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Full Length Research Paper

Asymbiotic germination of immature embryos of a medicinally important epiphytic orchid *Acampe papillosa* (Lindl.) Lindl.

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The immature embryos (28 weeks after pollination) were inoculated on M (Mitra et al., 1976), and PDA (Potato Dextrose Agar) media, with and without different growth additives. The seeds showed positive germination response in both the nutrient media but the frequency and onset of germination response and associated morphogenetic changes leading to seedling development varied with the nature of growth stimulus. In basal M medium, about 40.75±0.75% seeds germinated, while in basal PDA medium, 21.25±1.25% seeds reacted positively to germination (P<5%). M medium supplemented with coconut water (CW) (15%), supported early and highest germination (70.75±0.75%) and induced protocorm multiplication; complete seedlings were obtained in 131.50±1.73 days. Additional presence of activated charcoal (AC) (0.2%) in the PDA medium inhibited the seed germination, while use of CW (15%) or yeast extract (YE) (2 g/L) in the medium, favoured enhanced and early germination response and differentiation of protocorms. YE, favored development of profusely hairy protocorms formation and healthy seedlings were obtained within 176.25±1.25 days.

Key words: *Acampe papillosa*, immature embryos, *in vitro* asymbiotic germination, protocorms.

INTRODUCTION

Orchid seeds are unique in being exceedingly small, dust like in appearance, and more or less fusiform in shape; these lack endosperm and have undifferentiated embryos enclosed within transparent seed coats. Their germination in nature is dependent upon a suitable association with a mycorrhizal fungus. Their fungal requirement can, however, be compensated by supply of sugars and other mineral nutrients *in vitro*, and several orchid species from diverse habits and habitats have successfully responded to asymbiotic germination (Arditti et al., 1982a; Hossain et al., 2009; Pathak et al., 1992, 2001, 2011), much, however, still remains to be learnt about the nutrient requirements of commercially important and / or endangered orchid species, keeping in view of the large size of the orchid family.

Acampe papillosa, a species of epiphytic orchids, has ornamental potential for its evergreen, clustered foliage and multiflowered, pendulous racemes, with yellow green flowers. Its therapeutic utility is also well documented; its roots are used to cure rheumatism (Bi et al., 2005; Lawler, 1984). Its natural populations are on decline due to commercial collection and habitat destruction. The protocols for its mass propagation are, however, yet to be developed.

In the present study an attempt was made to assess the *in vitro* asymbiotic germination potential of immature embryos of *A. papillosa*, a medicinally important epiphytic orchid.

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MATERIALS AND METHODS

The green and undehisced capsules, harvested 28 weeks after pollination (WAP) from live plants served as source for young seeds with immature embryos. These were scrubbed with 'teepol' (1%), washed thoroughly under running tap water for 15 to 20 min, and surface sterilized for 7 min with $HgCl_2$ solution (0.1%), with 1 to 2 drops of 'teepol' as a wetting agent prior to washing with sterilized distilled water. These were also treated with streptomycin (0.03%) for 5 min and repeatedly washed with sterilized double distilled water so as to remove all the traces of sterilizing agents, and subsequently, these were dipped in 70% ethyl alcohol for 30 s, flame sterilized, and were split open longitudinally with a sterilized blade to scoop out the immature embryos, under aseptic conditions. The effect of two different media [M (Mitra et al., 1976), and PDA (Potato Dextrose Agar)] was tested on *in vitro* seed germination and subsequent seedling development in *A. papillosa*. Effect of different growth additives [AC (activated charcoal; 0.2%), CW (coconut water; 15%), YE (yeast extract; 2 g/L)] was also assessed during the experimentation. The seeds were inoculated on different media in cultures vessels, and incubated at 25±2°C under 12 h photoperiod provided by cool white fluorescent tubes (40 µmol m^{-2} s^{-1}). Eight replicates were used for each treatment. The cultures were examined regularly and the observations such as germination frequency, and on the number of days taken for onset of germination, spherule and protocorm formation, chlorophyll synthesis thereof, emergence of leaf as well as root primordia and seedling development were recorded. Subculturing was done at four week intervals. The well-developed seedlings with 2 to 3 leaves and 1 to 2 roots were gradually subcultured on hormone free and subsequently on one half and one fourth strength nutrient medium, respectively for 3 months as a part of hardening procedure. These were then removed from culture vessels and thoroughly washed with lukewarm water to remove adhering medium completely without causing damage to the roots. Subsequently, the seedlings were treated with a mild fungicide (Bavistine; 0.01%) solution and streptomycin (0.03%) for 5 min. These were then transferred to green house and potted in clay pots with potting mixture of charcoal pieces, Sphagnum moss, pine bark and brick pieces (1:1:1:1); these showed 70% survival rate.

One - way analysis of variance (ANOVA) which is used to compare two or more or means using the F- distribution was performed with respect to each response (average ± standard error against each additives is mentioned in Tables 1 and 2). The results of ANOVA showed no significant differences of additives at 5% level of significance. Various groups of additives showing identical/similar responses were statistically formed. To this end, Tukey test was performed at 5% level with respect to showing identical/similar responses are denoted in Tables 1 and 2 by putting the same alphabet as superscript on the respective means. In order to observe if the difference between means of the two groups is significant or not (two culture media), a t-test for independent samples was used.

RESULTS

The immature embryos in young seeds (28WAP) procured from the green undehisced capsules (Figure 1A) showed positive germination response in both the nutrient media that is M and PDA but the frequency and onset of germination response and associated morphogenetic changes leading to seedling development, varied with the nature of growth stimulus; a differential response was, however, observed on these media (Tables 1 and 2).

M medium

In basal M medium (control), about 40.75±0.75% seeds were germinated. During germination, the embryos swelled (Figure 1B), assumed spherical outline, and emerged out of the seed coats through apical/lateral slits as chlorophyllous spherules (Figure 1C to D), within 41.50±0.87 days. The germination entities (spherules) developed into protocorms (Figure 1E) which differentiated first leaf and root primordia in 91.50±1.73 and 121.25±2.50 days of inoculation respectively and complete seedlings were obtained in 156.25±2.50 days. Incorporation of CW (15%), supported early and highest germination (70.75±0.75%) and induced protocorm multiplication. The morphogenetic stages leading to seedling development were, however, in general advanced in medium containing additives; In the AC or YE enriched medium, protocorms differentiated 1st leaf and root primordia and complete seedlings with 2 to 3 leaves and 1 to 2 roots were obtained within 136.50±1.73 days (Figure 1F), CW, however, showed its pronounced effect and complete seedlings were obtained in 131.50±1.73 days. Rhizogenesis, however, took place in 108.75±2.50 days in medium supplemented with YE (2 g/L). Hairy protocorms were obtained on M medium supplemented with YE (2 g/L) or AC (0.2%), and complete seedlings were obtained after 136.50±1.73 days of inoculation, without significant differences from CW (131.50+1.73). M medium supplemented with CW proved to be an optimal combination for seed germination (70.75±0.75%) and seedling development (131.50±1.73 days) in *A. papillosa*, without significant differences from YE or AC added to M medium cultures, however with results significantly higher when the same additives were added to PDA medium cultures.

PDA medium

In basal PDA medium, only 21.25±1.25% seeds reacted positively to germination as compared to M medium (P< 5%). The embryos emerged out of the seed coats as spherules, acquired chlorophyll and developed into protocorms in 71.50±0.87 days. Leaf primordia were differentiated in 108.75±1.25 days old cultures. Roots, moreover took another 28 days to develop; complete rooted seedlings were obtained in 196.25±1.25 days. Additional presence of AC in the medium inhibited the seed germination. Additional use of CW or YE in the nutrient medium, favoured enhanced and early germination response and differentiation of protocorms (Figure 1G). In YE supplemented medium, favoured

Table 1. Effect of different nutrient media on the frequency and onset of seed germination, and spherule and protocorm formation during asymbiotic germination in *A. papillosa*.

Treatment	Nutrient media		Student-t test	
	M	PDA	*t - ratio*	*p* value
Germination frequency (%)				
-Average or control?	40.75±0.75[a]	21.25±1.25[b]	-8.275	0.001
AC (2 gl^{-1})	65.75±0.75[a]	0.00±0.00[b]	-2.564	0.04
CW (15%)	70.75±0.75[a]	31.25±1.25[b]	-6.537	0.001
YE (2 gl^{-1})	61.50±0.87[a]	40.50±0.87[b]	-7.635	0.001
Onset of germination (days)				
-	23.75±1.25[a]	31.25±1.25[b]	-16.803	0.001
AC (2 gl^{-1})	21.25±1.25[a]	0.00±0.00[d]	-2.372	0.05
CW (15%)	20.00±0.00[a]	22.50±1.25[b]	-23.524	0.001
YE (2 gl^{-1})	22.50±1.44[a]	21.25±1.44[b]	-20.631	0.001
Spherules (days)				
-	41.50±0.87[a]	51.50±0.87[b]	-23.568	0.001
AC (2 gl^{-1})	36.50±0.87[a]	0.00±0.00[b]	-2.497	0.04
CW (15%)	36.50±0.87[a]	36.50±0.87[a]	-60.852	0.001
YE (2 gl^{-1})	36.50±0.87[a]	41.50±0.87[b]	-32.669	0.001
Protocorms (days)				
-	61.25±1.25[a]	71.50±0.87[b]	-32.205	0.001
AC (2 gl^{-1})	54.00±0.58[a]	0.00±0.00[b]	-2.547	0.04
CW (15%)	51.50±0.87[a]	61.50±0.87[b]	-27.622	0.001
YE (2 gl^{-1})	54.00±0.57[a]	61.50±0.87[b]	-36.573	0.001

Entries in the columns nos. 2 and 3 are mean ± S.E.; same alphabetical letter in the superscript denotes that the corresponding means are in the same group using Tukey's multiple range test at 5%. a-b, Written horizontally as superscripts on right side of the data represent significantly difference between two media. AC, Activated charcoal; CW, coconut water; YE, yeast extract.

Table 2. Effect of different nutrient media on chlorophyll synthesis, emergence of leaf and root primordia and seedling development during asymbiotic germination in *A. papillosa*.

Treatment	Nutrient media		Student-t test	
	M	PDA	*t - ratio*	*p* value
Chlorophyll synthesis (days)				
-	41.50±0.87[a]	51.50±0.87[b]	-23.568	0.001
AC (2 gl^{-1})	36.50±0.87[a]	0.00±0.00[b]	-2.497	0.04
CW (15%)	31.50±0.87[a]	36.50±0.87[b]	-29.040	0.001
YE (2 gl^{-1})	36.50±0.87[a]	36.50±0.87[a]	-59.088	0.001
1st Leaf primordium (days)				
-	91.50±1.73[a]	108.75 ±1.25[b]	-30.022	0.001
AC (2 gl^{-1})	81.25±2.50[a]	0.00±0.00[b]	-2.579	0.04
CW (15%)	76.50±0.87[a]	101.50±0.87[b]	-18.283	0.001
YE (2 gl^{-1})	81.50±0.87[a]	101.50±0.87[b]	-23.156	0.001

Table 2. Continued.

1st Root primordium (days)				
-	121.25±2.50[a]	136.50±0.87[b]	-43.440	0.001
AC (2 gl^{-1})	111.50±1.73[a]	0.00±0.00[b]	-2.598	0.04
CW (15%)	111.50±1.73[a]	126.50±0.87[b]	-40.472	0.001
YE (2 gl^{-1})	108.75±2.50[a]	134.00±2.31[b]	-24.039	0.001
Seedlings (days)				
-	156.25±2.50[a]	196.25±1.25[b]	-23.180	0.001
AC (2 gl^{-1})	136.50±1.73[a]	0.00±0.00[b]	-2.607	0.03
CW (15%)	131.50±1.73[a]	161.50±0.87[b]	-25.361	0.001
YE (2 gl^{-1})	136.50±1.73[a]	176.25±1.25[b]	-20.328	0.001

Entries in the columns nos. 2 and 3 are mean ± S.E.; same alphabetical letter in the superscript denotes that the corresponding means are in the same group using Tukey's multiple range test at 5%. a-b, Written horizontally as superscripts on right side of the data represent significantly difference between two media.

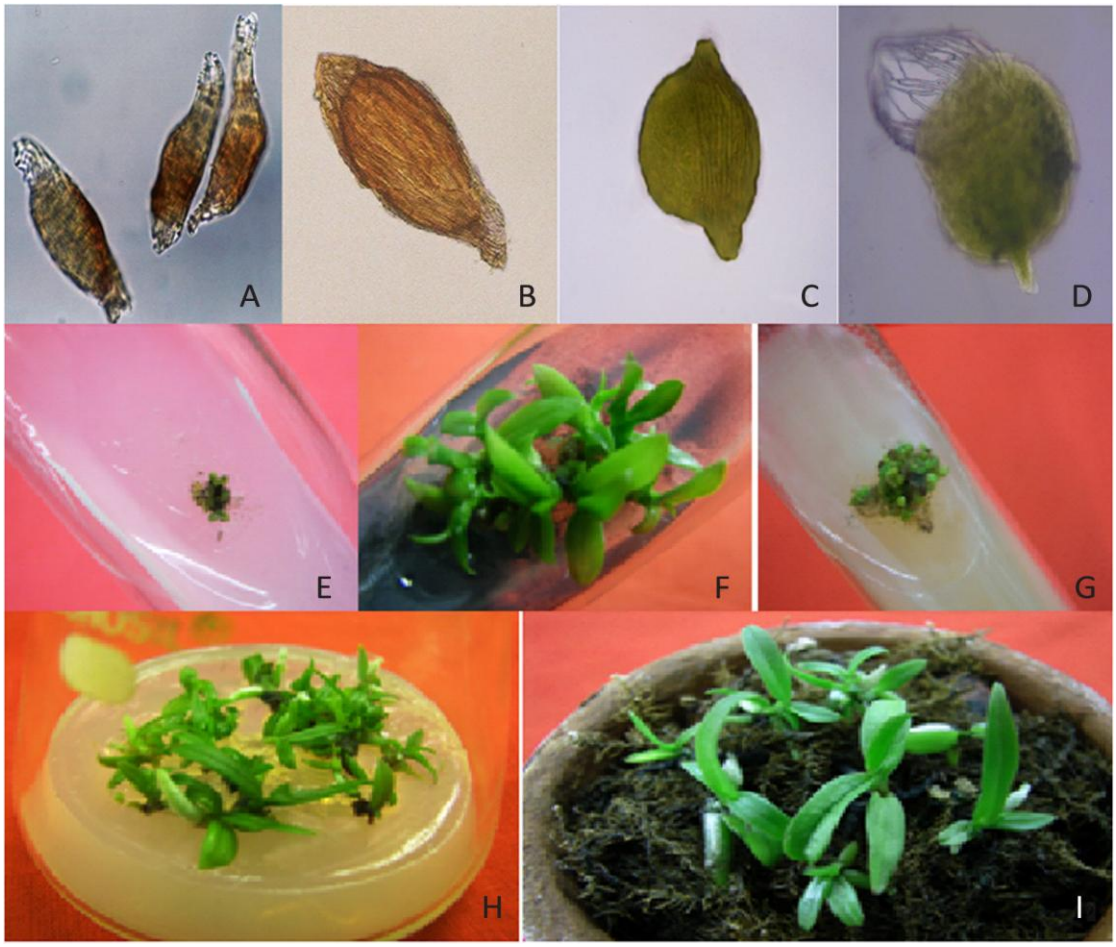

Figure 1. *In vitro* asymbiotic germination of immature embryos culture in *A. papillosa.* (A) Immature seeds at the time of inoculation (×20); (B) Swelling of embryo (×30); (C) Spherules emerging out by rupturing seed coats laterally (×20); (D) Differentiation of 1st leaf primordium (×10). (E) Hairy protocorms (basal M medium); (F) seedling development [M+AC (2g/L)]; (G) Protocorm multiplication and differentiation [PDA+CW (15%)]; (H) Healthy seedlings; (I) Seedlings transferred to a clay pot.

development of profusely hairy protocoms and healthy seedlings were obtained within 176.25±1.25 days. With CW in the medium, 31.25±1.25% seeds germinated and the protocorms were obtained in 61.50±0.87 days; this additive favoured better and early seedling growth and complete rooted seedlings were obtained in 161.50±0.87 days (Figure 1H), but with a significantly inferior germination frequency as compared to CW when added to M medium. Complete seedlings thus developed were transferred to green house and these subsequently showed 70% survival rate (Figure 1I).

DISCUSSION

Since the orchid seeds contain negligible quantity of reserve material for the development of embryos (Leroux et al., 1997) and further lack metabolic machinery (glyoxysomes) for conversion of their lipidaceous reserve food material into more utilizable forms (Harrison, 1977; Vij and Sharma, 1983), these require a symbiotic association with a mycorrhizal fungus, in nature (Bernard, 1904). Ever since Knudson (1921, 1922, 1925) germinated the seeds of *Cattleya, Laelia, and Epidendrum* on a sugar rich medium *in vitro*, thus bypassing their fungal requirement, the technique of asymbiotic seed germination has been positively tested in a large number of orchid species and hybrids (Arditti et al., 1982a, b; Buyun et al., 2004; Chang et al., 2005; Kauth et al., 2008; Pathak et al., 1992, 2005, 2011; Vij and Pathak, 1988). In the present investigation, the young seeds with immature embryos (28WAP) were used because it has been reported that upon maturation the seeds of many orchid species show decrease in viability or become dormant (Arditti et al., 1981). The fertilized ovules from few weeks old capsules are capable of germinating *in vitro* (Arditti et al., 1982a; Vij and Pathak, 1988). The technique reduces the time lapse between pollination and sowing of seeds, saves them from exposure to sterilizing agents and favours production of a large number of seedlings. Though literature studies reveal that the immature seeds germinate better than the mature ones, the earliest stage at which the embryos can germinate varies with the species, genus, hybrid and culture conditions in orchids (Arditti et al., 1982a). The easy germinability of immature seeds can be attributed to their distended testa cells, metabolically awakened embryos, and absence of dormancy factors (Linden, 1980; Yam and Weatherhead, 1988). In the presently investigated species, seeds successfully germinated on chemically defined (Mitra et al., 1976) and undefined [Potato Dextrose Agar (PDA)] media under asymbiotic conditions indicating thereby that their fungal requirement was compensated by providing an appropriate nutrient regime *in vitro*. Though the seeds showed positive germination response in both the nutrient media, but the frequency and onset of germination response and associated morphogenetic changes leading to seedling development varied with the nature of growth stimulus. M medium has been successfully used earlier in a number of orchid species (Pathak et al., 2001, 2011). M medium supplemented with AC, enhanced frequency of, and slightly advanced onset of germination, promoted growth and multiplication of protocorms, and advanced differentiation thereof and subsequent seedling development, whereas, its presence proved inhibitory for seed germination in PDA medium. Protocorms whenever obtained, develop absorbing hair. The absorptive potential of protocorms, however, varied with the chemical stimulus in the nutrient pool; protcorms obtained in YE enriched PDA medium were profusely hairy. The profuse growth of absorbing hair observed presently in YE enriched PDA supplemented cultures, and their profuse growth in dark incubated cultures reported earlier by Ichihashi (1990) in *Bletilla striata* seedlings indicates their relation with stress (nutritional as well as physical). Its impairing/inhibitory effects are, however, also on record in some species (Arditti and Ernst, 1984; Pathak et al., 2001; Weatherhead et al., 1986). Literature studies, however, reveal a beneficial effect of this darkening agent (AC) in a large number of orchid species (Arditti and Ernst, 1984; Ernst, 1976; Pathak et al., 2001; Vij and Pathak, 1988; Vij et al., 1988; Weatherhead and Harberd, 1980; Yam et al., 1989). Such beneficiary effects of activated charcoal on seed germination and protocorm development are attributed due to its high adsorption affinity to excessive and inhibitory compounds in the culture media (Fridborg et al., 1978; Weatherhead et al., 1978). Multiplication of protocorms was observed in CW supplemented cultures. Additional presence of CW in medium favoured germination [*Cattleya* (Kerbauy and Handro, 1981), *Cymbidium* (Chung et al., 1985), *Rhynchostylis retusa* (Nath et al., 1991), Dendrobium (Devi et al., 1990) and *Paphiopedilum purpuratum* (Yam and Weatherhead, 1988)], protocorm multiplication (Sagawa and Kunisaki, 1982) and for seedling growth [*Dendrobium* (Devi et al., 1990)]. In the present study, the best results during *in vitro* asymbiotic germination, protocorm multiplication and seedling development were achieved when M medium was supplemented with CW (15%) under 12 h photoperiod as compared to PDA medium ($P<5\%$), in *A. papillosa*.

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