab}	2.75 ± 0.4^{b}	0.70 ± 0.10^{bcd}
	7	100	5.60 ± 0.98^{ab}	0.57 ± 0.11^{ab}	7.75 ± 2.2^{a}	1.20 ± 0.20^{a}
	10	80	3.20 ± 0.86^{abc}	0.32 ± 0.04^{b}	1.75 ± 0.2^{b}	0.30 ± 0.07^{e}
Coconut water	1	80	2.40 ± 0.68^{bc}	0.37 ± 0.02^{ab}	1.25 ± 0.2^{b}	0.25 ± 0.0^{e}
	3	100	6.20 ± 0.58^{ab}	0.32 ± 0.02^{b}	2.50 ± 0.6^{b}	0.53 ± 0.1^{cde}
	5	80	3.20 ± 0.86^{abc}	0.72 ± 0.29^{a}	2.25 ± 0.2^{b}	1.00 ± 0.1^{ab}
	7	60	2.40 ± 1.03^{bc}	0.40 ± 0.04^{ab}	3.50 ± 1.2^{b}	0.80 ± 0.1^{bc}
	10	60	1.20 ± 0.58^{c}	0.37 ± 0.04^{ab}	2.00 ± 0.7^{b}	0.55 ± 0.0^{cde}

The values followed by different letters within columns are significantly different from each other at 5% level. Data represent means ± S.E.

number (7.75) and lengthier (1.20 cm) root formation were observed and was very much comparable to the root formation on IBA and NAA (Fig. 1D).

Similar beneficial effects of CW and/or banana homogenate and/or potato homogenate on seedling growth have often been reported in many orchid species like *Cattleya* seedlings [11], *Vanda spathulata* [6], *Dendrobium tosaense* [21]. BE promote higher percentage germination and early rooting and faster growth and development on *D. lituiflorum* [47]. The possible reasons could be that, bananas are a good source of K, Mg, Cu, and Mn and vitamin C or vitamin A [49] with natural plant growth regulators like IAA, GA_7, GA_x, zeatin, zeatin riboside and 2-iP [17,18,45].

In our experiment, addition of 3% CW produced a maximum of 6.20 shoots of stunted growth with 100% shoot regeneration ability. Increase in concentration to 5% of CW promotes elongated shoots (0.72 cm) compared to BP (Fig. 1E). Likewise, enhanced shoot development using CW has been in many *Dendrobium* [19,41,43,21,48,31] and in *Cymbidium pendulum* [15].

Molnar et al., [28] stated that CW was the most complex combination of compounds, contains a number of amino acids, organic acids, nucleic acids, several vitamins, sugars and sugar alcohols, plant hormones (auxins, cytokinins), minerals, and other unidentified substances and none of which alone is totally responsible for growth promoting qualities. In this study, without addition of any plant growth regulators, protocorm produced multiple shoots and roots more or less equally to cytokinins and auxins. These results suggest that complex natural supplements can efficiently support shoot induction, multiplication and multiple root formation.

3.3. Effect of auxins on shoot and root development

Shoots developed on IBA produced a maximum of 8.75 roots per shoot on IBA 5.0 mg l^{-1} than any other auxins and natural supplements tested (Fig. 1G; Table 3). Protocorms cultured on auxins IAA, 2,4-D and PIC failed to promote shoot or root where explants turned white and yellow. Similarly IAA, 2,4-D reported inhibitory in *Dactylorhiza* sp. [35], *Coelogyne punctulata* [42] but in *Dendrobium fimbriatum* the highest rate of shoot induction and highest shoot elongation were obtained in media that contained Picloram [14]. These results agreed with the statement by Davies [5] that the relative degree of activity of individual auxins in different growth processes is variable from plant to plant, organ to organ, tissue to tissue and cell to cell and moreover, also with the age and physiological state of the plant (tissue). The possible reasons could be that, IAA by its high instability nature, is usually less effective than synthetic auxins like 2,4-D or NAA [8], whereas, IBA is physiologically a more active auxin than NAA and IAA in root initiation, and it acts as a precursor for endogenous IAA [20].

Independently NAA with 1/2 MS medium at every concentration (1–10 mg l^{-1}) is able to produce multiple shoots and roots in *D. aqueum*. The maximum number of elongated shoots with multiple and lengthier roots were produced in a medium with NAA alone, and was comparatively higher than the cytokinins and organic supplement used in this study. At the maximum of 9.40 shoots with 100% shoot inducing ability was observed on NAA 3 mg l^{-1} and elongated shoots of 1.52 cm and lengthened roots of 1.48 cm were observed on NAA 7 mg l^{-1}. Similarly in *Dendrobium longicornu* [7] and in a species of *Dendrobium* [37], individual NAA was reported to produce multiple numbers of shoots than cytokinins and stimulates seedling growth in *C. punctulata* [42]. Multiple root inductions on shoot multiples have also been reported in *Dendrobium chrysanthum* [46] and in *Dendrobium* sp. [37].

Plantlets with well-developed roots from all the treatments, grew well following acclimatization and were transferred to pots *in vitro* (Fig. 1H and I).

3.4. Synergistic effect of NAA with cytokinins on shoot and root development

Overall, individual treatments revealed that NAA alone could develop complete plantlets from leafy shoot protocorms whereas, 2iP and TDZ have promoted multiple shoots and lengthier shoots. Thus, to evaluate the synergistic effect of these plant growth regulators, protocorms were cultured on 1/2 MS medium containing NAA 3.0 mg l^{-1} either with 2iP or TDZ at 1–10 mg l^{-1}. At the end of ten weeks of culture, significant difference on shoot growth was obtained. In all combinations tested, the regenerative response as well as root formation was significantly reduced or nil. However, NAA

Table 3 Effect of auxins on shoot and root development of *D. aqueum* after 15 weeks of culture.

Auxins	Conc. (mg l^{-1})	Freq. of response (%)	No. of shoots per explant	Avg. shoot length (cm)	No. of roots per explant	Avg. root length (cm)
IBA	1	80	1.20 ± 0.37^{c}	0.75 ± 0.28^{bc}	1.75 ± 0.63^{d}	0.30 ± 0.10^{cd}
	3	80	2.60 ± 0.75^{bc}	0.87 ± 0.11^{bc}	6.50 ± 0.87^{ab}	0.73 ± 0.13^{bc}
	5	80	1.40 ± 0.40^{c}	1.00 ± 0.09^{b}	8.75 ± 1.18^{a}	0.90 ± 0.18^{b}
	7	40	0.60 ± 0.40^{c}	0.72 ± 0.02^{bc}	3.75 ± 0.48^{bcd}	0.50 ± 0.29^{bcd}
	10	20	0.20 ± 0.20^{c}	0.62 ± 0.04^{bc}	1.50 ± 1.19^{d}	0.08 ± 0.05^{d}
NAA	1	80	2.00 ± 0.55^{c}	0.60 ± 0.14^{bc}	6.75 ± 1.70^{ab}	0.73 ± 0.14^{bc}
	3	100	9.40 ± 1.81^{a}	0.67 ± 0.07^{bc}	4.50 ± 0.50^{bcd}	0.88 ± 0.29^{bc}
	5	100	4.40 ± 0.51^{b}	0.47 ± 0.06^{c}	2.50 ± 0.96^{cd}	0.48 ± 0.2^{bcd}
	7	80	1.20 ± 0.37^{c}	1.52 ± 0.20^{a}	6.75 ± 0.85^{ab}	1.48 ± 0.13^{a}
	10	80	2.40 ± 0.68^{bc}	0.52 ± 0.06^{c}	5.50 ± 0.87^{bc}	0.48 ± 0.03^{bcd}

The values followed by different letters within columns are significantly different from each other at 5% level. Data represent means ± S.E.

Table 4 Synergetic effect of NAA with cytokinins on shoot and root development of *D. aqueum* after 15 weeks of culture.

1/2 MS	Conc. (mg l^{-1})	Freq. of response (%)	No. of shoots per explant	Avg. shoot length (cm)
NAA	1	66.67	1.74 ± 0.03^{c}	0.54 ± 0.01^{d}
3.0 + TDZ	3	83.33	2.00 ± 0.01^{b}	0.48 ± 0.01^{e}
	5	83.33	1.00 ± 0.02^{e}	0.53 ± 0.02^{de}
	7	50	1.08 ± 0.11^{e}	0.38 ± 0.01^{f}
	10	50	1.05 ± 0.05^{e}	0.63 ± 0.03^{ab}
NAA	1	83.33	2.11 ± 0.07^{b}	0.66 ± 0.02^{a}
3.0 + 2iP	3	50	1.32 ± 0.04^{d}	0.64 ± 0.01^{ab}
	5	50	4.99 ± 0.07^{a}	0.57 ± 0.02^{cd}
	7	50	1.00 ± 0.02^{e}	0.41 ± 0.01^{f}
	10	100	1.97 ± 0.04^{b}	0.60 ± 0.01^{bc}

The values followed by different letters within columns are significantly different from each other at 5% level. Data represent means ± S.E.

3.0 mg l^{-1} along with 2iP 5 mg l^{-1} produced a maximum of 4.99 shoots per explant with almost 50% regenerative response (Table 4). The highest shoot length of 0.66 cm and 0.64 cm was observed on NAA combined with 2iP 1 mg l^{-1} and 3 mg l^{-1} respectively. These results agreed with the previous studies on *Dendrobium aphyllum* (Roxb.) Fisch. and *D. moschatum* (Buch-Ham) SW., where NAA along with TDZ supported shoot elongation and did not increase the frequency of shoot regeneration [33]. Addition of NAA with 2iP lowered the efficiency of NAA or has a significant effect on shoot development.

Rooted plantlets were cultured on 1/2 MS basal medium for 4 weeks followed by a sucrose free medium for five weeks before being transferred to potting mixture. Plantlets grew slowly and well on small pots containing 1:1 (w/w) mixture of brick pieces and charcoal. Mosses laid over the potting mixture help moistening and the polyethylene bags contributed high humidity. Well acclimatized plantlets in *in vitro* successfully maintained and transferred to green house with the maximum survival of 96% for more than five months.

4. Conclusion

In conclusion, the present study reported micropropagation results in *D. aqueum* for the first time. Individual auxins, natural supplements and cytokinins represented effective growth promoting factors. Efficient shoot regeneration, multiplication and rooting by using individual treatments of auxins, natural additives and cytokinins were established from protocorm. Individual levels of NAA and IBA alone successfully produced healthy shoots and roots both in multiple numbers and in length than any other cytokinins from protocorms of *D. aqueum*. Results obtained on natural additives BP and CW are also noticeable in view of cost effective approach. The efficient production of protocorms and subsequent elongated multiple shoots and well rooted plants by individual auxins and organic additives provides a simple and cost effective protocol for mass propagation and conservation for this species and could be tried to valuable *Dendrobium* species.

Acknowledgements

We sincerely acknowledge the financial support from the Ministry of Environment, Forest and Climate Change, New Delhi, Government of India and Rajiv Gandhi National Fellowship (UGC).

References

[1] J. Arditti, R. Ernst, Micropropagation of Orchids, John Wiley and Sons, New York, 1993.
[2] M.J. Beck, J.D. Caponetti, Am. J. Bot. 70 (1983) 1–7.
[3] E. Bektas, M. Cuce, A. Sokmen, Turk. J. Bot. 37 (2013) 336–342.
[4] W.H. Chen, H.H. Chen, Orchid biotechnology, World Scientific Publishing, Singapore, 2007.
[5] P.J. Davies (Ed.), Plant Hormones, Regulatory Factors in Hormone Action: Level, Location and Signal Transduction, Kluwer Academic Publishers, Dordrecht, 2004.
[6] W. Decruse, A. Gangaprasad, S. Seeni, S.V. Menon, Indian J. Exp. Biol. 41 (2003) 924–927.
[7] S. Dohling, S. Kumaria, P. Tandon, AoB Plants (2012), http://dx.doi.org/10.1093/aobpla/pls032.

[8] E.F. George, M.A. Hall, G.J.D. Klerk, Plant Propagation by Tissue Culture: Volume 1. The Background, third ed., Springer, 2008, ISBN 978-1-4020-5005-3.
[9] R.M.P. Gutierrez, J. Med. Plants Res. 4 (2010) (2010) 592–638.
[10] A. Huetteman, E.J. Preece, Plant Cell, Tissue Organ Cult. 33 (1993) 105–119.
[11] M.O. Islam, S. Matsui, S. Ichihashi, Lindleyana 15 (2000) 81–88.
[12] IUCN. IUCN Red List Categories, as approved by the 51st meeting of the IUCN Council Gland, Switzerland, 2000.
[13] D.A. Johansen, Plant microtechnique, McGraw Hill Book Co., New York, 1940.
[14] M.F. Kabir, M.S. Rahman, A. Jamal, M. Rahman, M. Khalekuzzaman, J. Anim. Plant Sci. 23 (2013) 2013.
[15] S. Kaur, K.K. Bhutani, Hort. Sci. (Prague) 39 (2012) 47–52.
[16] N.V. Ket, E.J. Hahn, S.Y. Park, D. Chakrabathy, K.Y. Paek, Biol. Plantarum 48 (2004) 339–344.
[17] R.A. Khalifah, Plant Physiol. 76 (1966) 280–283.
[18] R.A. Khalifah, Nature 212 (1966) 1471–1472.
[19] C.K. Kitsaki, S. Zygouraki, M. Ziobora, S. Kintzios, Plant Cell Rep. 23 (2004) 284–290.
[20] C. Liu, J. Zhu, Z. Liu, L. Li, R. Pan, L. Jin, Plant Growth Regul. 38 (2002) 37–43.
[21] S. Lo, S. Nalawade, C. Kuo, C. Chen, H. Tsay, *In vitro* Cell. Dev. Biol. 40 (2004) 528–535.
[22] J.P. Luo, C. Wawrosch, B. Kopp, Sci. Hortic. 12 (2009) 258–262.
[23] G. Mahendran, V.N. Bai, Sci. Hortic. 119 (2009) 203–207.
[24] R.B. Malabadi, S.G. Mulgund, K. Kallappa, J. Plant Physiol. 162 (2005) 473–478.
[25] K.P. Martin, J. Madassery, Sci. Hortic. 108 (2006) 95–99.
[26] K.M. Matthews, The flora of Tamil Nadu Carnatic, St. Josephs College, Tiruchirappalli, Tamil Nadu, India, 1983.
[27] H.J. Meyer, J.V. Staden, Hort. Science 25 (1988) 1070–1071.
[28] Z. Molnar, E. Virag, V. Ordog, Acta Biolo. Szeged. 55 (2011) 123–127.
[29] T. Mondal, S. Aditya, N. Banerjee, Plant Tissue Cult. Biotechnol. 23 (2013) 251–261.
[30] T. Murashige, F.A. Skoog, Physiol. Plant. 15 (1962) 473–497.
[31] N. Nambiar, C.S. Tee, M. Maziah, Plant Omics 5 (2012) 10–18.
[32] K.M. Nasiruddin, R. Begum, S. Yesmin, Asian J. Plant Sci. 2 (2003) 955–957.
[33] N.R. Nayak, S.P. Rath, S. Patnaik, Sci. Hortic. 71 (1997) 243–250.
[34] N.R. Nayak, S. Sahoo, S. Patnaik, S.P. Rath, Sci. Hortic. 94 (2002) 107–116.
[35] K.W. Novotna, H. Vejsadova, P. Kindlmann, Biol. Plantarum 51 (2007) 198–200.
[36] S. Parthibhan, J.H.F. Benjamin, M. Muthukumar, N.A. Sherif, T. Senthil Kumar, M.V. Rao, Afr. J Plant Sci. 6 (2012) 383–393.
[37] M.S. Parvin, M.E. Haque, F. Akhter Moniruzzaman, A.B.M. Khaldun, Bangladesh J. Agric. Res. 34 (2009) 411–416.
[38] J.E. Preece, M.R. Imel, Sci. Hortic. 48 (1991) 159–170.
[39] C.S. Reddy, C. Pattanaik, M. Murthy, K. Reddy, EPTRI – ENVIS Newsletter 11(4) (2005).
[40] J.P. Robinson, V. Balakrishnan, J. Britto, Bot. Res. Int. 2 (2009) 99–102.
[41] J. Roy, N. Banerjee, Sci. Hortic. 97 (2003) 333–340.
[42] S.K. Sharma, P. Tandon, in: S.P. Vij (Ed.), Biology, Conservation and Culture of Orchids Influence of growth regulators on asymbiotic germination and early development of Coelogyne punctulata Lindl, Affiliated East West Press, New Delhi, India, 1986, pp. 441–451.
[43] S.S. Sheelavanthmath, H.N. Murthy, B.P. Hema, E.J. Hahn, K.Y. Paek, Sci. Hortic. 106 (2005) 395–401.
[44] J.A. Teixeira da Silva, Floriculture Ornamental, Floricult. Ornament. Biotechnol. 7 (2013) 1–52.
[45] J. Van Staden, J. Stewart, Z. Pflanzenphysiol. 76 (1975) 280–283.
[46] S.P. Vij, P. Pathak, J. Orchid Soc. India 3 (1989) 25–28.
[47] S. Vyas, S. Guha, M. Bhattacharya, I. Usha Rao, Sci. Hortic. 121 (2009) 32–37.
[48] S. Vyas, S. Guha, P. Kapoor, I. Usha Rao, Sci. Hortic. 123 (2010) 551–557.
[49] M.W. Wall, J. Food Compost. Anal. 19 (2006) 434–445.
[50] P.S. Wesley, B. Chitra Devi, B. Sahaya Shibu, S. Moin, Asia Pac. J. Mol. Biol. Biotech. 21 (2013) 26–32.
[51] P. Zhao, W. Wang, S.F. Feng, F. Wu, J.Q. Yang, W.J. Wang, Plant Cell, Tissue Organ Cult. 90 (2007) 131–139.