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A new report of *in vitro* flowering and multiple shooting in a wild epiphytic orchid *Oberonia recurva* Lindl. from asymbiotically germinated seedlings

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Abstract

Anthropogenic activities are sparingly high from past many decades that are a major threat for the genetic diversity. *Oberonia recurva* is distributed in South West of India mainly in the areas of Western Ghats. The Western Ghats were once covered with dense forests. But now, a large part of the area has been logged or converted to agricultural land or cleared for livestock grazing, reservoirs and roads. The growth of populations around protected areas and other forests has also led to habitat destruction, increased fragmentation, wildlife poaching and human-wildlife conflict. Major threats to this species are from deforestation and habitat destruction. Endemism and particular habitat makes it prone to threats. Therefore, the aim is to set up *in vitro* protocol for this delicate species. Asymbiotic seed germination, seedling development and effect of cytokinins on germination and seedling development were investigated in *O. recurva*. Five different media (Vacin and Went, Modified Knudson C, ½ MS, Lindemann and BM1) were examined for their effectiveness in promoting seed germination. Three light treatments for seed germination (0/24, 16/8, 24/0 h light/dark cycle) and three light treatments for seedling development were examined (8/16, 12/12, 16/8 h light/dark cycle). Effect of cytokinins (BAP, KN, ZEA) were also examined along with five media and percentage seed germination was also observed. Out of all the five media tried, BM1, LM and ½ MS showed high germination percentage 86.66%, 84.23% and 80.75% respectively, but the seedling development was high in BM1 and ½ MS. The best responded light treatment was 16/8 for germination and the same results were observed for the seedling development too. Effect of cytokinins was observed on germination and seedling development. Higher concentrations of cytokinins were used for multiple shooting and *in vitro* flowering. KN and BA were the best responded cytokinins for the multiple shooting and *in vitro* flowering.

Keywords: *Oberonia recurva*; cytokinin; *in vitro* flowering; seed germination; seedling development.
Abbreviations : BAP_6-Benzylaminopurine; BM_1 -Terrestrial orchid medium; KC_Knudson C; KN_Kinetin; LM_Lindemann; MS_Murashige and Skoog; VW_Vacin and Went; ZEA_Zeatin.

Introduction

Orchids are the largest group amongst angiosperms. The Royal Botanical Garden (RBG) of Kew listed 880 genera and nearly 22,000 accepted Species, but the exact number is unknown (perhaps as many as 25,000) because of taxonomic disputes (Chase et al., 2003). According to the estimates, the family Orchidaceae includes 800 genera and 25,000 species (Dressler, 1993; Stewart and Griffiths, 1995; Mabberley, 1997; Koopowitz, 2001; Cribb et al., 2003, Dressler, 2005; Souza and Lorenzi 2008). As other important Orchid growing countries, India is blessed with a wealth of Orchid flora, and about 1300 species are estimated to occur in our country (Jain, 1980; Kumar and Sasidharan, 1985). *Oberonia* is a large genus of primarily epiphytic orchids, rarely lithophytic in habit, comprises about 330 species widely distributed in East Africa, India, and Indonesia (Bose et al., 1999). The plant grows well in a moist atmosphere and in temperatures ranging 15.5°- 26.5°C. Sometimes plants occur on the rocks, boulders and cliff faces exposed to the full heat of the sun, but orchids grow on the trunks and branches of rainforest trees, occasionally extending their range into drier sites. *Oberonia recurva* is minute, epiphytic herb with fleshy leaves (Fig. 1a). Racemes are straight or recurved. Capsules are sub-rotund to obovoid. (Fig. 1b). The native species of *Oberonia* have tiny flowers but flowers are not self-pollinating. Capsules are produced sporadically and in groups along the racemes and the flowers are believed to by splash-pollinated by raindrops dislodging the pollen from the anthers. Reproduction in Oberonia is solely from seed. Seed dispersal takes 4-6 months from pollination. The plants are facing threat because of habitat destruction. Plants were collected from hill base of Panhala forest, Kolhapur District, Maharashtra (India) (21^0 30'58.33" N; 79^0 48' 28.86" E elevation 1159 ft). Western Ghats are protected but not all the habitats of the species. Symbiotic germination is the major way of seed germination in nature but symbiotic germination should not help us to understand the nutrient needs of the plant, therefore *in vitro* germination provides the way to understand the plant species and requirements for growth. Many Orchidologists believe that asymbiotic seed germination is an efficient way to increase the multiples of endemic orchids and also helps in their conservation (Van Waes and Debergh, 1986; Malmgren, 1992; Stewart and Kane, 2006; Timothy et al., 2007; Dutra et al., 2008). No earlier work was observed about the symbiotic or asymbiotic germination of *Oberonia recurva* and other species of *Oberonia* except *O. ensiformis*. The present investigation is a new report for the asymbiotic seed germination and seedling development of the *O. recurva*. Malabadi et al., 2012

investigated *in vitro* seed germination of *O. ensiformis* (Rees) Lindl, from the Western Ghats and used smoke saturated water (SSW) for promoting seed germination. Authors observed 85% germination on Mitra et al., (1976) basal medium supplemented when supplemented with 10% (v/v) SSW. Nevertheless Malabadi et al., 2012 had discussed *in vitro* germination in *O. ensiformis* but have not discussed the different media, effects of light and cytokinins on the same genus. Therefore the main objectives of this research were 1) To increase the seed germination percentage, 2) To study the need of plants about nutrition and photoperiod. 3) To study the effects of three cytokinins on seed germination and seedling development.

Results

Effect of media and cytokinins on seed germination

All the five media were observed for seed germination and seedling development with and without cytokinins. Seeds began swelling within 3rd week after the inoculation and germination commenced from 4th week (Fig. 1 c, d) after inoculation but on the media without cytokinins, the swelling began late (6th week) and germination was observed from 8th week. The germination percentage was not significantly different on both (with and without cytokinins) but germination was delayed on medium without cytokinins. Visual contamination rate of cultures was 5%. Out of all the five media, BM1, LM and ½ MS showed germination and early protocorm development 86.66%, 84.23% and 80.75% respectively and earliest protocorm development on BM1 and ½ MS. LM showed higher germination percentage but the seedling development was quite low as compare with BM1 and ½ MS (Fig. 3). Nevertheless on BM1 medium, highest seed germination and seedling development was recorded but seedlings became pale in stage 5 whereas on ½ MS, both seed germination and seedling development was high and no yellowing of seedlings with time was observed. Germination percentage on KC (72.22%) and VW (76.06 %) was also good but seedling development was low. On VW medium, seedling development was recorded upto stage 2 because further development of protocorm was arrested. Interestingly, seed germination percentages and protocorm developing period was not constant to all media, but at 6th week, stage 2 was highest in ½ MS (58.2%) and followed by BM1 (30.2%) and stage 1 in VW (92.3%), KC (83.33), BM1(65.47%), LM (88.7%) and ½ MS (41.76%) (Fig. 3). However, BM1, ½ MS supported protocorm development to a complete plant development (Stage 5) at 15 weeks (Fig. 3) incubation. LM and KC supported seed development to Stage 5 but growth rate was slow.

Photoperiod effect on germination and development

Photoperiod is an important factor for germination and development of seedlings. In this experiment, it was observed that the photoperiod is playing important role in the germination. Germination percentage was significantly different in three photoperiods 0/24, 16/8, 24/0 are 76.26%, 90.37%, 89.69% respectively on MS medium. All developmental stages (Stage 0, 1, 2, 3, 4, and 5) were observed in all three photoperiods irrespective of the time (Fig. 4). Three photoperiods (8/16, 12/12, 16/8 h light/dark cycle) were analysed for further development of the seedlings and significant difference were observed. On 12th week Stage

Table 1. Comparative mineral salt content of six asymbiotic Orchid Seed germination media**. [**Vacin and Went (VW), Kundson C (KC), BM-1 Terrestrial orchid medium (BM1), Lindemann (LM) and ½ MS Murashige and Skoog (½ MS)].

	VW	KC	BM1	LM	½ MS
Macronutrients (mM)					
Ammonium	3.78	10.02		15.14	10.31
Calcium	0.4	2.12		2.12	1.5
Chlorine		3.35	0.0021	14.08	3.1
Magnesium	1.01	1.01	0.83	0.49	0.75
Nitrate	5.19	8.37		2.12	19.7
Potassium	7.02	5.19	2.2	15.07	10.89
Phosphate	2.47	1.84	2.2	0.99	0.63
Sulfate	5.3	4.92	1.1	8.1	0.86
Sodium	0.1		0.2		0.1
Micronutrients (µM)					
Boron			161.7	16.4	50
Cobalt			0.11		0.053
Copper			0.1	0.1	0.5
Iron	183	90	100.2	17.96	50
Iodine				0.6	2.5
Manganese	33	37	147.9	37.63	50
Molybedenum			1.03		0.52
Zinc			34.8	3.5	14.95
Nickel				0.24	
Organics (mg/l)					
D-Biotin			0.05		
Casein hydrolysate			500		
Folic acid			0.5		
L-Glutamine			100		
Glycine			2	2	2
myo-Inositol			100	100	100
Nicotinic acid			5	1	0.5
Pyridoxine. HCl			0.5	1	0.5
Thaimine. HCl			0.5	10	0.1
Total mineral salt concentration (mM)	25.27	36.82	6.53	58.11	47.84
Total N	8.97	18.39	0	17.26	30.01
NH4:NO3	0.73	1.12	0	7.14	0.52

Fig. 1. Seed source and asymbiotic germination of *Oberonia recurva*. (A) Plant in habitat. (B) Close up of fruits (C, D) Seed germination and Protocorm development. (E) Complete *in vitro* developed seedling. Scale Bars, 1 cm.

Table 2. Seed germination and protocorm development in *Oberonia recurva*

Stage	Description
0	Hyaline embryo, testa intact
1	Embryo swollen, rhizoids present (=germination)
2	Continued embryo enlargement, testa ruptured
3	Emergence of first leaf
4	Elongation of first leaf and further development
5	Complete developed plant

Fig 2. *In vitro* development of *Oberonia recurva*. (A, B) Initiation of multiple shooting. (C, D) Effect of Kinetin. (E) Effect of BA. Scale Bars, 1cm.

3 was highest 29.76% in ½ MS on 16/8 and 28.98% in 12/12, nevertheless in 8/16 stage 3 was not initiated (Fig. 4).

Effect of cytokinins

In this experiment cytokinins were investigated for the seed germination and development of the plant (Table 3). The best results of seed germination were observed in KN (13.95 µM, 95.01%), ZEA (9.12 µM, 94.08%) and BAP (4.44 µM, 85.04%) (Fig.5). In order to find out the effects of cytokinin (BAP, KN) on seedling development, complete developed seedlings were transferred to different concentrations of BAP (4.44 – 22.2 µM) and different concentrations of KN (4.65 – 23.25 µM) for *in vitro* flowering and multiple shooting (Table 3, Fig 2). Multiple shooting was higher in 4.65, 9.30, and 23.25 µM of KN. Lower concentrations (4.65, 9.30 µM) showed multiple shoots but with lesser shoot height (1.83,1.96 cm respectively) and lesser leaf width (0.33, 0.26 cm respectively) (Table 3) on the other hand, 23.25 µM showed higher shoot number with high shoot height (2.3 cm) and larger shoot width. BAP showed multiple shooting in 4.44 – 22.2 µM, out of all concentrations, best responded was 4.44 µM that showed higher shoot number with larger shoot height (3 cm that was higher as compare with KN). Rooting was also observed in the same concentrations of KN and BAP (Fig. 2 E) therefore no other rooting hormones were tested; probably endogenous auxins are promoting root development in *O. recurva*. Early *in vitro* flowering was also observed in some concentrations of Kin and BA. Highest *in vitro* flowering was observed in 13.95 µM of KN and 4.44 µM BAP (Fig. 2). In the present investigation, *in vitro* flowering and multiple shooting was highest in 4.44 µM BAP. *In vitro* pollination was not possible because flowers were minute.

Discussion

O. recurva is a taxon on that no previous work has been reported regarding *in vitro* propagation and seed germination (asymbiotic and symbiotic). Some reports are there that are concerned with taxonomic work. Different media were used to study the nutrient needs of the epiphytic orchids for asymbiotic germination. The asymbiotic media used in this experiment contain similar components (carbohydrates, minerals salts, gelling agent); nevertheless media vary in specific mineral salts, additives and vitamins. All media used in the current study contain a nitrogen source, either as inorganic nitrogen or organic nitrogen. Some media also have mixture of both inorganic and organic nitrogen source (Table 1). Knudson C and VW contain inorganic sources of nitrogen only, although BM1 contains an amino acid as the sole source of nitrogen in the media. Nevertheless LM and ½ MS contain a mixture of inorganic nitrogen sources and an amino acid. LM contains the lowest concentration of nitrogen (2.12 mM) as prepared in this study. Nitrogen source has shown different effects on different orchid species (Anderson, 1996; Stewart Kane, 2006; Van Waes and Debergh, 1986). All media initiated seed germination and supported protocorm development at least upto Stage 2, suggesting that nitrogen in any form and concentration should support asymbiotic seed germination in *O. recurva*. However, LM contained the lowest concentration of nitrogen and still supported seed germination and protocorm development. This suggests that *O. recurva* relies on lower concentration of nitrogen for the initiation of seed germination and protocorm development, and more upon ready sources of carbohydrate and moisture to support seed germination. Interestingly, BM1, ½ MS initiated seed germination and supported advanced protocorm development (Stage 5). But developed seedlings became pale and some of them died in BM1 but on the other hand, seedlings were green in ½ MS. MS contains glycine being the sole nitrogen source and BM1 contains glutamine along with glycine. Van Waes and Debergh (1986); Malmgren (1992, 1996); Anderson (1996) had reported improvement in seed germination of orchids by the addition of amino acids to the media and the reduction of inorganic nitrogen sources. These researchers have suggested that organic sources of nitrogen (i.e., amino acids) are more readily available to the seed or plant in spite of inorganic nitrogen source, because of this reason seed germination and seedling development was observed on BM1 and ½ MS but for further development, seedling needs higher concentration of nitrogen as inorganic source that is absent in BM1 and present in ½ MS probably this is the reason for yellowing of seedlings in BM1. Sporadic information is available regarding the absolute role of organic nitrogen sources in asymbiotic orchid seed germination media. In general, orchid seeds vary in their light requirement for germination and development (Darnell, 1952). Several experiments on the effect light on orchid seed germination were recorded (Kauth et al., 2006; Dutra et al., 2008, 2009; Vasudevan and Van Staden, 2010; Wang et al., 2011). Seed germination is inhibited by light incubation in terrestrial orchids (Arditti et al., 1981; Ernst, 1982; Van Waes and Debergh, 1986; Yamazaki and Miyoshi, 2006). But such inhibitory response was not observed in epiphytic orchids. In

Table 3. Effect of cytokinins on *in vitro* seedling development and flowering.

Effect of BAP & KN on *In vitro* Seedling							
Hormone (µM)	No of Multiple Shoots	Height of Shoots	Root length	Fresh Weight	Width of leafs	No. of Inflorescence	Flower Buds
KN							
4.44	20.33 ± 1.45 a	1.83 ± 0.03 d	0.76 ± 0.03 b	1.32 ± 0.28 bc	0.33 ± 0.03 b	1.33 ± 0.33 bc	48.6 ± 0.88 a
8.88	15.66 ± 1.20 b	1.96 ± 0.08 cd	0.4 ± 0.05 c	0.14 ± 0.01 d	0.26 ± 0.03 b	2 ± 0.57 b	44.33 ± 5.78 a
13.32	10 ± 1.15 c	2.23 ± 0.14 bc	1.1 ± 0.06 a	0.85 ± 0.09 c	0.33 ± 0.03 b	5.3 ± 0.33 a	54 ± 2 a
17.76	5.33 ± 0.88 d	2.7 ± 0.05 a	1.1 ± 0.1 a	2.21 ± 0.05 a	0.86 ± 0.43 a	1.3 ± 0.33 bc	50 ± 2.88 a
22.2	23 ± 1.73 a	2.3 ± 0.08 b	0.56 ± 0.14 bc	1.47 ± 0.23 b	0.66 ± 0.18 a	0.33 ± 0.33 c	52 ± 0.57 a
BAP							
4.65	29 ± 0.57 a	3 ± 0.11 a	1.23 ± 0.14 a	7.06 ± 0.17 a	0.73 ± 0.03 a	6.33 ± 0.33 a	62.33 ± 3.28 a
9.30	6 ± 0.58 b	2.5 ± 0.28 ab	1.06 ± 0.06 a	2.68 ± 0.60 b	0.53 ± 0.17 a	0.66 ± 0.66 b	51 ± 2.64 a
13.95	7.33 ± 0.33 b	2.3 ± 0.05 b	0.8 ± 0.05 b	1.89 ± 0.09 bc	0.46 ± 0.16 a	0	0
18.6	5 ± 1.15 b	2.5 ± 0.28 ab	0.66 ± 0.03 b	1.21 ± 0.22 c	0.63 ± 0.06 a	0	0
23.25	6 ± 1.52 b	2.3 ± 0.08 b	0.56 ± 0.03 b	0.79 ± 0.13 c	0.66 ± 0.03 a	0	0

Values with the same letter are not significantly different within stages (α= 0.05)

present investigation, effects of photoperiod on both asymbiotic seed germination and subsequent *in vitro* seedling development were observed. Highest seed germination and seedling development was observed in 16/8 h light/dark cycle. Photoperiod 24/0 h light/dark cycle also played an important role in seed germination and seedling development. In general orchid seeds respond to cytokinins either increase or decrease in germination (Arditti and Ernst, 1984). The particular response varies with the species. In present investigation, seed germination was observed without cytokinin also but the rate of germination was slow. For the seed germination of *O. recurva* different cytokinins were used in variable concentrations. Lower concentrations of BAP and ZEA responded better in comparison to lower concentrations of KN. But the best response was in 13.95 µM KN and even seedling development was also good. Similar results were observed by De Pauw et al., (1995) in *Cypripedium*, in that germination was enhanced by lower concentrations of BAP and higher concentration of KN. In present investigation different concentrations of KN and BAP were used that helped in developing multiple shooting and *in vitro* flowering. KN and BAP have been reported to be responsible in multiple shooting in number of orchid species (Yan et al., 2006; Thomas and Michael, 2007; Kishor and Devi, 2009). In this work, BAP responded better in comparison to KN for multiple shooting and even for *in vitro* flowering. Cytokinins are believed to be important signals for flowering (Bernier et al., 1993; Lejeunne et al., 1994; Bonhomme et al., 2000). Cytokinin is also a common requirement for *in vitro* flowering (Nitsch and Nitsch, 1967; Bernier, 1988; Peeters et al., 1991). In the present study, *in vitro* flowering was observed in KN and BAP. But 4.44 µM BA had responded the best. Similar results with BAP were observed by Sim et al., (2007, 2008); Kim et al., (2007).

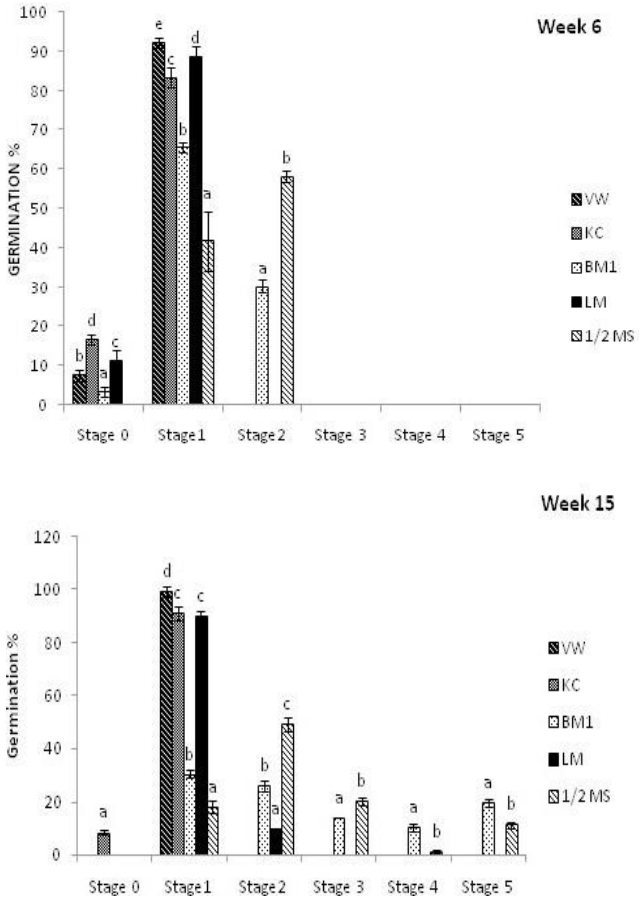

Fig 3. Effects of culture media on percent seed germination and protocorm development after 6 and 15 weeks. [Histobars with the same letter are not significantly different within stages (α= 0.05)].

Materials and Methods

Plant Material and sterilization

Seeds were inoculated from mature capsules before dehiscence. Seeds were surface sterilized for 3 minutes in 0.1% Mercuric Chloride and 70% ethanol for 30 seconds. Surface sterilized seeds were rinsed three times for 2 minutes each in sterile distilled water.

Media preparation and Inoculation

Five basal media (Table 1) were prepared viz Vacin and Went (1949) (modified by Phyto Technology V891), modified Knudson C (Knudson, 1946), ½ MS (Murashige and Skoog, 1962), Lindemann (LM; Lindemann et al., 1970) and BM1 (Van Waes and Debergh, 1986). Media were adjusted to pH 5.8 with 0.1N NaOH. Approximately 30 sterilized seed were placed into the centre of flask and the seeds were evenly spread on the medium. Ten replicates were inoculated per medium type. Germination and protocorm development were scored on a scale of 0–5 (Table 2).

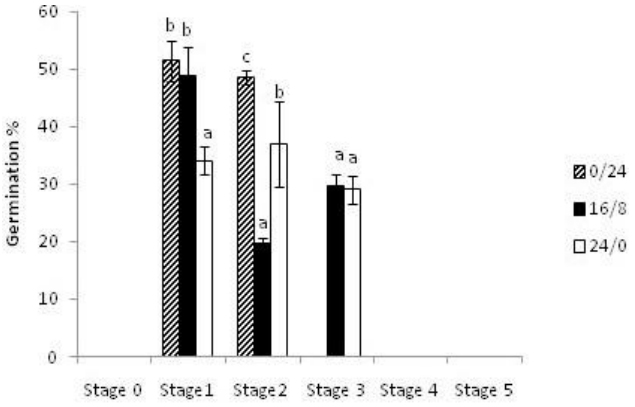

Fig 4. Effects of different photoperiod on percent seed germination and protocorm development in ½ MS Media on Week 12. [Histobars with the same letter are not significantly different within stages (α= 0.05)].

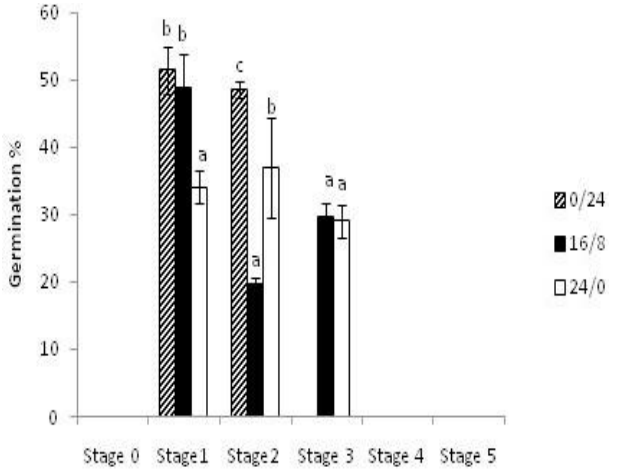

Fig 5. Effects of different cytokinins on seed germination. [Histobars with the same letter are not significantly different within stages (α= 0.05)].

Experiments

The effects of three photoperiod treatments (0/24, 16/8, 24/0 h light/dark cycle) on asymbiotic seed germination were observed. Seed germination and protocorm development were scored regularly. The effects of three photoperiods (8/16, 12/12, 16/8 h light/dark cycle) on subsequent *in vitro* seedling development of *O. recurva* were evaluated. Cultures were maintained at 25±5°C. Effects of different cytokinins (BAP, KN, and ZEA) on asymbiotic germination and seedling development at different concentrations (BAP – 4.44, 8.88, 13.32 µM; KN – 4.65, 9.30, 13.95 µM and ZEA-4.56, 9.12, 13.68 µM) were examined. All the five media were supplemented with different concentrations of cytokinins (BAP, KN, and ZEA). The best responded medium (½ MS) was used with higher concentrations of KN and BAP for *in vitro* flowering and multiple shooting.

Statistical Analysis

Germination percentages were calculated by dividing the number of seeds in each individual germination and development stage by the total number of viable seeds in the sample. Data were subjected to one-way analysis of variance (ANOVA), and the means were compared by Duncan's multiple comparison test (α = 0.05) using DSAASTAT version 1.1. Means are presented with standard error (±SE).

Conclusions

It is concluded that if an orchid is critically dependent on a compatible mycorrhiza for germination in nature, the loss of that mycorrhizal fungus *in situ* ultimately results in non establishment of the orchid in new habitat. Therefore, current study presents a first look at the asymbiotic germination and development of *O. recurva*. Investigation revealed that ½ MS is the best medium for multiplication and development. Data also includes that how cytokinins help in germination of the *O. recurva*. Multiple shooting and *in vitro* flowering was also observed in the presence of high concentrations of cytokinins.

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