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Effects of Different Media and Organic Additives on Seed Germination of Geodorum Densiflorum (Lam) Schltr. – An Endangered Orchid

Agriculture

KEYWORDS : Geodorum densiflorum, Endangered Orchid, Seed germination, Additives, Media

S. Muthukrishnan	Department of Plant Science, Bharathidasan University, Tiruchirappalli- 620 024, Tamil Nadu, India.
T. Senthil Kumar	Department of Industry University Collaboration, Bharathidasan University, Tiruchirappalli- 620024, Tamil Nadu, India.
M. V. Rao	Department of Plant Science, Bharathidasan University, Tiruchirappalli- 620 024, Tamil Nadu, India.

ABSTRACT

Interaction of different media and organic additives showed significant effects on seed germination of G. densiflorum. Among the different organic additives tomato extracts significantly enhanced seed germination and was found to be the most suitable one for enhance the seed germination, It was also the best among treatments for production of viable protocorms. Highest seed germination was recorded in 5% tomato extract fortified with ½ MS medium than compared to mMS, MS, KC medium. In this study, Tomato was selected to be the best organic additive and ½ MS medium as the suitable medium for seed germination of G.densiflorum when compared to other organic additives like potato and coconut water and different media.

Introduction

Orchids are experiencing a steady decline in tropical countries due to destruction of natural forest areas. Some species are disappearing due to extensive collection and habit destruction by anthropogenic activities. It is essential to take measures for the conservation and propagation of these endangered orchid species. Geodorum densiflorum (Lam) Schltr., is an endangered terrestrial orchid appearing above the ground only during the rainy season (Datta et al., 1999). The species is horticulturally important for its attractive dense cluster of pink to white flowers, as well as for the anti-diabetic property of its underground pseudobulb (Lakshminarasimhan and Sharma 1991; Hegde 1996). Because of damage of its natural habitats by continuous destruction of forest for land reclamation and indiscriminate collection by orchid lovers, this species has now become endangered. But the demand of such orchids is increasing day by day in local and foreign markets. As orchid seeds do not possess mature endosperm, their natural germination is limited and need a symbiotic association with specific mycorrhizal fungus. Orchids also propagate vegetatively, but the conventional methods of propagation of orchids are very slow and laborious. For these reasons the price of orchids is very high. The discovery of in vitro germination and micropropagation contribute immensely to alleviate their scarcity. Sheelavantmath et al. (2000) and Bhadra and Hossain (2003) reported a protocol for rhizome based propagation of G. densiflorum. It is important to study the different aspects of in vitro propagation using both seeds and explants.

For conservation and mass multiplication of threatened and endangered orchids, asymbiotic seed germination is considered much faster and effective method, successfully reported in G. densiflorum (Bhadra and Hossain 2003; Roy and Banerjee 2002; Sheelavantmath et al., 2000).

Experiments on seed germination including those referred above have reported only the effects of basal media on germination only; further seedling development was experimented together with the addition of growth regulators or organic additives. In general, orchid seeds have sufficient growth hormones to germinate and develop into seedlings in nature (Arditti, 1979). Therefore mass propagation of the seedlings in vitro could be achieved with a suitable medium devoid of growth regulators. Also, seedlings raised through this growth regulator free experiment would help as a source of explants to produce multiples of desired healthy plantlets. It is suggested that Terrestrial species can germinate both in the light and dark and are seem to require light for the induction or improvement of shoot and or root formation (Arditti, 1979). The present investigation was undertaken with a view to develop efficient in vitro asymbiotic seed germination for using media and different additives, for G. densiflorum.

MATERIALS AND METHODS

Seed source, sterilization procedure

Green, mature capsules (undehisced) were collected from Kolli hills, Eastern Ghats, Tamil Nadu, India. The seeds measured ca. 273-340 x 61-106 µm in size and numbered around 1000-1200 seeds per one mg weight. The embryos were elliptical and yellowish in colour.

A single capsule was washed in running tap water with 1-2 drops of soap solution (Teepol) for 15 min. Then the capsule was transferred to sterilized laminar hood. The capsule was air dried for while and dipped in 70% alcohol for few seconds and burnt off in seconds. This was repeated two to three times. Finally the capsule cut opened longitudinally with a sterile surgical knife and the seeds were carefully extricated and sown on media.

Seed viability test

Seed viability was tested by using TTC (2, 3, 5-triphenyl tetrazolium chloride) test (Waes, 1986). 10 ml of 1% TTC solution with the pH 7.0 was taken in a screw cap tube with the seeds. Then the solution was kept in dark at 20±2^{0}C for 24 hrs. After 24 hrs, the solution was drained and the seeds were washed in sterile distilled water for 3 times. Red stained viable seeds and yellowish unstained seeds were counted under a light microscope with several samples to get the percentage.

Asymbiotic media selection and culture condition

Fourteen basal media viz, Murashige and Skoog (1962) medium (MS), Burgeff (1936) medium (N3F), Curtis (1936) medium (Curtis), Ernst (1982) medium (RE), Fast (1976) medium (F), Ichihashi and Yamashita (1979) medium modified by Phytotech(NP), Knudson (1946) medium (KC), Liddell (1953) medium (LIddl.), Mitra et al., (1976) medium (Mitra), Vacin and Went (1949) medium (VW), Wolter (1968) medium (W&S) were used in the present study. MS medium diluted and modified into ½ macro strength (½ MS), MS vitamin replaced with B_5 vitamin (mMS, m ½ MS,). Every medium was prepared without any of its prescribed growth regulators and the organic additives. All the media were set with their appropriate pH and sugars and solidified with 8% agar before autoclaved at 121^{0}C and 1.5 kg cm^{-2} for 20 min. The seeds were sown onto the surface of the medium and the plates were wrapped with 2-3 layers of parafilm. Cultures were maintained under white fluorescent lamp with 16 hrs photoperiod at a temperature of 23±2^{0}C.

Effect of Additives

Organic additives like Coconut water, tomato juice, potato juice 5, 10 and 15ml/l (w/v) were supplemented to seed germination medium like MS, ½ MS, mMS, KC to assess the enhancement of seed germination rate.

Experimental design and data collection
For both the experiments, a minimum of eight replicates with about 100-400 seeds were treated. The germination process was evaluated by constant observation under light microscope every 24 h from the time of sowing day, and seeds were classified according to Pierik et al. (1988). The germination % (G) was calculated.

Data related to orchid seed germination stages was adopted from Stenberg and Kane (1998) and Mariat (1952). Comparisons between treatments were made with DMRT (Duncan, 1955).

Results and Discussion
The seed germination rate and duration varied with the medium used. Maximum germination % and the appearance of protocorms were achieved on ½ MS medium.

Effect of media on seed germination and PLB development
Seed viability was about 95.76 % as confirmed by TTC test. Among the media tested, half strength MS medium supplemented with 3% sucrose observed highest in % of germination of about 95.54% (Table 1). This was almost similar to the viability tested and thus the TTC viability test proved best for this species. Significant germination was also observed on m ½ MS media fortified with B5 vitamins (87.60%), KCa (91.57%). Seed germination on KC and B5 media has also been reported for other orchids (Dohling et al. 2008) despite these media being suitable for Dendrobium chrysanthum (Hajong et al., 2010). The promoting and inhibitory effects of KC medium on the seed germination of many orchid species have been reported (Nongrum et al. 2007).

Whereas MS (87.90%) because the presence of ammonium nitrate in MS medium may explain the high germination rate because NH_4^+ is readily assimilated during the initial stages of development and greatly influences growth and differentiation (Raghavan and Torrey, 1964). PLBs are the globular green cell masses considered as the early stages of seedling development in orchids. PLBs were observed in all media (Fig. 1) except in KP, Liddell, Curtis, Fast, Mitra NP and W&S medium. The embryos formed in these media were in different in colours due to insufficient nutrients to produce chlorophylls. (Fig 1e, f.), and no further development could be observed. This may be caused by the deficiency of nutrients in those media. The size of the PLBs varied with respect to nutrient media used (Table 1). The largest PLBs were observed in ½ MS media (588.65µm) which was 4 times larger than the normal embryo size, followed by mMS medium (507.25 µm) which was 3 times larger, and also the germination rate was high than compared to MS medium. The high germination in ½ MS medium could be attributed to the fact that this medium is also especially rich in both macro- and micro-nutrients. The nitrogen source has previously been shown to affect germination of various orchid species (Anderson, 1996; Stewart and Kane, 2006).

The smallest PLBs were measured (214.15µm) in RE and W&S medium. The PLBs on major nutrient medium developed up to first 4 stages the seedling development was failed in all media. The color of the germinating seeds and the PLBs differed between media. Different coloration was observed during the germination viz., green, white, brown and black (Table 1). The yellow colored seeds turned to green and developed PLBs on MS, mMS, ½ MS, KC and m ½ MS media. The reason could be that the nutrients at macro and a micro level along with vitamins were sufficient for development. Whereas, the seeds on RE, KP, and Curtis media turned to pale green. On KC, V&W and WS media seeds and PLBs remained yellow and turned brown. NP, Fast and Mitra media seemed to be not suitable for G. densiflorum where the seeds turned white and yellowish brown color respectively. Inconsistencies in germination of the seeds amongst various media may represent insufficiency of the nutrients. All media were used here contributed to PLBs development but failed in further seedling development. This may be because of the inappropriate nutrient level or compositions. The embryos swelled and resulted in the rupture of testa either partially or completely in about 36-46 days in all media. NP, Liddell, Curtis, Fast, Mitra and W&S media were shown no further development into PLBs. Some of the media (V&W, KC, RE and N_3F, / 108, 110, 125, and 145 days respectively) took more time to develop PLBs and also struggled to promote further development. The Protocorms grew and developed primordium (Fig. 5c) with numerous rhizoidal hairs within 12-15 weeks in $^1/_2$ MS, m$^1/_2$ MS, m 1/4 MS. Different stages also observed in seed germination (Fig. 5k-m). In the present study, germination did not require the inclusion of plant growth regulators, suggesting that sufficient endogenous hormones were already present (Lo et al., 2001).

Effect of organic additives
In this study, the seed germination was carried out on MS, ½ MS, mMS, and KC medium supplemented with homogenates of various organic additives like local tomato, potato and coconut water. The type and concentrations of organic additives influenced the response of seeds in terms of germination.

It was onserved that potato homogenate added to ½ MS media at 5, 10, and 15% was not effective in enhancing the growth of PLBs in the end of week four (Table 4). Seeds in media containing 5, 10, and 15% of Potato homogenate began to form some discoloration of the surface that eventually caused the whole tissue to change into light green colour (Fig. 5h). Although Potato homogenate has been widely used in plant tissue culture, addition of organic additive was not found to be a good option to improve PLB proliferation from seed. Based on, coconut water at all concentrations in four media the seed germination was occur in reduced percentage of all the tested media at the end of week 8 except for the control(Table 2, Fig. 4). Likewise Talukdar (2001) and Kanjilal (1999) discovered that the addition of 15% of CW in ½ MS medium stimulated the in vitro seed germination and protocorm development of D. aphylumm. At concentration 10 -15%, it enhanced seed germination of many orchid species (Shantz, 1952).

It appears from the present study that among the different organic additives at various concentrations, tomato at 5% was the most effective in germination of seed and growth of PLBs (Fig. 3, Fig. 5i). Seed germination was increased when tomato homogenate was used (Table 3). However, tomato has vitamin C, K, mineral nutrients and lycopene that affect the growth development of cells. Seeds cultured in media that had 10 or 15% of tomato homogenate began to produce more number of germination and up to IV th stage to show signs of regeneration. Lower concentration of tomato homogenate was found to be inhibitory towards early germination of seeds (Fig. 3). PLB grown in tomato homogenate treatment is shown in (Fig 5i). PLBs in 5% of tomato homogenate were observed to be greener and denser but did not produce shoots. Also in this study, average time were calculated in the seeds cultured on potato homogenate, tomato homogenate and coconut water was determined after a two month of culture. Previously, we mentioned that based on this study, tomato homogenate in half strength MS medium was the best organic additive in enhancing the germination of seeds.

Conclusion
The present study is the first report a successful and efficient protocol for asymbiotic seed germination of G.densiflorum. For conservation of plants, the seeds are, in general, preferred for propagation because they maintain maximum genetic diversity. In vitro seed germination ensures germination in this hard-to-germinate taxon and this enabled us to study morphogenetic changes during seedling development. The mass propagation of orchids through asymbiotic seed germination was achieved and can now be considered as a viable tool in the conservation of the declining native orchid population. It offers a ready means for raising the large numbers of plants needed for ex situ conservation of this threatened orchid.

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Table 1. Effect of different media on seed germination of G. densiflorum

Media	Germination%	Colour of PLB's	Size of the Protocorms (µm)
MS	87.90	Yellowish green	473.25
½ MS	95.54	Light green	588.65
mMS	95.48	White color	507.25
m ½ MS	87.60	Dark green	469.15
V&W	77.92	Yellowish white	398.75
RE	83.66	Brownish/yellowish	214.25
N_3F	81.09	Dark green	330.25
KC	91.57	Light green	307.25
Mitra	41.78	Light yellowish colour	250.75
Curtis	24.75	Light yellowish	215.25
Liddel.	25.11	Light yellowish green	230.15
W&S	20.63	Brownish	214.15
Fast	26.14	Yellowish brown	310.25
NP	25.98	Brownish/yellowish	275.65

Table 2. Effect of coconut water in seed germination on selected media

Media	Additives Coconut water (ml) %	Germination %	Colour of PLB's	Size of the PLB,s (W&L)
MS	5	25.00	Light green	436-486.4
	10	43.18	Light yellowish	304-334.4
	15	47.88	Light white	298-301
½ MS	5	36.30	Yellowish green	304-334.4
	10	44.78	Light green	298-301
	15	34.94	White yellowish	245-295
mMS	5	24.39	Light green	309-321
	10	47.32	Light green	425.6-440
	15	34.09	Yellowish green	298-301
KCa	5	51.21	Yellowish green	416-423
	10	52.73	Pale yellowish	426-443
	15	38.62	Light white	321-396

Table 3. Effect of tomato in seed germination on selected media

Media	Additives Tomato (ml) %	Germination %	Colour of PLB,s	Size of the PLB,s (W&L)
MS	5	50	Light yellow green	460-377.6
	10	62.55	Light white	542-542
	15	50.42	White yellowish	430-425
½ MS	5	100	Dark green	625.6-625.6
	10	63.80	Light yellowish	547.2-547.2
	15	55.23	Light yellowish	436-486.4
mMS	5	60.78	Yellowish green	304-334.4
	10	27.61	Pale yellowish	298-301
	15	61.40	Light white	409-421
KCa	5	46.80	Light green	273.6-456
	10	53.84	Light white	216.4-436
	15	43.42	Light white	186-356

Table 4. Effect of potato in seed germination on selected media

Media	Additives potato (ml) %	Germination %	Colour of PLB's	Size of PLB's (W&L)
MS	5	37.5	Light green	450-577.6
	10	57.39	Light white	532-532
	15	50.42	White yellowish	430-525
½ MS	5	60.19	Light green	425.6-440.8
	10	68.53	Light yellowish	547.2-547.2
	15	49.41	Light yellowish	436-486.4
mMS	5	47.32	Yellowish green	304-334.4
	10	23.20	Pale yellowish	298-301
	15	42.74	Light white	309-321
KC	5	41.80	Light green	273.6-456
	10	61.11	Light white	316.4-466
	15	46.47	Yellowish green	286-456

Fig. 1 Days of responses of seed germination on different media

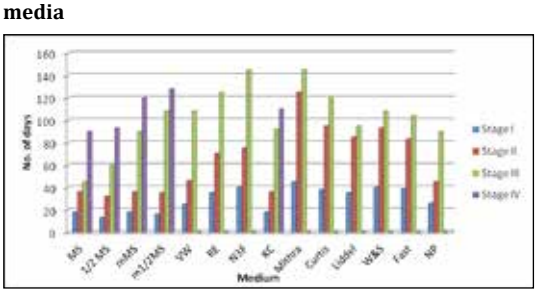


Fig. 2 Effect of potato on seed germination

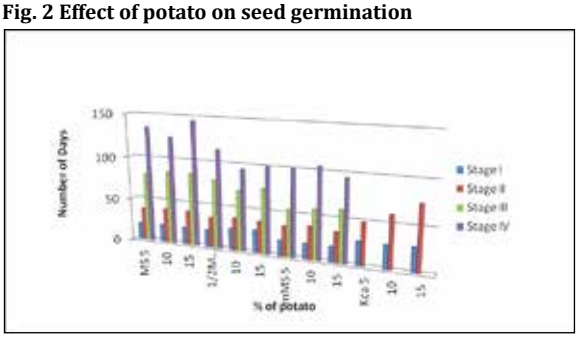


Fig. 3 Effect of tomato on seed germination

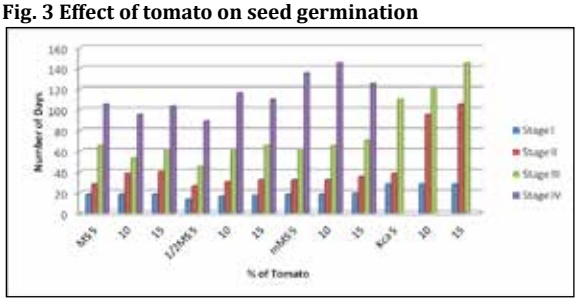


Fig. 4 Effect of coconut water on seed germination

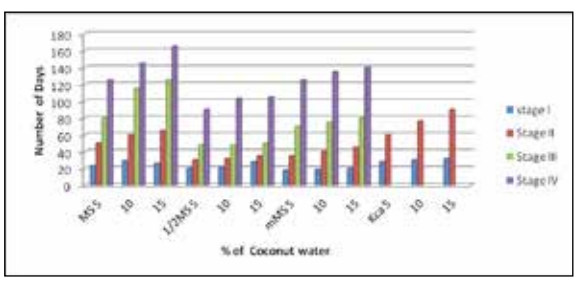
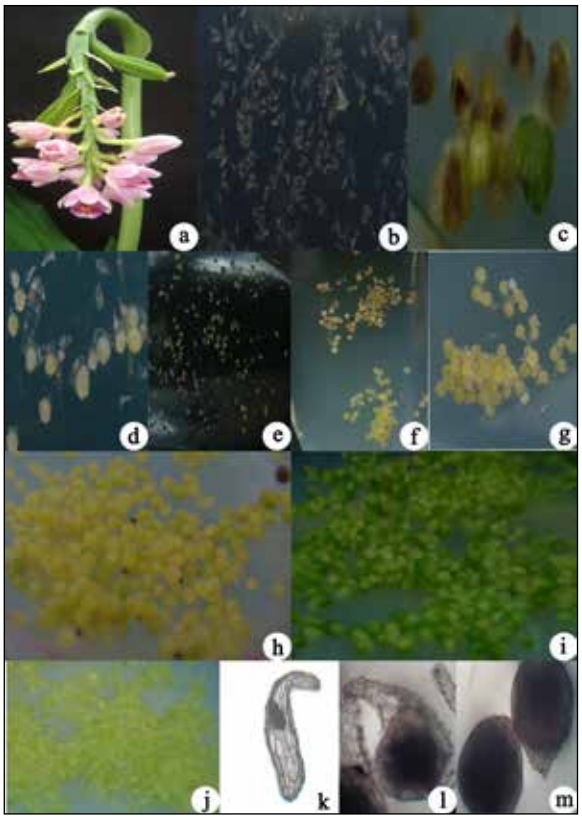


Fig. 5 Effect of media& additives on seed germination of G.densiflorum



a. Flower with pod; b. seeds soaked on media; c. PLB's with hairs; seed germination: d-KC medium; e-W&S medium; f-N_3F; g- ½ MS medium; h. Seed germination in Potato; i. seed germination in tomato; j. seed germination in Coconut water; k-m stages.

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