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Induced xenogamy enhances capsule formation, seed viability, *in vitro* seed germination, and seedling growth in the threatened orchid *Diplomeris hirsuta*: A strategy for conservation

Tshering Chomu Bhutia[1, 2], Laxuman Sharma[2]*, Siddhartha Sankar Biswas[1*], Rampal[1, 3], S. Manivannan[2, 4], Tshering Lhamu Bhutia[5], Suman Natta[1] and L.C. De[1]

*[1]ICAR-National Research Centre for Orchids, Pakyong, Sikkim, India. [2]Department of Horticulture, Sikkim University, Sikkim, India. [3]ICAR-Directorate of Floricultural Research, Regional Station, Vemagiri, Andhra Pradesh, India. [4]Department of Horticulture, Central University of Tamil Nadu, Thiruvarur, India. [5]ICAR Research Complex for NEH Region, Sikkim Centre, Tadong. *E-mail: lsharma@cus.ac.in, siddssac20475@gmail.com*

Abstract

Diplomeris hirsuta, a medicinally important yet threatened terrestrial orchid species, is vulnerable to extinction as per the IUCN Red List. This study aimed to investigate the influence of different pollination methods on *D. hirsuta* seed viability, seed germination in tissue culture media and their subsequent plant growth. A field experiment (on pollination systems) conducted in North Sikkim, Phamtam, revealed significant positive effects of different pollination treatments on capsule formation, with induced xenogamy demonstrating superior results. Cross-pollination techniques, particularly induced xenogamy, increased the percentage of capsule formation by 190% and enhanced capsule dimensions compared to natural pollination methods. Induced xenogamy resulted in seeds with higher viability (85.7% higher) and germination rates (737% higher), contributing to the better vigour and growth of *D. hirsuta* offspring compared to the control treatment, with the most promising results in BM-1 terrestrial media. Tissue culture experiments with BM-1 terrestrial media demonstrated the most successful seed germination and subsequent seedling growth. Thus, induced xenogamy treatment can be recommended for improving capsule formation, seed viability, and seed germination of *D. hirsuta* orchids. Additionally, BM-1 terrestrial media can be used for the successful mass multiplication of these orchids.

Key words: *Diplomeris hirsuta,* Snow orchid, threatened orchid, pollination strategies, tissue culture, basal media

Introduction

The genus *Diplomeris* comprises terrestrial orchids that are primarily distributed across the Eastern Himalayan region, which includes parts of China, India, and Nepal. Among these, *Diplomeris hirsuta,* commonly known as the snow orchid, is popularly known for its medicinal value and ecological significance (Chaudhuri, 2007; Gowthami *et al.*, 2021). This orchid is native to specific regions, including Central China, Nepal, Western Bhutan, and Eastern India (Moktan, 2021). The name "*Diplomeris*" is originated from the Greek word "*diplo*" and "*merus*, " meaning "two" and "part, " respectively, referring to the divided stigma projections that resemble appendages (Pearce and Cribb, 2002). The term "*hirsuta*, " derived from Latin, which highlights the plant's distinctive hairy stems, leaves, and ovaries (Jalal, 2012).

D. hirsuta inhabits the damp, shaded microhabitats, often found on mossy rocks next to natural streams or roadsides. This species is known for having a sparse distribution pattern, usually found in a small geographic area. This species is known for its sparse distribution, preferring high humidity, shady and forested areas, or watercourse-adjacent environments (Moktan, 2021; Bhutia *et al.*, 2023). The IUCN Red Data Book listed the orchid as vulnerable due to its specific habitat requirements and limited distribution (Nayar and Sastry, 1987). The species is protected under CITES Appendix II, signalling its global concern for its conservation. *D. hirsuta* is listed as a threatened species in national legislation, the Biological Diversity Act 2002, with recommendations for inclusion in Schedule-38 alongside other orchid taxa (Agarwal and Singh, 2013).

The first records of *D. hirsuta's* vulnerabilities date back to the mid-1970s, also, Pradhan (1974) attributed these vulnerabilities and extension to landslides and habitat encroachment as the environmental causes. The ongoing threats to this orchid include habitat degradation and loss, fragmentation of habitat, overharvesting and uncontrolled collection, invasive species competition, loss and disturbance of pollinators, and issues associated with orchid seed germination (Fay, 2018). Orchid seeds are minute, spherical clumps of embryonic cells without endosperm or cotyledons. To successfully germinate the orchid seeds in their natural habitat, a symbiosis with specific mycorrhizal fungi is required for the essential supply of water and nutrients (Biswas *et al.*, 2021). In nature, this type of symbiosis is uncommon, which explains why orchid seeds hardly ever germinate. Orchid mass proliferation thus depends exclusively on tissue culture. Because of this, orchids are more susceptible to going extinct.

The primary locations of *D. hirsuta* orchid growing sites in the Sikkim Himalayan region include Phamtam, North Sikkim, and Assam lingzey in Pakyong, Sikkim. These areas are situated close to national highways and roadsides and are subjected to various natural and human-induced disturbances. Activities such as road construction, railway expansion, and heavy rainfall exacerbate the threats to the orchid's habitat. Despite these known challenges, very little research has been conducted on pollination techniques and tissue-culture-based seed germination in *D. hirsuta.*

Self-compatibility has been reported in numerous terrestrial orchids (Thakur and Dongarwar, 2012). For instance, in *Dactylorhiza fuchsia*, self-pollination was observed when the pollinarium bends to facilitate autogamous seed and fruit formation (Talalaj *et al.*, 2019). *D. hirsuta* flowers indicated that self-compatibility might potentially be feasible in this flower. The proximity between pollen and stigma can increase the likelihood of spontaneous self-pollination, especially when environmental triggers such as wind or raindrop impact are present. This can lead to pollinia detaching and adhering to the stigmatic surface, even without external pollinator interaction. While self-pollination ensures some level of reproductive success in the absence of pollinators, it often comes at a cost. Studies have shown that self-pollination can result in lower capsule formation rates, reduced seed viability, and the production of less vigorous offspring compared to cross-pollination strategies (Klein *et al.*, 2007; Krauss *et al.*, 2005). This highlights the need to explore alternative pollination methods that can enhance reproductive outcomes and ensure greater genetic diversity in vulnerable orchid species. Given these limitations, alternative pollination techniques such as induced xenogamy could enhance seed viability, promote healthier offspring, and help address these challenges while supporting orchid conservation.

Additionally, identifying suitable tissue culture media is crucial for successful seed germination and plant growth, enabling large-scale propagation and conservation of *D. hirsuta.* Therefore, this research aims to bridge the conservation gap for *D. hirsuta* by assessing the impact of various pollination methods on capsule formation, seed viability, and germination rates. It will compare natural and induced pollination strategies to identify the most effective method for enhancing reproductive success. Additionally, the study seeks to determine the optimal tissue culture media for *in vitro* germination and seedling growth, essential for mass propagation due to the species' dependence on rare mycorrhizal associations. These findings will inform strategies to boost genetic diversity and ensure the sustainable conservation of this vulnerable orchid.

Materials and methods

Study site: Pollination system research for *D. hirsuta* was conducted in Phamtam, North Sikkim (27.279164 N, 88.615680 E) due to its accessibility, the abundance of *D. hirsuta* populations, and the minimal human disturbance to the natural ecosystem. This subtropical region, characterized by an average annual temperature of 25–28°C, provides an ideal natural habitat for this species. During the dormant period from October to March, plants remain dormant on rock surfaces. Subsequently, vegetative growth commences in April, leading to flowering in late June. Robust flower buds were carefully selected from this site for experimental studies, ensuring the collection of healthy and representative samples for further analysis.

Treatment details: In 2019, populations from North Sikkim (27.43287 N, 88.60193 E) and Assam Lingzey were used as plant resources. A Randomised Block Design (RBD) was applied with seven treatments, each replicated three times across 105 plants (7 treatments × 3 replications × 5 plants per replication). The treatments included: T_1: Flower buds left in their natural habitat for natural pollination (natural pollination/ control), T_2: Emasculated flower buds left unpollinated (agamospermy), T_3: Flowers bagged with butter paper bag without disturbing the pollen source (spontaneous autogamy), T_4: Pollen gathered from a flower and used to pollinate the same flower, with a butter paper bag (Induced Autogamy), T_5: Pollen collected from two distinct blooms of the same plant and used to pollinate another flower of the same plant, bagged afterwards (Artificial Geitonogamy), T_6: Pollen taken from plants in a separate area (and used to pollinate the flowers another experimental site (Artificial Xenogamy) and T_7: Pollen collected from a plant at the experimental site and used to pollinate the flowers, of the same site (Induced Xenogamy). Pollen was collected from multiple plants located at the same experimental site (North Sikkim), although these plants were not part of the experimental design. This pollen was then used to pollinate the flowers subjected to the treatment, and the flowers were subsequently covered with butter paper bags, inducing xenogamy. Throughout the experiment, maintaining a consistent pollen supply was essential. The pollinia were carefully collected using forceps and applied according to the respective treatments (Johnson *et al.*, 2009; Sipes *et al.*, 1995). Capsule formation rates, capsule length, and diameter were recorded.

Seed collection and viability test: Mature capsules collected from the experimental site at 30 days post-pollination were used for seed extraction. The extracted seeds were then dried for 24 hours using a silica desiccant. Following Lakon's 1949 Protocol, 100 seeds (weighing approximately 5 mg each) were placed in cryovials, submerged with a tetrazolium chloride solution at around 35–37°C, and left for 48 hours to determine the viability of the seeds. Under a compound microscope, the number of viable seeds containing viable embryos were counted, and the percentage of viable seeds was calculated. Non-viable embryos appeared white in colour, while viable embryos stained in red colour. The tetrazolium chloride test was chosen due to its reliability in assessing seed viability. This test operates by allowing seeds to absorb the tetrazolium chloride solution, which dehydrogenase enzymes in living tissues convert to red formazan, an insoluble coloured compound that highlights viable tissues (Deswal and Chand, 1947). This method also served as an indicator of embryo maturity, as deep red coloration is associated with fully developed embryos (Van Waes and Debergh, 1986; Hosomi *et al.*, 2011, 2012; Lallana and Garcia, 2013; Soares *et al.*, 2014; Macedo *et al.*, 2014). Despite the smaller size of *D. hirsuta* seeds, compared to many other orchid seeds, a 20x resolution scan was performed to ensure accurate evaluation. To enhance contrast, a light blue background was placed inside the scanner cover, facilitating the visibility of stained seeds.

Tissue culture experiment and asymbiotic germination of seeds: The tissue culture experiment focused on the four best seed sources selected based on the results of the seed viability test. In 2019 and 2020, the study was conducted at the ICAR-NRC for Orchids' tissue culture unit in Pakyong, Sikkim. The present study included four treatment groups: (T_1) control, in which flower buds were left undisturbed in their natural habitat to facilitate natural pollination; (T_3) flower bagged without disturbing the pollen source, resulting in spontaneous autogamy (SA); (T_4) Induced autogamy (IA), where pollen was collected from a flower and used to pollinate the same flower, followed by bagging with a butter paper bag; and (T_7) induced xenogamy (IX), where pollen was collected from plants not included in the experimental design but from the same experimental site (Assam Lingzey) and used to pollinate the treated flowers, followed by bagging with a butter paper bag. Each treatment had five replications, with each tissue culture bottle containing 100 seeds from the respective treatment. Our objective was to determine the optimal tissue culture medium for *D. hirsuta* seed germination and growth. Mature seed capsules from the chosen treatments were surface sterilized for three minutes using a solution of 100 mL containing 5 mL ethanol, 5 mL 6.0% sodium hypochlorite, and 90 mL sterile distilled water. The sterilized capsules were then rinsed three times with sterile distilled water for 30 seconds each.

Five asymbiotic seed germination media were tested for their efficacy in promoting germination and plant development: (1) BM-1 terrestrial medium, (2) Malmgren modified terrestrial medium, (3) Gamborg's B5 medium, (4) White's medium, and (5) Murashige and Skoog (MS) medium. All media were adjusted to a pH of 5.8 before autoclaving at 121°C and 117.7 kPa for 40 minutes. Each culture bottle received 50 mL of the sterilized medium. Seeds from the surface-sterilized capsules were aseptically inoculated onto the sterile medium. the culture bottles were incubated at 25°C±3°C, Observations included the percentage of seed germination, time to seed germination, time to first leaf and root development, shoot length, leaf length, and leaf width at 10 days after the formation of the first leaf, as well as plant biomass at the second leaf formation stage.

Statistical analysis: The data analysis from the laboratory tissue culture experiment was conducted under a Completely Randomized Design (CRD) and the field experiment involving various pollination systems under a Randomized Block Design (RBD), using Analysis of Variance (ANOVA). The statistical analysis was performed using the online data analysis tool OPSTAT (Sheoran, 2010). $P \leq 0.05$ was used as the significance level for determining the least significant differences between the treatment means for both studies (Gomez and Gomez, 1984).

Result

Pollination treatment and capsule formation: Seven distinct pollination treatments were applied to five healthy flower buds from each plant/ replication in accordance with the treatment plan. Prior to anthesis, five healthy plants per replication were chosen and subjected to the designated pollination treatments. The *D. hirsuta* plants showed signs of capsule production after 5-7 days post-treatment, marked by the development of a small, ovoid capsule at the base of the petals. After 30 days, fully developed, robust, dark green capsules were developed across treatments. The statistical analysis revealed that the pollination treatments had a substantial impact on capsule formation. No capsule formation was observed under agamospermy (T_2). Maximum capsule formation was recorded under induced xenogamy (T_7) and induced autogamy (T_4), showing an approximately 190%, 147%, and 16% increase compared to the control (T_1), spontaneous autogamy (T_3), and induced autogamy (T_4), respectively (Fig. 1).

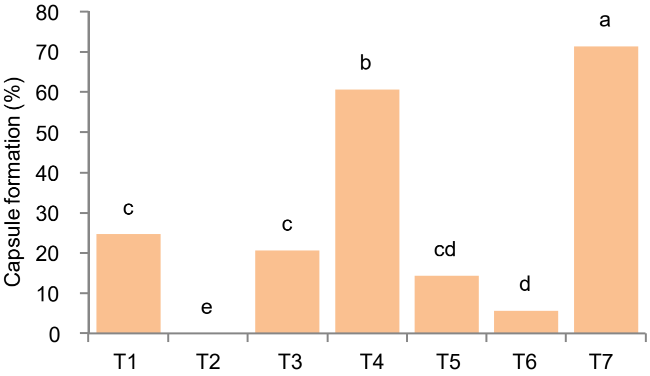

Fig. 1. Capsule formation of *D. hirsuta* under different pollination systems. Statistically significant differences among treatment means are indicated by unique lowercase letters, as established by the LSD ($P < 0.05$) following the Duncan multiple range test. T1: control, natural pollination, T2: agamospermy, T3: spontaneous autogamy, T4: induced autogamy, T5: artificial geitonogamy, T6: artificial pollination with pollen from different location, T7: induced xenogamy.

Both the induced xenogamy and the induced autogamy produced capsules that had no statistically significant difference between them, and produced capsules that were dark green and healthy. The development of capsules during the spontaneous autogamy treatment (T_3) demonstrated that *D. hirsuta* orchid pollination is independent of pollinators.

The dimensions of the capsule were also significantly impacted by the pollination procedures. Under induced xenogamy (T_7) treatment, the maximum capsule length and breadth (Fig. 2) were recorded. Capsules from this treatment were approximately 34.4%, 25.2% and 6.7% longer and approximately 99.5%, 71.4%, and 4.7% wider than those obtained from control (T_1), spontaneous autogamy (T_3), and induced autogamy (T_4) treatments, respectively.

Seed viability under different pollination treatment: The seed viability was notably influenced by the different pollination techniques applied. In Fig. 3 the induced xenogamy treatment (T_7) significantly shows higher seed viability compared to the other treatments. Seed viability under T_7 was approximately 85.7%, 42.8%, and 14.2% greater than that recorded for the control (T_1), spontaneous autogamy (T_3), and induced autogamy (T_4) treatments, respectively.

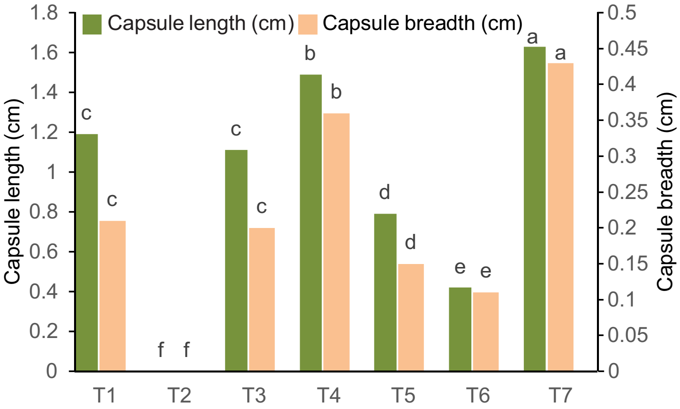

Fig. 2. *D. hirsuta* capsule length (A) and Capsule breadth (B) under different pollination systems. Statistically significant differences among treatment means are indicated by unique lowercase letters, as established by the LSD ($P < 0.05$) following the Duncan multiple range test. T_1: control, natural pollination, T_2: agamospermy, T_3: spontaneous autogamy, T_4: induced autogamy, T_5: artificial geitonogamy, T_6: artificial pollination with pollen from different location, T_7: induced xenogamy.

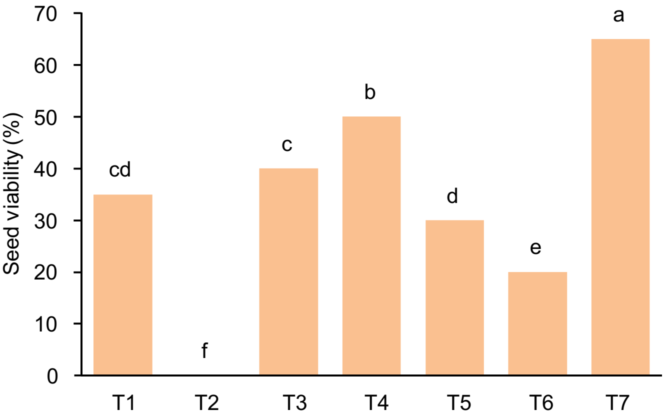

Fig. 3: *D. hirsuta* seed viability under different pollination systems. Statistically significant differences among treatment means are indicated by unique lowercase letters, as established by the LSD ($P< 0.05$) following the Duncan multiple range test

Seed germination rate in different basal media: Among the five basal media tested, BM-1 terrestrial media was found to be the most effective for germinating seeds from all four pollination treatments used in the *D. hirsuta* tissue culture experiment (Table 1). While White's and M.S. media also proved to be effective in germination of *D. hirsuta* seeds; however, these media were only capable of supporting seed germination obtained through induced xenogamy (T_7) treatment. These media failed to initiate germination for seeds from other treatments. The highest germination percentage was observed for seeds from the induced xenogamy (T_7) treatment when cultured on BM-1 terrestrial media. Germination under this treatment was approximately 737%, 268.5%, and 38.8% higher than that of the control (T_1), spontaneous autogamy (T_3), and induced autogamy (T_4) treatments, respectively. Additionally, compared to White's and M.S. media, seeds from the T_7 treatment showed a germination percentage that was 4516% and 4507% higher, respectively, when grown on BM-1 terrestrial media.

Table 1. *D. hirsuta* seed germination (%) in different tissue culture media

Treatment	Seed germination (%) in different tissue culture media				
	Media 1	Media 2	Media 3	Media 4	Media 5
Control	5.40c	0a	0a	0b	0b
SA	3.30c	0a	0a	0b	0b
IA	19.9b	0a	0a	0b	0b
IX	45.2a	0a	0a	1.95a	5.49a

Statistically significant differences among treatment means are indicated by unique lowercase letters, as established by the LSD ($P< 0.05$) following the Duncan multiple range test.

Time taken by seeds to germinate: After four weeks in culture, the seeds began to show morphological changes, appearing round and swollen with a visible light green colour. By the eighth week, a significant number of seeds had germinated and developed into round, swollen, green protocorm-like bodies (PLBs). More precisely, the first seed germination was recorded in BM-1 terrestrial media for the seeds from the induced xenogamy (T_7) treatment, and the seed germination began on the 58th day (Table 2) of culturing. When compared to the seeds from the control (T_1), spontaneous autogamy (T_3), and induced autogamy (T_4) treatments, the seeds from the induced xenogamy (T_7) treatment took approximately 19.7%, 11.3%, and 6.7% less time in BM-1 terrestrial media. In contrast, the induced xenogamy (T7) treatment seeds in BM-1 terrestrial media germinated 14.3% and 15.6 % faster than in White's and M.S. media, respectively.

Table 2. Time taken by *D. hirsuta* seeds to germinate in different tissue culture media

Treatment	Time taken (Days) for seed germination				
	Media 1	Media 2	Media 3	Media 4	Media 5
Control	72.4a	NA	NA	NA	NA
SA	67.5a	NA	NA	NA	NA
IA	64.2b	NA	NA	NA	NA
IX	58.1c	NA	NA	67.8a	68.9a

Statistically significant differences among treatment means are indicated by unique lowercase letters, as established by the LSD ($P< 0.05$) following the Duncan multiple range test.

Time taken for first leaf formation: In BM-1 terrestrial media under induced xenogamy (T_7) treatment, leaf development in germinated seeds began on the 96th day following inoculation (Table 3). First leaf formation under induced xenogamy (T_7) treatment in BM-1 terrestrial medium took approximately 14.0%, 10.6%, and 1.76% fewer days than under control (T_1), spontaneous autogamy (T_3), and induced autogamy (T_4) treatments, respectively. When comparing different basal media, the seeds from the induced xenogamy (T_7) treatment took approximately 6.06% and 9.25 % less days for the seeds to germinate in the BM-1 terrestrial media than in the White and M.S. media, respectively.

Table 3. Time taken by *D. hirsuta* plants for first leaf formation in different tissue culture media

Treatment	Time taken (Days) for first leaf formation				
	Media 1	Media 2	Media 3	Media 4	Media 5
Control	113a	NA	NA	NA	NA
SA	111a	NA	NA	NA	NA
IA	101b	NA	NA	NA	NA
IX	97.1c	NA	NA	103a	107a

Statistically significant differences among treatment means are indicated by unique lowercase letters, as established by the LSD ($P< 0.05$) following the Duncan multiple range test. Murashige and Skoog Media

Time taken for the first root formation: In BM-1 terrestrial media, the first root formation was observed on the 156th day after inoculation during the induced xenogamy (T_7) treatment (Table 4). Root initiation under the induced xenogamy (T_7) treatment began approximately 17.0%, 7.44%, and 1.5% earlier than in control (T_1), spontaneous autogamy (T_3), and induced autogamy (T_4) treatments, respectively.

Table 4. Time taken by *D. hirsuta* plants for first root formation in different tissue culture media

Treatment	Time taken (Days) for first root formation				
	Media 1	Media 2	Media 3	Media 4	Media 5
Control	188a	NA	NA	NA	NA
SA	185a	NA	NA	NA	NA
IA	174b	NA	NA	NA	NA
IX	156c	NA	NA	165a	166a

Statistically significant differences among treatment means are indicated by unique lowercase letters, as established by the LSD ($P< 0.05$) following the Duncan multiple range test.

Plant biomass at the second leaf formation stage: In BM-1 terrestrial media, under induced xenogamy (T_7) treatment, plant biomass reached a record high during the second phase of leaf formation stage (Table 5). Compared to spontaneous autogamy (T_3) and induced autogamy (T_4), the biomass under induced xenogamy (T_7) treatment was approximately 39.1%, 20.2%, and 2.89% higher, respectively. Among the basal media tested, BM-1 terrestrial medium produced significantly greater plant biomass compared to the other two effective media. Specifically, BM-1 generated approximately 17.7% and 18.7% more plant biomass at the second leaf formation stage than White's and M.S media, respectively.

Table 5. *D. hirsuta* plants biomass (mg) at 2nd leaf formation stage in different tissue culture media

Treatment	Plant biomass (mg)				
	Media 1	Media 2	Media 3	Media 4	Media 5
Control	0.069c	NA	NA	NA	NA
SA	0.071c	NA	NA	NA	NA
IA	0.083b	NA	NA	NA	NA
IX	0.096a	NA	NA	0.079	0.078

Statistically significant differences among treatment means are indicated by unique lowercase letters, as established by the LSD ($P< 0.05$) following the Duncan multiple range test.

Shoot length, leaf length and leaf width after 10 days of first leaf formation: Ten days following the first leaf formation, shoot length, leaf length, and leaf width were observed to be the highest under induced xenogamy (T_7) treatment (Table 6). Compared to the control (T_1), spontaneous autogamy (T_3), and induced autogamy (T_4) treatments, the shoot lengths, leaf lengths, and leaf widths in BM-1 terrestrial media under induced xenogamy (T_7) treatment were approximately 24.8%, 12.4%, and 2.82% higher, respectively. Leaf length showed increases of approximately 42.4%, 21.2%, and 6.06%, while leaf width was 60%, 40%, and 10% higher under the same comparisons. When comparing basal media, BM-1 terrestrial media outperformed White's and M.S media. It produced approximately 4.52% and 5.42% greater shoot length, 17.0% and 23.4% greater leaf length, and 25% and 18% greater leaf width, respectively.

Table 6. *D. hirsuta* Shoot length, leaf length, and leaf width after 10 days of first leaf initiation in different tissue culture media

Treatment	Shoot length (mm) after 10 days of first leaf initiation				
	Media 1	Media 2	Media 3	Media 4	Media 5
Control	1.77c	NA	NA	NA	NA
SA	1.82c	NA	NA	NA	NA
IA	1.99b	NA	NA	NA	NA
IX	2.21a	NA	NA	2.11a	2.09a
Leaf length (mm) after 10 days of first leaf initiation					
Control	0.33c	NA	NA	NA	NA
SA	0.35c	NA	NA	NA	NA
IA	0.40b	NA	NA	NA	NA
IX	0.47a	NA	NA	0.39a	0.36a
Leaf width (mm) after 10 days of first leaf initiation					
Control	0.10c	NA	NA	NA	NA
SA	0.11c	NA	NA	NA	NA
IA	0.14b	NA	NA	NA	NA
IX	0.16a	NA	NA	0.12a	0.13a

Statistically significant differences among treatment means are indicated by unique lowercase letters, as established by the LSD ($P< 0.05$) following the Duncan multiple range test.

Discussion

There are several possible advantages to induced xenogamy over natural pollination. It entails the purposeful facilitation or promotion of cross-pollination between genetically dissimilar individuals. This process enhances genetic variation, which is crucial for the adaptation of progeny to shifting environmental conditions. Pimm *et al.* (2014) suggested that a higher level of genetic diversity led to more robust progeny capable of withstanding environmental stressors more effectively. This genetic variability is critical for enhancing the resilience of plants to various environmental challenges such as drought, disease, and fluctuating temperatures. By ensuring efficient fertilisation and seed development, cross-pollination through induced xenogamy improves fruit and seed formation. It has been found that, in comparison to self-pollination, cross-pollination increases fruit size, yield, and nutritional quality in various plant species (Klein *et al.*, 2007). In addition to improving fruit characteristics, cross-pollination through induced xenogamy also contributes to superior seed quality. Traits such as flavour, colour, texture, and nutritional content can be enhanced through genetic recombination resulting from cross-pollination (Dwivedi *et al.*, 2015). Our findings align with these results, as both the percentage of capsules formed and their size were significantly greater under the induced xenogamy treatment (T_7) compared to the natural pollination (control treatment, T_1) (Dwivedi *et al.,* 2015) with a suitable basal medium like BM-1 terrestrial, can significantly enhance the growth and development of *D. hirsuta* plants, offering potential benefits for both conservation and cultivation efforts.

This study provides valuable insights into the reproductive biology, propagation, and conservation of *Diplomeris hirsuta* orchids, focusing on the combined effects of growth media and pollination treatments. Induced xenogamy has proven to be an effective strategy, significantly improving seed viability, seedling vigour, and capsule development compared to natural and self-pollination. By facilitating cross-pollination between genetically diverse individuals, induced xenogamy not only enhances genetic diversity but also leads to the production of more robust progeny capable of withstanding environmental stressors. This technique also offers a promising solution to mitigating the risks of inbreeding depression, which is a critical challenge in maintaining the health and sustainability of orchid populations.The study further highlights the pivotal role of BM-1 terrestrial media in supporting seed germination and early seedling growth. As a balanced growth medium, BM-1 effectively accommodates the nutritional and hormonal requirements of developing seedlings, promoting the successful initiation of roots, elongation of shoots, and overall seedling vigour. This medium's adaptability and efficiency make it a versatile resource for orchid tissue culture and propagation studies, offering a reliable platform for both experimental and conservation-focused applications. Together, these findings emphasize the importance of adopting innovative techniques, such as induced xenogamy and optimized growth media, for the conservation and propagation of *D. hirsuta* orchids. These strategies not only facilitate the survival and growth of this species but also provide a framework for addressing broader challenges in orchid conservation, particularly in the face of habitat loss and environmental change. Future research should aim to explore the long-term effects of induced xenogamy on genetic diversity and plant fitness, as well as its potential for large-scale propagation. Additionally, further studies on the optimization of growth media tailored to the specific needs of orchids could enhance propagation efficiency and plant quality. Such efforts will be instrumental in developing sustainable management practices for *D. hirsuta* and other vulnerable orchid species, ensuring their preservation and ecological resilience in their native habitats.

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References

Agarwal, D.K. and P. Singh, 2013. Legislations for orchid conservation in India and development of national red list as per IUCN criteria. *J. Orc. Soc*., 11(1): 27-35.

Biswas, S.S., D.R. Singh, L.C. De, N.S. Kalaivanan, R. Pal and T. Janakiram, 2021. A comprehensive scenario of orchid nutrition-A review. *Plant Nutr.,* 44: 905917.

Bhutia, T..L., N.T. Bhutia, Ngura Sailo, Rampal and T.C. Bhutia, 2023. *Ex situ* conservation of rare and threatened orchid: *Diplomeris hirsuta* (Lindl.). *J. Appl. Hortic.*, 25(2): 120-122. https://doi.org/10.37855/jah.2023.v25i02.21.

Deswal, D. and U. Chand, 1947. Standardization of the tetrazolium test for viability estimation in rice bean (*Vigna umbellata* (Thunb.) Ohwi and Ohashi) seeds. *Seed Sci. Tech*., 25: 409-417.

Davies, P.J. 2010. *Plant Hormones: Biosynthesis, Signal Transduction, Action,* Springer SSBM.

Duvick, D.N. 1999. Heterosis: Feeding People and Protecting Natural Resources. *Genetics*, 267-273

Dwivedi, S.L., K.L. Sahrawat, H.D. Upadhyaya, A. Mengoni, M. Galardini, M. Bazzicalupo, *E.g.* Biondi and M. Hungria, 2015. Advances in host plant and rhizobium genomics to enhance symbiotic nitrogen fixation in grain legumes. *Adv Agron.,* 129: 1-116.

Fay, M.F. 2018. Orchid conservation: How can we meet the challenges in the twenty-first century? *Bot. Stud.,* 59: 1-6.

Gomez, K.A. and A.A. Gomez.1984. Statistical Procedures for Agricultural Research. *John Wiley and Sons.*

Gowthami, R., N. Sharma, R. Pandey and A. Agrawal, 2021 Status and consolidated list of threatened medicinal plants of India. *Genet. Resour. Crop Evol.,* 68: 2235-2263.

George, E.F., M.A. Hall and G.J. De. Klerk, 2008. *Plant Propagation by Tissue Culture the Background*, 3rd Edition, Springer, Dordrecht, Vol. 1.

Hosomi, S.T., C.C. Custódio, P.T.Seaton, T.R. Marks and N.B. Machado-Neto, 2012. Improved assessment of viability and germination of *Cattleya* (Orchidaceae) seeds following storage. *In Vitro Cellular & Developmental Biology-Plant*, 48: 127-136.

Hosomi, S.T., R.B. Santos, C.C. Custodio, P.T. Seaton, T.R. Marks and N.B. Machado-Neto, 2011. Preconditioning *Cattleya* seeds to improve the efficacy of the tetrazolium test for viability. *Seed Sci. Technol*., 39: 178-189.

Jalal, J.S. 2012. The Snow orchid (*Diplomeris hirsuta* (Lindl.) is in distress in the Western Himalaya of India. *The Mc Allen Orc. Soc. J.* 13: 11-15.

Johnson, T.R., L.S. Stewart, P. Kauth, E.M. Kane and N. Philman, 2009. Confronting assumptions about spontaneous autogamy in populations of *Eulophiaalta* (Orchidaceae) in south Florida: Assessing the effect of pollination treatments on seed formation, seed germination and seedling development. *Bot. J. Linn.,* 161: 78-88.

Klein, A.M., B.E. Vaissière, J.H. Cane, I. Steffan-Dewenter, S.A. Cunningham, C. Kremen and T. Tscharntke, 2007. importance of pollinators in changing landscapes for world crops. *Proc Biol Sci B: Biological Sciences*, 274: 303-313.

Krauss, S.L., E.A. Sinclair and J.D. Bussell, 2005. Effects of pollination on reproduction and longevity in the grassland perennial, *Rutidosis leptorrhynchoides* (Asteraceae). *Ann. Bot.*, 95: 1031-1037.

Lallana, V.H., L.F. Garcia, 2013. Efecto de pretratamientosen la prueba de viabilidad de semillas de *Trichocentrum jonesianum* (Orchidaceae). [Effect of pretreatments on *Trichocentrum jonesianum* seeds viability test]. *Investigacion Agraria*, 15:120-132.

Lakon, G. 1949. The topographical tetrazolium method for determining the germination capacity of the seed. *Plant Physiol.,* 24: 389-394.

Macedo, M.C., D.B.C.J. Rosa, M.B. Tatara, J.S. Soares, N.T.K. Hofmmann, Y.B.C.J. Rosa, 2014. Armazenamento de sementes e aclimatização de *Brassavola tuberculata* Hook. [Seed storage and acclimatization of *Brassavola tuberculata*]. Semina: *Ciências Agrária*s, 35: 2883-2894.

Moktan, S., D.Boral, P. Rai, 2021. The fate of *Diplomeris hirsuta* (Lindl.) Lindl.: A vulnerable orchid in Darjeeling region of eastern Himalaya, India. *Ecology, Environment and Conservation,* 27(3): 1174-1179

Murashige, T., F.Skoog, 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures*. Physiol. Plant.,* 15(3): 473-497.

Nayar, M.P. and A.R.K. Shastry, 1987. Red Data Book of Indian Plants. *Botanical Survey of India*, Calcutta.

Pearce, N.R. and P.J. Cribb, 2002. Flora of Bhutan: The Orchids of Bhutan. Royal Botanic Garden, Kew, London.

Pimm, S.L., C.N. Jenkins, R. Abell, T.M. Brooks, J.L. Gittleman, L.N. Joppa, P.H. Raven, C.M. Roberts and J.O. Sexton, 2014 The biodiversity of species and their rates of extinction, distribution, and protection. *Science,* 344: 1246752.

Pradhan, U.C. 1974. *Diplomeris hirsuta* (Lindl.) a survey. *American Orc Soc Bulletin,* 43: 525-528

Robertson, J.L. and R. Wyatt, 1990. Reproductive biology of the yellow-fringed orchid, *Platanthera ciliaris*. *Am. J. Bot.,* 7**7**: 388-398.

Sheoran, O.P. 2010. Online Statistical Analysis (OPSTAT). Software developed by Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana, India. http://www.hau.ernet.in/opstat.html.

Sipes, S. and V.J. Tepedino, 1995. Reproductive biology of the rare orchid, *Spiranthes diluvialis*: breeding system, pollination and implications for conservation. *Conserv. Biol.,* 9: 929-938.

Soares, J.S., Y.B.C.J. Rosa, M.B. Tatara, J.C. Sorgato and C.S.R. Lemes, 2014. Identificação da viabilidade de sementes de orquídeaspelo teste de tetrazólio. [Viability identification of orchid seeds by the tetrazolium test]. *Semina: Ciências Agrárias*, 35: 2275-2284.

Sydes, M.A. and D.M. Calder, 1993. Comparative reproductive biology of two sun-orchids; the vulnerable *Thelymitra circumsepta* and the widespread *T. ixioides* (Orchidaceae). *Aust. J. Bot.,* 41: 577-589.

Taiz, L. and E. Zeiger, 2010. *Plant Physiology* (5th ed.). Sinauer Associates.

Talalaj, I., J. Kotowicz, E. Brzosko, B. Ostrowiecka and O. Aleksandrowicz, 2019. Spontaneous caudicle reconfiguration in *Dactylorhiza fuchsii*: A new selfpollination mechanism for Orchideae. *Pl. Syst .Evol.,* 305: 269-280.

Thakur, U. and N. Dongarwar, 2012. Artificial pollination and in-vitro asymbiotic seed germination in garden orchid *Spathoglottis plicata Blume* (Orchidaceae). *Recent Res. Sci. Tech.,* 4(2): 13-18.

Van Waes, J.M. and P.C. Debergh.1986. *In vitro* germination of some Western European orchids. *Physiol. Plant.*, 67: 253-261.

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