ISSN 1817-3721, E-ISSN 1818-8745

Plant Tissue Cult. & Biotech. **30**(1): 65-75, 2020 (June)
©Bangladesh Assoc. for Plant Tissue Culture & Biotechnology

PTC&B

Influence of Cytokinins on Rhizome Mediated Growth and Morphogenesis of an Endangered Medicinal Orchid *Geodorum densiflorum* (Lam.) Schltr.

Soumi Bhattacharyya and Nirmalya Banerjee*

Laboratory of Cytogenetics and Plant Biotechnology, Department of Botany, Visva-Bharati University, Santiniketan-731235, West Bengal, India

Key words: Asymbiotic seed germination, Diagravitropic, Protocorm like bodies

Abstract

Effects of different nutrient media, vitamins and peptone on *in vitro* asymbiotic seed germination of *Geodorum densiflorum* (Lam.) Schltr. were studied. *In vitro* developed rhizomes were used to determine the influence of cytokinins on growth and morphogenesis. Seed germination and survival rate of protocorms were highest in MS medium compared to other basal media. The protocorms raised through seed germination directly proliferated into rhizomes in later phase of growth and development. Nodal portions of rhizomes further exhibited growth through formation of direct and callus mediated protocorm like bodies. In general, three types of rhizome movements were noticed; *viz.* positively geotropic, negatively geotropic and diagravitropic movements. In the PGR free control and in the presence of NAA alone rhizomes exhibited positively geotropic movements. On the contrary, presence of cytokinin either alone or in combination with NAA exhibited diagravitropic movements. Application of TDZ completely suppressed the positively geotropic movement and enhanced the frequency of negatively geotropic movement of rhizomes followed by shoot bud formation. BAP was most effective cytokinin for large scale rhizome mediated multiplication of *Geodorum densiflorum* and TDZ could be effectively employed for rapid leafy shoot regeneration.

Introduction

Geodorum densiflorum (Lam.) Schltr. is an endangered terrestrial orchid (Datta et al.1999).This herbaceous, land orchid possesses pseudo-bulb as a storage organ, which is having serious implications for survival in the adverse and nutrient limited conditions. The pseudo-bulb of *Geodorum densiflorum* is ethnomedicinally known for the treatment of

*Author for correspondence: <nirmalya.banerjee54@gmail.com>.

DOI: https://doi.org/10.3329/ptcb.v30i1.47792

various human diseases. The root paste is used as insecticide and wound healing agent. The pseudo-bulbs are used to regularize menstrual cycle (Dashet al.2008), for controlling diabetes (Patil and Patil2005) and also applied externally to cure carbuncles (Nathet al.2011). It also possesses antimicrobial activity (Akter et al.2010).

G. densiflorum is distributed in India, Nepal, Australia, Bangladesh, Srilanka, China, Bhutan, Papua New Guinea and Himalayas (Roy and Banerjee 2001,Theng and Korpenwar 2014). In India it is distributed in Karnataka, Kerala, Tamilnadu and Maharashtra (Theng and Korpenwar 2014). This plant is also floriculturally important for the presence of dense cluster of pinkish white long lasting flowers. Increasing commercial demand for its ornamental as well as medicinal importance is the prime cause of the continuous exploitation of this plant from its natural habitat and as a result of which this species has now become rare and endangered (Datta et al.1999). To overcome this critical situation, asymbiotic seed culture and micropropagation techniques are extremely useful for rapid clonal propagation of the species.

Like other members of Orchidaceae, seeds of *G. densiflorum* are extremely minute and non-endospermic. In nature, association with a specific fungal partner is a pre-requisite for orchid seed germination (Arditti 1967, Mitra 1986, Roy and Banerjee 2001). Vegetative propagation is quite common in many members of Orchidaceae. However, the developmental process of this conventional method is rather a slow process which is expensive as well. The discovery of *in vitro* asymbiotic seed germination and micropropagation protocol contributed immensely to resolve this problem. Sheelavantmath et al. (2000) reported a protocol for rhizome based *in vitro* propagation of *G. densiflorum*.

Plant growth regulators play a key role for developing specific modes of growth in the cultured cells and tissues, particularly the cytokinins and auxins are of importance in *in-vitro* culture as the latter is primarily concerned with root formation (Vuylsteke 1989 ,Bohidar et al. 2008) and the former plays a crucial role in shoot bud regeneration (North et al. 2012). Combination of auxins and cytokinins in the nutrient media is an ideal situation which determines successful plant regeneration (North et al. 2010).

In view of this, the present investigation was undertaken for developing an efficient *in vitro* protocol for asymbiotic seed germination, rhizome development and rhizome mediated micropropagation using suitable plant growth regulators.

For optimization of rhizome mediated *in vitro* propagation protocol of *G. densiflorum,* a comparative study of the role of different cytokinins either singly or in combination with auxins has been critically evaluated.

Materials and Methods

Asymbiotic seed culture experiment was performed with seeds taken from 29 weeks old mature green capsule of *G. densiflorum*. For initiation of asymbiotic seed culture utilizing the microscopic seeds, the standardized method developed in our laboratory

has been adopted (Roy and Banerjee 2001, 2002). Five replicate culture tubes were used per treatment, each containing 20 ml of semi solid culture medium. Seed suspension was spread with the help of Pasteur pipette in each culture tube containing semisolid medium. The cultures were maintained at 25± 2°C under 10 hrs photoperiod of 2500-3000 lux irradiance.

Five basal media were used in asymbiotic seed culture experiment. Those were Knudson's C medium (KC) (Knudson 1946), Modified Knudson's C medium (MKC), Murashige and Skoog's medium (MS) (Murashige and Skoog 1962), Vacin and Went medium (VW) (Vacin and Went 1949) and Lindeman Orchid medium (LM) (Lindemann et al. 1970). In MKC medium the original iron source was replaced with iron-EDTA. In all the basal media, 2% (w/v) sucrose served as carbon source. In addition, 0.1% peptone (w/v) and 0.1% vitamin mixture (w/v) were used as organic additives. The vitamin mixture used in the media consisted of nicotinic acid, pyridoxine and thiamine HCl at 1:1:10 ratio. The pH of the culture medium was adjusted to 5.7 prior to autoclaving. The medium was solidified with 0.9% (w/v) agar. The culture tubes were plugged with non absorbent cotton and autoclaved at 121^0C for 20 min under 15 lb $inch^{-2}$.

After establishing the primary seed culture, the cultures were examined every week for initiation of germination. Emergence of the embryo from the seed testa was considered as initiation of germination (De Pauw et al. 1995, Roy and Banerjee 2002). Embryo swelled up after 3 months of inoculation but, the growth of the embryo was mostly restricted to globular stage till 5th months.

Based on the performance of germination and survival of the initial culture, MS basal medium was selected for further study of growth and development of explants. For this purpose, half strength MS containing 3% (w/v) sucrose as carbon source was used as subculture medium, where globular stage protocorms were further advanced into rhizome after 4 months. The same basal medium was used as PGR free control. Of the various PGR treatments, NAA as auxin was used with four cytokinins in various concentrations and combinations. For each treatment, 3 replicate flasks containing 100ml of medium were used. Small nodal segments of aseptically grown rhizomes were taken as explants for initiation of organ culture. Five explants were inoculated in each replicate flask. Data regarding rhizome growth, development and leafy shoot emergence in the PGR treatments were gathered after 3 months of culture.

The mean values of different morphogenetic responses were analyzed by ANOVA along with DMRT (α = 0.05). All statistical analyses were performed according to Little and Hills (1978).

Initially 10 well-rooted plantlets, each with a healthy pseudo-bulb, were washed in slow running tap water to remove the traces of agar and subsequently treated with 5g/l sulfur (80% WG) fungicide for 7 min. Fungicide treated plants were subsequently transferred to pots containing soilrite and kept at 25 ± 2°C in laboratory condition for about 1 month under regular observation and watering. After commencement of new

growth, evidenced by emergence of new leaves, the plantlets were shifted to clay pots containing a mixture of garden loam and sand in 2:1 ratio and kept in a moist, shady place in the experimental orchidorium for about two months. Finally healthy plantlets were transplanted to their natural habitat during rainy season to prevent possible desiccation.

Results and Discussion

Seed germination began after about three weeks of inoculation. Interestingly, in majority of orchid species, mature seeds packed in pods contain immature embryo axis and dry testa. During germination these immature embryo axis first attains mature stage, called the globular stage or protocorm. In majority of orchid species such protocorms of stage-1 pass through subsequent developmental stages, *viz.* stage-2 or leaf primordia stage, stage-3 or leafy stage and stage-4 or rooted stage (Arditti 1967). However, in case of *Geodorum densiflorum* the protocorms directly formed rhizomes of irregular shape with distinct nodes and internodes. Sheelavantmath et al. (2000) also reported that *in vitro* asymbiotic seed germination resulted in development of protocorms, which were later transformed into rhizomes, instead of shoots and roots (Fig. 1A-D). The results of various treatments shown in Table 1, clearly depicted that in *G. densiflorum,* MS exhibited best response for germination and protocorm survival. Highest percentage of globular stage protocorm was also obtained in MS. Similarly, KC basal medium supplemented with vitamin also showed high frequencies of germination and formation of globular stage protocorms though necrosis was slightly higher. LM basal medium was more productive compared to vitamin and peptone supplementation. Thus, in *G. densiflorum,* supplementation of the medium with peptone and vitamin was not essential for initiation of seed germination. Roy and Banerjee (2001) also reported that *G. densiflorum* has no stringent nutritional requirements for initiation of germination unlike other terrestrial orchids.

The protocorms raised through asymbiotic seed cultures further proliferated into branched rhizomes with distinct nodes and internodes in half strength of MS medium (Fig. 1B-D).

In the second phase of the study, nodal portions of rhizomes were used as explants for achieving micropropagation through organogenesis. The morphogenetic responses of explants as well as the influences of different cytokinins with or without NAA are presented in Table 2. The explants either proliferated through the formation of direct PLBs or callus mediated PLBs. Axillary branch formation was very common phenomenon of the rhizome. Initially, the growth of rhizomes was horizontal along the medium surface (diagravitropic) (1 - 5 mm). Subsequently, the rhizome tips curved either downward or upward or continued earlier diagravitropic movement. For these tropic movements, rhizomes that were 5 mm or more in length and exhibited either positively or negatively geotropic or diagravitropic movements were taken into considerations.

Hence, the gravitropic responses actually represented the second phase of rhizome growth.

Rhizome movements of *Geodorum densiflorum* have been reported by Roy and Banerjee (2002) but, they reported only two types of rhizome movements- positively geotropic and diagravitropic. However, in the present study, three types of rhizome movement were noticed, *viz.* positively geotropic, negatively geotropic and diagravitropic movements (Fig. 1B-D). Depending upon the exogenous PGR supplementation, movement of rhizomes varied (characteristics features of rhizomes were shown in Table 2).In the PGR free control and in the presence of NAA alone,

Fig. 1.Seed germination and development of rhizome of *Geodorum densiflorum*: (A) germinated seeds restricted at globular stage protocorms; (B) rhizome proliferation showing positively geotropic movement and axillary branch formation; (C) root regeneration from diagravitropic rhizomes; (D) negatively geotropic rhizome showing leafy shoot regeneration.

positively geotropic rhizome movement occurred. In contrast to that, presence of cytokinin (BAP and 2iPA) either alone or in combination with NAA exhibited diagravitropic movement of rhizomes. Application of TDZ however completely suppressed the positively geotropic movement of rhizomes and enhanced the frequency

of negatively geotropic movement of rhizomes followed by shoot bud formation (Fig. 1D). Negatively geotropic rhizomes always formed leafy shoots after attaining a certain length. In contrast to this, higher concentrations of TDZ and BAP characteristically induced direct shoot development (Table 3). Positively geotropic or diagravitropic rhizomes also formed shoots when both NAA and cytokinins (BAP/Kinetin/2iPA) were present in the medium. Combined application of auxin and cytokinins increased the frequency of leafy shoot emergence (Fig. 1D).

Table 1. Effects of different organic supplements in different culture media on seed germination of *Geodorum densiflorum* (Lam.) Schltr. after three months of seed culture.

Treatment MS Basal	Germination of seeds (%) ± SE*	Necrosis of explants (%) ± S.E.*	Globular stage (%) ± S.E.*	Leaf primordia (%) ± S.E.*	Survival of explants (%) ± S.E.*
MS Basal	85.81±2.38^{f}	14.19±2.38^{a}	85.81±2.38^{f}	0^{a}	85.81±2.38^{e}
MS+peptone	61.88±4.46^{ab}	38.12±4.46^{de}	61.88±4.46^{ab}	0^{a}	55.90±4.88^{a}
MS+vitamin	54.39±5.40^{a}	45.61±5.40^{e}	54.39±5.40^{a}	0^{a}	53.14±5.25^{a}
KC basal	68.67±1.63^{bc}	31.33±1.63^{cd}	68.67±1.63^{bc}	0^{a}	67.75±1.83^{abc}
KC+peptone	74.62±2.78^{cde}	25.38±2.78^{bc}	72.53±3.34^{bcde}	2.08±0.80^{b}	74.08±2.78^{bcde}
KC+vitamin	81.86±2.75^{ef}	18.14±2.75^{ab}	81.55±2.68^{ef}	0.30±0.29^{a}	81.86±2.75^{de}
MKC basal	69.73±4.46^{bcd}	30.27±4.46^{cd}	67.48±4.58^{bc}	2.25±0.91^{b}	68.66±4.39^{abcd}
MKC+peptone	70.14±3.33^{bcd}	29.85±3.33^{cd}	70.14±3.33^{bcd}	0^{a}	67.18±3.91^{abc}
MKC+vitamin	76.38±4.83^{cde}	23.62±4.83^{bc}	76.38±4.83^{cde}	0^{a}	73.75±5.38^{bcde}
LM basal	80.07±2.09^{def}	21.19±2.25^{abc}	80.07±2.09^{def}	0^{a}	78.81±2.25^{cde}
LM+peptone	76.13±3.12^{cde}	23.87±3.12^{bc}	76.13±3.12^{cde}	0^{a}	74.96±3.52^{bcde}
LM+vitamin	74.59±3.43^{cde}	25.41±3.43^{bc}	74.59±3.43^{cde}	0^{a}	64.77±8.93^{abc}
VW basal	71.31±3.39^{bcd}	28.68±3.39^{cd}	71.31±3.39^{bcde}	0^{a}	59.60±9.39^{ab}
VW+peptone	68.79±2.48^{bc}	31.20±2.48^{cd}	68.61±2.41^{bc}	0.19±0.18^{a}	68.80±2.47^{abcd}
VW+vitamin	70.63±0.80^{bcd}	29.37±0.80^{cd}	68.66±0.64^{bc}	1.97±0.85^{b}	67.34±2.02^{abc}

SE: Standard error; Data shown are the mean of Five replicates ± SE. *Mean values followed by the same letter are not significantly different at 0.05 level (DMRT).

Table 2. Characteristic features of rhizomes.

Characteristics	Positively geotropic rhizome	Negatively geotropic rhizome	Diagravitropic rhizome
Occurrence	In the PGR free control and in the presence of NAA alone	In the presence of TDZ mainly. Leafy shoot emergence occurred in the presence of cytokinins along with NAA	In the presence of cytokinins mainly BAP and 2iPA, either alone or along with NAA
Diameter	Thick	Initially thin then thick	Comparatively thin
Surface	Hairy	Smooth	Smooth
Appearance	Light to dark green. Presence of distinct nodes and internodes	Green. After a certain length emergence of leafy shoot with or without pseudobulbs	Dark green. Nodes not always distinct

The concentration of exogenous cytokinin appeared to be the primary factor affecting shoot multiplication (Ngomuo et al. 2013). The significant role of BAP on the formation of axillary branch, indirect multiple shoot buds or PLBs was critically recorded, compared to other cytokinins tested (2iPA, TDZ and Kn). Formation of rhizomes was very common in each treatment. The effects of four cytokinins on rhizome mediated growth and morphogenesis were shown in Table 3 and Table 4. Highest (100%) axillary branch formation (Fig. 2C) was recorded in half strength of MS + 2µM BAP + 2µM NAA and half strength of MS + 4µM BAP + 1µM NAA. These findings indicated that BAP (2 and 4 µM) was more effective in the formation of axillary branches or multiple shoot buds. Similar result was also reported by Bhadra and Hossain (2003). Highest callus frequency (93.33%) (Fig. 2B) was recorded in half strength of MS + 2µM BAP + 2µM NAA, while 100% direct PLB formation without callus phase (Fig. 2A) was achieved inhalf strength

Fig. 2. Rhizome mediated growth and developmental pathways of *Geodorum densiflorum*: (A) Direct PLB formation; (B) Callus mediated PLB formation; (C) Axillary branch formation; (D) Direct and callus mediated PLB formation showing differentiation of shoots; (E-F) Stepwise acclimatization of *in vitro* regenerated plantlets. (E) Transplanted healthy plantlets under laboratory condition and initiation of daughter plant (F) transferred plant in natural habitat (Departmental garden).

of MS supplemented with 0.5µM BAP and 2µM NAA and also 0.5µM TDZ and 1µM NAA. Thus, BAP and TDZ were more effective than Kn and 2iPA for the formation of callus and direct PLBs. Higher concentration of BAP significantly enhanced the proliferation of shoot bud, which was corroborated by Hussein (2012). However, TDZ was found responsible for leafy shoot formation. Gubbuk and Pekmzci (2004) reported rapid proliferation and elongation of shoots with TDZ compared to BAP in *Musa* spp. Highest percentage of leafy shoot formation in rhizomes was detected in half strength

Table 3. Effects of different cytokinins and NAA on rhizome mediated growth and developmental pathways of *Geodorum densiflorum* (Lam.) Schltr.

Treatment		Rhizome formation (%) ± S.E	Axillary branch formation (%) ± S.E	Callus formation (%) ± S.E	Direct PLB formation (%) ± S.E
NAA (µM/l)	Cytokinin (µM/l)				
0	0	86.67 ± 6.67	46.67 ± 6.66	0	53.33 ± 13.33
1	0	100 ± 0	76.67 ± 1.67	0	80 ± 19.99
2	0	88.89 ± 11.11	65.56 ± 8.67	0	51.66 ± 25.87
0	BAP0.5	100 ± 0	60 ± 11.54	33.33 ± 6.66	53.33 ± 13.33
1	BAP0.5	100 ± 0	60 ± 11.54	0	53.33 ± 6.66
2	BAP0.5	100 ± 0	60 ± 30.54	0	100 ± 0
1	BAP1	93.33 ± 6.66	93.33 ± 6.66	0	80 ± 11.54
2	BAP1	86.67 ± 6.66	86.67 ± 6.66	80 ± 11.54	0
0	BAP2	93.33±6.66	93.33 ± 6.66	85 ± 7.63	6.67 ± 6.66
2	BAP2	100 ± 0	100 ± 0	93.33 ± 6.66	6.67 ± 6.66
0	BAP4	93.33 ± 6.66	93.33 ± 6.66	86.67 ± 6.66	0
1	BAP4	100 ± 0	100 ± 0	53.33 ± 6.66	13.33 ± 6.66
0	TDZ0.5	35 ± 4.99	70 ± 9.99	15 ± 7.63	78.33 ± 11.66
1	TDZ0.5	58.33 ± 8.33	66.67 ± 8.33	0	100 ± 0
1	TDZ1	60 ± 11.55	53.33 ± 6.66	0	80 ± 0
2	TDZ1	33.33 ± 6.67	66.67 ± 13.33	0	26.67 ± 13.33
1	TDZ2	40 ± 0	66.67 ± 13.33	0	0
2	TDZ2	53.33 ± 17.64	86.67 ± 23.09	26.67 ± 26.66	20 ± 11.54
0	TDZ4	53.33 ± 6.67	0	13.33 ± 13.33	26.67 ± 13.33
1	TDZ4	40 ± 0	73.33 ± 6,66	6.67 ± 6.66	26.67 ± 13.33
2	TDZ4	46.67 ± 6.67	73.33 ± 13.33	0	26.67 ± 6.66
0	2iPA0.5	86.67 ± 6.67	66.67 ± 6.67	86.67 ± 6.67	0
1	2iPA0.5	60 ± 11.55	56.67 ± 3.33	46.67 ± 17.64	13.33 ± 6.67
2	2iPA0.5	80 ± 0	53.33 ± 6.67	80 ± 0	6.67 ± 6.67
1	2iPA1	73.33 ± 13.33	58.33 ± 10.14	60 ± 23.09	26.67 ± 13.33
2	2iPA1	73.33 ± 6.67	73.33 ± 6.67	73.33 ± 6.67	20 ± 0
1	2iPA2	86.67 ± 6.67	26.67 ± 6.67	86.67 ± 6.67	6.67 ± 6.67
2	2iPA2	73.33 ± 6.67	33.33 ± 6.67	73.33 ± 6.67	6.67 ± 6.67
0	2iPA4	86.67 ± 6.67	6.67 ± 6.67	86.67 ± 6.67	6.67 ± 6.67
1	2iPA4	66.67 ± 6.67	46.67 ± 6.67	66.67 ± 6.67	0
0	Kn0.5	93.33 ± 6.67	36.67 ± 8.82	40 ± 20	36.67 ± 8.82
1	Kn0.5	66.67 ± 17.64	82.22 ± 9.69	6.67 ± 6.67	82.22 ± 9.69
0	Kn1	90 ± 5.77	65 ± 5	6.67 ± 6.67	65 ± 5
1	Kn1	60 ± 0	77.78 ± 11.11	22.22 ± 11.11	77.78 ± 11.11
2	Kn1	73.33 ± 17.64	78.33 ± 11.67	6.67 ± 6.67	78.33 ± 11.67
0	Kn2	80 ± 11.55	51.11 ± 8.89	24.44 ± 4.44	51.11 ± 8.89
2	Kn2	93.33 ± 6.67	63.33 ± 8.82	6.67 ± 6.67	63.33 ± 8.82
0	Kn4	80 ± 11.55	71.67 ± 17.40	13.33 ± 6.67	71.67 ± 17.40
1	Kn4	66.67 ± 6.67	47.22 ± 13.89	30.55 ± 2.78	47.22 ± 13.89
2	Kn4	86.67 ± 6.67	61.67 ± 21.67	8.33 ± 8.33	61.67 ± 21.67

Data shown are the mean of three replicates ± Standard error (SE).

Table 4. Effects of different Cytokinins and NAA on rhizome length and necrosis of *Geodorum densiflorum*.

Treatment		Rhizome length (mm) ± S.E.	Necrosis ± SE
NAA (µM/l)	Cytokinin (µM/l)		
0	0	2 ± 0.12	0
1	0	3.87 ± 0.06	13.33 ± 6.66
2	0	7.6 ± 0.31	20 ± 11.54
1	BAP0.5	3 ± 0.11	0
2	BAP0.5	2.7 ± 0.15	0
2	BAP1	1.87 ± 0.06	13.33 ± 6.66
1	BAP2	1.6 ± 0.09	6.67 ± 6.66
2	BAP2	1.36 ± 0.08	0
0	BAP4	1.5 ± 0.17	6.67 ± 6.66
1	BAP4	1.93 ± 0.13	0
0	TDZ0.5	1.9 ± 0.21	6.67 ± 6.66
1	TDZ0.5	2.27 ± 0.39	13.33 ± 6.66
2	TDZ0.5	1.6 ± 0.09	0
1	TDZ1	2.17 ± 0.16	0
2	TDZ1	1.77 ± 0.14	0
1	TDZ2	2 ± 0.11	0
2	TDZ2	2.7 ± 0.60	0
1	TDZ4	2.13 ± 0.06	0
2	TDZ4	2.07 ± 0.06	0
1	2iPA0.5	0.967 ± 0.03	26.67 ± 6.66
2	2iPA0.5	1 ± 0.11	13.33 ± 6.66
1	2iPA1	2.16 ± 0.20	13.33 ± 13.33
2	2iPA1	0.367 ± 0.03	13.33 ± 6.66
0	2iPA2	0.6 ± 0.05	6.667 ± 6.66
1	2iPA2	1 ± 0	6.667 ± 6.66
2	2iPA2	1.3 ± 0	6.667 ± 6.66
1	2iPA4	0.4 ± 0.09	13.33 ± 6.66
2	2iPA4	1.2 ± 0	6.667 ± 6.66
0	Kn0.5	0.6 ± 0.05	0
1	Kn0.5	0.3 ± 0	33.33 ± 17.63
2	Kn0.5	0.5 ± 0	20 ± 11.54
1	Kn1	0.6 ± 0.05	40 ± 0
2	Kn1	1 ± 0.11	26.67 ± 17.63
1	Kn2	0.6 ± 0.05	13.33 ± 13.33
2	Kn2	1 ± 0.11	6.67 ± 6.66
1	Kn4	0.5 ± 0	33.33 ± 6.66
2	Kn4	0.36 ± 0.33	13.33 ± 6.66

Data shown are the mean of three replicates ± Standard error (S.E.).

of MS supplemented with various concentrations and combinations of NAA and TDZ (Fig. 2D). In addition to this, TDZ alone or in combination with NAA also exhibited elongation of rhizomes. On the contrary, explants cultured in Kinetin failed to show any

positive response of growth, which was also reported by Sheelavantmath et al. (2000). 2iPA failed to exhibit any inductive growth and development. On the contrary, necrosis was comaparatively high in 2iPA and kinetin treatments compared to rest of the cytokinins..

Thus, the concentration and combination of auxins and cytokinins present in the nutrient media are important factors which determine successful plant regeneration (North et al. 2010). TDZ promoted leafy shoot regeneration with the formation of basal pseudo bulbs. Low concentrations of NAA induced root development (Sheelavantmath et al. 2000).

Initially 10 well rooted plantlets were transplanted to pots containing soilrite under laboratory condition and kept under proper observation. More than 80% of the plantlets survived and initiated growth after about 2-3 weeks (Fig. 2E). After commencement of new growth evidenced through the formation of daughter pseudo-bulb at the axillary position, the parent plant gradually turned necrotic. Finally these plants were transferred to their natural habitats (Fig. 2F) following proper acclimatization protocol. 70% survival was recorded after 6 months of transfer.

Considering all the results it can be concluded that BAP, among all the tested cytokinins, was the most effective one for large scale rhizome mediated multiplication of *Geodorum densiflorum* and TDZ could be effectively employed for rapid leafy shoot regeneration.

Therefore, this paper reports a simple procedure for efficient production of plantlets through rhizome node cultures of *Geodorum densiflorum* (Lam.) Schltr. which could be exploited for large scale clonal propagation and *ex- situ* conservation of this medicinally important endangered orchid. Additionally the differential role of cytokinins on rhizome mediated growth and morphogenesis of this orchid species has been highlighted.

References

Akter S, Imam MZ and**Akter T** (2010) Antimicrobial activity of different extrcts of *Geodorumdensiflorum* (Lam.) Schltr. pseudobulb. Stamford J. Pharm.Sci. **3**: 47-48.

Arditti J (1967) Factors affecting the germination of orchid seeds. Bot.Rev. **33**: 1.

Bhadra SK and **Hossain MM** (2003) *In vitro* Germination and Micropropagation of *Geodorumdensiflorum* (Lam.) Schltr., an Endangered Orchid Species. Plant Tissue Cult. **13**: 165-171.

Bohidar S, Thirunavoukkarasu M and **Roa TV** (2008) Effect of Plant Growth Regulators on *in vitro* micro propagation of 'Garden Rue' (*R. graveolens*L.)," Int. J. Integrated Biol.**3**: 36-43.

Dash PK, Sahoo S and **Bal S** (2008) Ethnobotanical Studies on Orchids of Niyamgiri Hill Ranges Orissa, India. Ethnobot.leaflets**12**:70-78.

Datta KB, Kanjilal B and **De Sarkar D** (1999) Artificial seed technology: development of a protocol in *Geodorum densiflorum* (Lam) Schltr -An endangered orchid. Curr. Sci.**76**: 1142.

De Pauw MA,Remphrey WR and **Palmer CE** (1995) Thecytokinin preference for *in vitro* germination and protocorm growth of *Cypripedium candidum*. Ann. Bot. **75**: 267.

Gubbuk H and **Pekmezci M** (2004) *In vitro* propagation of some new banana types (*Musa* spp.). Turkish J. Agric. Forestry. **28**: 355-361.

Hussein N (2012) Effects of nutrient media constituents on growth and development of banana (*Musa* spp.) shoot tips cultured *in vitro*. Afri. J. Biotech.**11**: 9001-9006.

Knudson L (1946) A new nutrient solution for germination of orchid seed. Amer. Orc.h Soc. Bull. **15**: 214-217.

Lindemann EGP,Gunckel JE and **Davidson OW** (1970) Meristem culture of Cattleya. Amer OrchSoc. Bull. **39**: 1002- 1004.

Little TM and **Hills FJ** (1978) Agricultural experimentation (John Wiley and Sons, New York). 61.

Mitra GC (1986) *In vitro* culture of orchid seeds for obtaining seedlings. Biology,conservation and culture of orchids. pp. 401.

Murashige T and **Skoog F** (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiologia Plantarum. **15**: 473-497.

Nath M, Dutta BK and **Hajara PK** (2011) Medicinal plants in major diseases by Dimasatribes of Barak valley. Assam University J. Science and Technol.: Biology and environmental Sciences.**7**: 18-26.

Ngomuo M, Mneney E and **Ndakidemi P** (2013) The effects of auxins and cytokinin on growth and development of (*Musa* Sp.) Var. "Yangambi"explants in tissue culture. Amer. J. Plant Sci. **4**: 2174-2180.

North J, Ndakidemi P and **Laubscher CP** (2010) Thepotential of developing an *in vitro* method for propagatingStrelitziaceae. Afri. J. Biotech. **9**: 7583-7588.

North J, Ndakidemi P and **Laubscher CP (**2012) Effects of antioxidants, plant growth regulators and wounding on phenolic compound excretion during micropropagationof *Strelitziareginae*. Int. J. Phys. Sci. **7**: 638-646. http://dx.doi.org/10.5897/IJPS11.786.

Patil MV and **Patil DA** (2005). Ethnomedicinal practices of Nasik district, Maharashtra. Indian j traditional knowledge. **4**: 287-290.

Roy J and **Banerjee N** (2001) Cultural requirements for *in vitro* seed germination, protocorm growth and development of *Geodorumdensiflorum* (Lam.) Schltr. Indian J. Expt. Biol. **39**: 1041-1047.

Roy J and **Banerjee N** (2002) Rhizome and shoot development during *in vitro* propagation of *Geodorumdensiflorum*(Lam.) Schtr. Sci. Hort.**94**: 181-192.

Sheelavantmath SS, Murthy HN, Pyati AN, Kumar HGA and **Ravishankar BV** (2000) *In vitro* propagation of the endangered orchid, *Geodorumdensiflorum*(Lam.). Schltr. through rhizome section culture. Plant Cell Tiss.Org. Cult. **60**: 151-154.

Theng PA and **Korpenwar AN** (2014) Studies on phytochemical, pharmacognostic and physicochemical investigations of an endangered orchid- *Geodorumdensiflorum*(Lam.) Schltr. Int. J. Bioassays. **3**: 1771-1774

Vacin EF and **Went FW** (1949) Use of tomato juice in the asymbiotic germination of orchid seeds. Bot. Gaz. **111**: 174- 183.

Vuylsteke D (1989) Shoot-tip culture for the propagation, conservation and exchange of *Musa germplasm*, IBPGR, Rome.

(Manuscript received on 28 April, 2020; revised on 7 May, 2020)