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ASYMBIOTIC SEED GERMINATION OF AN ANTICANCEROUS TERRESTRIAL ORCHID *Eulophia nuda* Lindl. AND ITS ADULTERANT *Geodorum densiflorum* (Lam) Schltr.

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ABSTRACT

***Eulophia nuda* Lindl. corms are used to cure cancer and is overexploited by the traditional medicine practitioners and medicinal plant traders. The corms of *Geodorum densiflorum* look morphologically similar to the corms of *Eulophia nuda*, hence it is used as an adulterant to the corms of *Eulophia nuda*, consequetly, the population of *G. densiflorum* is also facing the exploitation pressure. In present study, asymbiotic seed germination of both the terrestrial orchids was achieved using six media like Vacin and Went (WV) medium, Malmgren modified (MM) terrestrial orchid medium, modified Kundson C medium, ½ MS medium, Lindemann medium (LM) and BM1 medium along with three photoperiods (0/24, 16/8, and 24/0 h L/D). *Eulophia nuda* showed highest asymbiotic seed germination on MM medium (80.55 %) and the best light treatment was 0/24 h L/D. The highest asymbiotic seed germination of *G. densiflorum* was on VW (84.07%), LM (83.29%) and BM 1(81.02%) and the best light treatment was 24/0 h L/D.**

Keywords: Adulteration, anticancer properties, asymbiotic seed germination, *Eulophia*, *Geodorum*, *Ex situ* conservation

INTRODUCTION

Eulophia nuda Lindl., commonly named as 'Amarkand', is a terrestrial orchid which has become endangered due to its over-exploitation for its valued anticancerous properties (Singh and Duggal, 2009, Nagulwar and Nandgave, 2016 ; Bhatt *et al.*, 2018; Hada *et al*., 2020). The corm of *E. nuda* are rich in antioxidants phenanthrenes. Phenanthrenes are bioactive molecules which helps scavenging oxygen under oxygen stress conditions (Tuchinda *et al.*, 1988; Kshirsagar *et al.*, 2010). Due to rich medicinal properties *E. nuda* corms are overexploited from its natural habitat by medical practitioners with the help of tribal people (Thomas *et al*., 2006; Jagtap *et al*., 2008; Narkhede *et al*., 2016). Owing to less availability of *E. nuda* corms in natural habitats, the collectors also collect the corms of *Geodorum densiflorum* from wild sources to be used as an adulterant and thus posing a serious threat to this plant as well (Narkhede *et al*., 2016). *G. densiflorum* is a terrestrial orchid which emerges after the first rain shower. The morphological characters of both the species are almost similar, thus rendering the correct identify of both the species difficult until flowering.

G. densiflorum corms are not only collected as an adulterant but also collected for its own medicinal properties like antimicrobial, antidiabetic, rich in antioxidant, thrombolytic activity, analgesic, sedative and anxiolytic properties (Hossain *et al*., 2012; Khatun *et al*., 2013; Keerthiga and Anand, 2014; Gunawan *et al*., 2020; Kabir *et al*., 2020). As a result, both the species needs conservation priority.

E. nuda and *G. densiflorum* produces millions of seeds in a single capsule and even vegetative multiplication is also an alternative way of multiplication, still very a small number of plants are there in nature. Orchid seeds are very minute and non-endospermic, and always need a fungal symbiotic partner to provide initial nutrition until plants become photosynthetically independent. In present study, asymbiotic seed germination of both the orchid species have been carried out by providing nutrition from the nutrient medium. Asymbiotic seed germination of *E. nuda* and *G. densiflorum* was tried by using six variable media and three variable light treatments. Many researchers have worked on the asymbiotic seed germination experiment of *E. nuda* and *G. densiflorum* individually but this research gives an insight about the nutritional need of both the species, since six media as well as variable light treatments were assessed to have the best treatments for highest seed germination.

MATERIALS AND METHODS

Sample collection

In present study the plant samples were collected from Kolhapur, Amravati and Chandrapur districts of Maharashtra state. Collected plant samples were stabilised in earthen pots in green house, providing all the necessary conditions in which plants were growing in nature. *G. densiflorum* flowered every year in greenhouse but no flowering was observed in *E. nuda* plant. Artificial pollination was tried with *G. densiflorum* to get the seeds for asymbiotic seed germination experiment.

Artificial pollination

G. densiflorum plants grow in rainy season and possess 5-7 white coloured flowers in each inflorescence. *G. densiflorum* flowers are trimerous having three sepals and three petals. Third petal is modified into pinkish yellow lip. Fully opened flowers are identified for pollinia and stigma. Pollinia is protected by anther cap. During pollination, anther cap was removed carefully and pollinia transferred to the receptive stigma as per self and cross pollination. After successful pollination, flowers become senesced and lead to capsule development. Artificial pollination was tried in *G. densiflorum* for three consecutive years and capsules were used in asymbiotic seed germination experiment. Flowering was not observed in *E. nuda* in green house and the seed were collected from the nature for asymbiotic seed germination.

Asymbiotic seed germination

Mature seed samples of *E. nuda* and *G. densiflorum* were collected from the field and green house. The seed samples were collected in clean filter paper and stored at 4°C in a refrigerator for asymbiotic seed germination experiment (Magrini *et al.,* 2019). Harvested seeds were surface sterilized thrice with distilled water (for 2 min each), with 0.1% mercuric chloride dip for 3 min, followed by 70% ethanol treatment for 30 sec and finally rinsed with distilled water three times. Sterilised seeds (100 seeds) were inoculated with the help of spatula on test tubes of different culture media for asymbiotic seed germination. The six media assessed were VC medium (Vacin and Went, 1949), MM medium (Malmgren, 1996), KC medium (Knudson, 1946), ½ MS medium (Murashige and Skoog, 1962), LM medium (Lindeman *et al*., 1970) and BM1 medium (Van Waes and Debergh, 1986b). The best germination medium was used for light treatment study. The freshly prepared medium in test tubes were tried for three light treatments *viz*., 0/24 h L/D (complete dark for 24 h), 16/8 h L/D (16 h light 8 h dark) and 24/0 h L/D (complete light for 24h). The temperature of growth culture was maintained

at 25 ± 5°C. Five replicates were used for each parameter. The experiment was conducted in a completely randomized design.

Data analysis

Asymbiotic seed germination (%) was calculated by dividing the number of seeds germinated with total number of seeds inoculated in each medium. Data was analysed by performing one-way analysis of variance and the means compared by Duncan's multiple comparison test ($\alpha = 0.05$) using DSAASTAT version 1.1.

RESULTS AND DISCUSSION

E. nuda and *G. densiflorum* are terrestrial orchids and appear after the first rain shower in the months of June or July. *G. densiflorum,* a self-compatible orchid, depends upon pollinators for its fruit set. Self-compatibility has widely been reported in orchid flowers considering it as an adaptation to limited number of pollinators (Galetto *et al*., 1997). Artificial pollination did not only increase the fruit set in this study but also helped in the conservation of the species. Increased fruit set increased the chances of the survival of species. In present study 308 flowers were pollinated with successful pollination percentage of 93.9 % (Table 3; Fig. 1). Artificial pollination was tried with other orchid species earlier (Dongarwar and Thakur, 2014), but there is no report about artificial pollination in *G. densiflorum*. Artificial, pollination was not tried in *E. nuda* because flowering was not achieved in this orchid.

Table 1: Asymbiotic seed germination of test orchids on different media

S. No.	Orchids	Nutrient media**	Seed germination (%)
1.	*Eulophia spectabilis*	VW	14.99 ± 3.46^{d}
		KC	33.33 ± 8.81^{cd}
		BM 1	50.01 ± 5.77^{bc}
		LM	61.66 ± 7.26^{ab}
		MM	80.55 ± 7.09^{a}
		½ MS	15.00 ± 2.88^{d}
2.	*Geodorum densiflorum*	VW	84.07 ± 2.59^{a}
		KC	58.03 ± 8.48^{c}
		BM 1	81.02 ± 5.86^{ab}
		LM	83.29 ± 3.35^{a}
		MM	78.26 ± 3.73^{abc}
		½ MS	62.00 ± 9.16^{bc}

*Means ± standard deviation mentioned in the column followed by the same letter are not significantly different as per Duncan's multiple range test at $\alpha = 0.05$.

**Nutrient media used: VC - Vacin and Went, MM – modified Malmgren medium, KC - Knudson medium, ½ MS - Murashige and Skoog, LM - Lindeman medium, BM1 - Van Waes and Debergh medium

In present study asymbiotic seed germination of *E. nuda* and *G. densiflorum* was observed in all the six media (Table 1). *G. densiflorum* seed began to swell in 4 weeks and took 10 weeks for germination. *G. densiflorum* showed highest seed germination on VW medium (84.07%), followed by media like KC (58.03%), BM-1(81.02%), LM (83.29%), MM (78.26% and ½ MS (62%) (Table 1; Fig. 2). All the media were observed for seedling development which revealed that BM1 and MM media supported better seedling growth than VW and LM media. VW and LM media showed very slow growth with respect to the seedling development. *E. nuda* exhibited highest seed germination on MM medium (80.55%), followed by LM medium (61.66%), BM1 medium (50.01%), KC medium (33.33%), VW medium (14.99%) and ½ MS medium (15%). The seed germination and seedling development was good in MM, LM and BM1 media. Seedling development was very slow in VW, KC and ½ MS media. Complete plants were developed in 33 weeks in *E. nuda* and in 22 weeks in *G. densiflorum* (Table 1, Fig. 2). All the six test media variably supported seed germination and seedling development

Fig. 1: *Geodorum densiflorum* A) Dissected flower B) Removal of anther cap C) Pollinia in anther cap D) Pollinia E) Pollination H) Capsule development

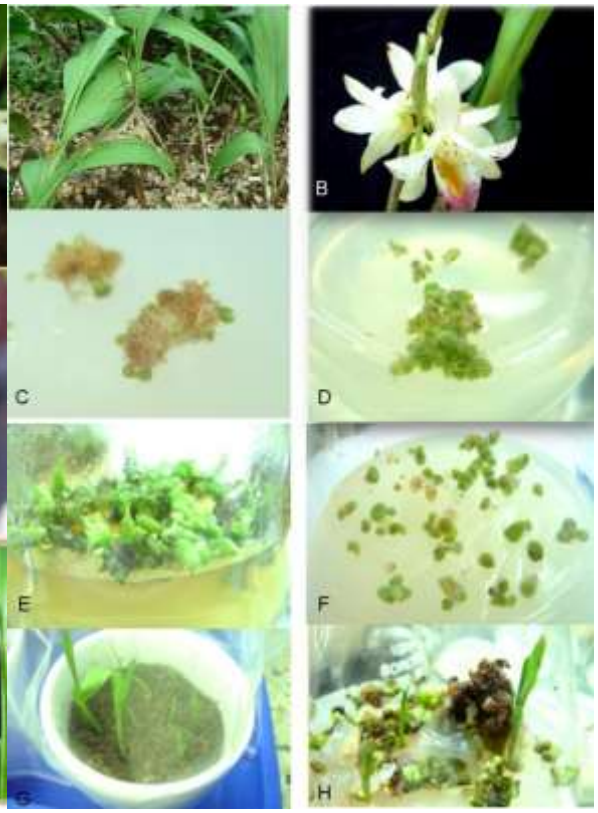

Fig. 2: Morphology of *Eulophia* (A) and *Geodorum* (B); Seed germination of *Eulophia* (C) and *Geodorum* (D); Protocorm development of *Eulophia* (E) and *Geodorum* (F); Developed seedling of *Eulophia* (G) and *Geodorum* (H)

because of variable media composition. Asymbiotic seed germination of *E. nuda* was studied on MS medium and *in vitro* flowering was also observed (McAlister and Van Staden, 1998; Chang *et al*., 2010). Dawande and Gurav (2015) used Knudson C medium for seed germination along with BA and IBA and achieved 90% seed germination in 10 weeks. Similarly, Roy and Banerjee (2001) studied seed germination of *G. densiflorum* in KC, VW and ½ MS media supplemented with plant growth regulators; while Muthukrishnan *et al*. (2013) reported 87% seed germination while using 14 different media.

Table 2: Effect of light on asymbiotic seed germination of *E. nuda* and *G. densiflorum*.

Light treatment	*Eulophia nuda* on MM medium	*Geodorum densiflorum* on VW medium
0/24 h L/D	80.55 ± 7.09a	68.78 ± 3.73a
16/8 h L/D	79.29 ± 1.54a	74.77 ± 2.78a
24/0 h L/D	69.64 ± 3.52a	84.07 ± 2.59a

*Means ± standard deviation mentioned in the column followed by the same letter are not significantly different according to Duncan's multiple range test at α=0.05. L/D- Light / Dark treatment

Sucrose is the main source of carbohydrate in all the test media, which supports the seed germination and seedling growth until plants are photo-synthetically independent. Comparing nitrogen content, inorganic form of nitrogen is present in KC and ½ MS media and organic form of nitrogen are present in all the six media. Nitrogen in any form supported

seed germination in present study; however, some researchers has observed more growth when media were supplemented with more organic form of nitrogen (Johnson *et al*., 2007). Photoperiod plays a crucial role in seed germination and development.

Table 3: Artificial pollination in *Geodorum densiflorum*

Pollination year	Total flowers bloomed (No.)	Self- pollinated flower	Cross pollinated flower (No.)	Capsule developed (No.)	Pollination percentage (%)
1st year	76	24	52	70	92.10
2nd year	124	25	99	118	95.16
3rd year	108	48	60	102	94.44
Mean	102.7	32.3	70.3	96.7	93.90

The best germinating media observed were VW and MM media for *G. densiflorum* and *E. nuda*, respectively, and these media were used for light treatment studies (0/24, 16/8, 24/0 h L/D) for seed germination. Highest germination in *G. densiflorum* was observed on 24/0 h L/D (84.07%), followed by 16/8 h L/D (74.77%) and 0/24 h L/D (68.78%) (Table 2). In *E. nuda* highest seed germination was observed in 0/24 h L/D treatment (80.55%), followed by 16/8 h L/D (79.29%) and 24/0 h L/D (69.64%) treatment (Table 2) (Zettler and McInnis, 1994). In present study, *E. nuda* showed enhanced seed germination when kept in continuous darkness, Similar observations have been made by other researchers in terrestrial orchids suggesting light induced seed dormancy (Arditti *et al*., 1981; Van Waes and Debergh, 1986; Yamazaki and Miyoshi, 2006). In contrary, *G. densiflorum* showed light enhanced seed germination, similar to those reported earlier (Rasmussen *et al*., 1990; Zettler *et al*., 1994). Overall effect of light on seed germination is species specific.

Conclusion: *Eulophia nuda* and *G. densiflorum*, both are rich in antioxidants and showing medicinal properties, but overuse of these plants from Nature make them more vulnerable. During this study a protocol of multiple propagation was developed so that plant can be multiplied and reintroduced in the nature.

Author contributions: Dr. Nitin Dongarwar (ND) gave the research plan and Ms. Uma Thakur (UT) executed tissue culture/laboratory work and statistical analysis of data. UT and ND contributed in manuscript write-up and its revision work.

Conflict of interests: The authors declare that they have no conflicts of interest.

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