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# *In vitro* propagation of some important medicinal and ornamental Dendrobiums (Orchidaceae): A review

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## Abstract

*Dendrobiums* are highly valued in the floriculture industry and have important medicinal properties used in preparing herbal medicines worldwide. Numerous anthropogenic factors are leading to the rapid loss of natural stands of germplasm. Plant tissue culture, specifically *in vitro* propagation, maybe the only viable solution for preserving and reintroducing RET plants back into the wild. An efficient protocol for *in vitro* seed germination and propagation through various explants of *Dendrobiums* was established. The protocols developed will not only help alleviate the pressure on the natural population under stress but also help meet its demands in pharmaceutical and ornamental industries and form the basis for conservation. A perusal of available literature reveals that micropropagation has been achieved using immature or mature seeds/embryos, protocorms, shoot tips, TLCs, leaf explants, pseudobulbs and nodal segments in *Dendrobium*s. This review provides a short synopsis of the advances made thus far in the *in vitro* propagation of ornamental and medicinal *Dendrobiums*.

**Key words:** *Dendrobium*, *In vitro*, orchids, plant growth regulators, propagation

## Introduction

Orchids are the most beautiful ornamental species having global importance as cut flowers and potted plants. This plant group is most diverse among the flowering plants having 800 genera and 35000 species (Aktar *et al.*, 2007). The demand for their cut flowers increased due to the higher number of flowers per inflorescence and beautiful, long-lasting flowers exhibiting an incredible diversity in size, shape, structure and colour. The genus *Dendrobium* is the third largest group among the Orchidaceae, comprising about 1400 species worldwide (Jin *et al.*, 2009) and has both ornamental and medicinal importance. *Dendrobium* is one of the most popular orchids worldwide because of the beauty of the flowers, the high number of flowers per inflorescence, year-round production and long-lasting inflorescence are advantages of *Dendrobium*. Among the total cut flowers orchid species of the world, the *Dendrobiums* contribute about 85% to the floriculture industry (Cheamuangphan *et al.*, 2013). Though *Dendrobiums* are grown primarily as ornamentals, some are employed as herbal medicines and food by many different cultures and tribes. Many orchid species, including *Dendrobiums* are threatened with the danger of extermination through deforestation, environmental pollution and indiscriminate collection. Meanwhile, many orchid species have become extinct and many others are on the verge of becoming rare and endangered. Many orchids are listed in the Red Data Book prepared by the International Union for Conservation of Nature and Natural Resources (IUCN). The entire family is now included in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), where international trade is strictly controlled and monitored (Pant, 2013). Applications of *in vitro* techniques might be the best solution for the mass propagation and conservation of this versatile group of plants. Orchids are used in traditional medicine systems worldwide, from subsistence to commercial levels of exploitation. In many countries like China, India, some parts of Europe, America, Australia and Africa, orchids have been used as traditional drugs for a long time. The ethnomedicinal values of several orchids were also discussed in "Charaka Samhita" a classical Indian medical system written by Charaka in Sanskrit a thousand years ago. Scientists traced the importance of orchids in the medicinal system. Chinese were the first to cultivate and describe orchids and they were the first to describe orchids for therapeutic use. Various orchid genera are used in traditional medicine. In traditional Chinese medicine (TCM), Herba Dendrobi represents the amalgamation of the stems of different species of *Dendrobiums*. TCM's most predominately used orchids are various *Dendrobium* species (*D. catenatum, D. chrysanthum, D. chrysotoxum, D. fimbriatum*, *D. loddigesii, D. moniliforme, D. nobile, and D. officinale*) used to make the drug Shihu.

### *Techniques in micropropagation of Dendrobiums*

**Asymbiotic seed germination and Protocorm development:** *In vitro* asymbiotic seed germination is a powerful tool to preserve RET, native and over-collected orchid species, producing many plants. It also makes it possible to increase the efficiency of conservation and breeding programmes since *in vitro* seed germination rates higher than 70% are commonly reported in many taxa (Kunakhonnuruk *et al.*, 2018), while in ex-vitro conditions under natural environmental conditions, these rates hardly exceed 5% germinated seeds (Ashok Pyati, 2019). In orchid seeds, the zygotic embryo is poorly differentiated, composed of 80-100 cells, meristem and cotyledons are usually not present at the time of seed dispersal, non-endospermic and contain almost no nutrients. In nature, germination and early developmental

stages must be symbiotic with highly specialized fungi (Swarts and Dixon, 2009). Although orchid seeds are produced in large numbers, the development of asymbiotic seed germination techniques followed the formulations of Knudson B and C media (1946) and shoot tip culture by Morel (1960) has revolutionized the concept of orchid cultivation. Many investigators have used immature seeds from unripe green pods for germination in aseptic cultures. Many species of orchids, including Dendrobium seeds from mature pods, have been found to exhibit poor germination, attributed to the dormancy of seeds. Germination of seeds generally depends on their nutritional reserves. Several variations in reserve food composition between immature and mature seeds have been reported by many researchers (Lal and Singh, 2020). The culture of immature seeds, often called green pod culture or embryo culture, has been used extensively for the successful germination of several taxa, including *Dendrobiums,* by several investigators. The high levels of ABA and a formation of a thick carapace with phenolic deposits will have a negative impact on asymbiotic seed germination. By selecting the green capsules at a certain time of fruit development increases the rate of asymbiotic seed germination. This phenomenon can now be explained by the fact that the embryo has completed the histo-differentiation program by forming a blueprint for protocorm formation. Also, at this time, the ABA level is low and only a thin cuticle is present around the embryo properly. By using ELISA and immune-staining techniques, endogenous ABA level has been reported in *Calanthe* and *Cypripedium.* ABA level remains low during early seed development and rises considerably during seed maturation (Lee *et al.*, 2007 and 2015). Fluoridone an ABA biosynthesis inhibitor, was injected into the developing capsules, enhancing the germination of mature seeds.

Lowering the ABA level imposes success in asymbiotic seed germination in orchids. The first visible sign of orchid seed germination is the swelling of embryos turning green and emergence out of the sphere-shaped structure, which subsequently develops into protocorms. These protocorms later differentiate into shoots and roots. Physical barriers such as thick seed coats and carapace have not yet fully developed. All these features facilitate asymbiotic seed germination of immature seeds. The accumulation of high levels of ABA and secondary metabolites are recognized as key negative factors in asymbiotic germination of mature seeds (Yeung, 2017). Seed dormancy, temperature, photoperiod and culture media may influence asymbiotic seed germination. An analysis of the morphogenetic changes in the orchid embryos before their seedlings indicates that an intervening protocorm stage usually accompanies orchid seed germination and subsequent development into seedlings. The orchid protocorm is a unique structure designed to establish symbiotic association in nature with a compatible mycorrhizal fungus and with a primary goal to form a shoot apical meristem for plantlet growth.

**Effect of nutrient media and PGRs on asymbiotic seed germination:** The impact of many mineral-based media has been tested for *in vitro* germination of *Dendrobium* seeds. Both seed germination and seedling development of *Dendrobiums* are strongly affected by the basal media's mineral composition and various PGRs (Table 1). A universal orchid medium is yet to be formulated since much is not known about the minimum mineral requirements of all the species. Some *Dendrobium* species prefer a low mineral content nutrient media, while others prefer high mineral composition for seed germination. The stimulation of *Dendrobium* seed germination and protocorm formation on some nutrient media namely, MS, Mitra *et al.* medium, $B_5$, *etc.,* may be due to their high total mineral contents (Paul *et al.*, 2011; Parmer and Acharya, 2016; Hajong *et al.*, 2010; Dohling *et al.*, 2008).

Similarly, some species of *Dendrobiums* were well responded to when cultured in low mineral content nutrient media, namely K.C., VW, Burgeff's $N_3$f *etc.* (Utami *et al.*, 2017; Mary and Divakar, 2015). Based on the literature surveyed, the nutrient regime for various orchid culture, including *Dendrobiums* is species-specific and no single medium is universally applicable for all the orchid species. The choice of culture medium strongly affected germination, presumably because of differences in the balance and supply of organic and inorganic nutrients. The importance of $NH_4^+$ and $NO_3^-$ ions during *in vitro* germination

Table 1. *In vitro* seed germination and seedling development in *Dendrobium* species

| Sl. No. | Species | Explant | Media composition | Results | References |
|---|---|---|---|---|---|
| 01 | *D. fimbriatum* | Immature seeds | MS + NAA (0.5 mg/L) + BAP (0.5 mg/L) | PLBs | Parmer and Acharya, 2016 |
| 02 | *D. ovatum* | Mature seeds | Modified White Medium + CW (200 ml/l) + P (4.0 g/l) | PLBs | Gurudeva, 2019 |
| 03 | *D. antennatum* | Mature seeds | Grow more medium + CW (10%) | PLBs | Nugroho *et al.*, 2018 |
| 04 | *D. macrostachyum* | Mature seeds | PM + BAP (3.0 mg/L) + NAA (1.0 mg/L) | PLBs | Parveen *et al.*, 2016 |
| 04 | *D. primulinum* | Mature seeds | MS + BAP (0.5 mg/L) + NAA (0.5 mg/L) | PLBs | Adhikari and Pant, 2019 |
| 06 | *D. aqueum* | Mature seeds | ½ MS | PLBs | Parthibhan *et al.*, 2012 |
| 07 | *D. aggregatum* | Seeds | MS and Phytomax media | Protocorms | Hossain, 2013 |
| 08 | *D. jenkinsii* | Immature seeds | MS | PLBs | Barman *et al.*, 2020 |
| 09 | *D. taurulinum* | Mature seeds | KC + AC (2.0 mg/L) | PLBs | Nurfadilah, 2016 |
| 10 | *D. officinale* | Seeds | ½ MS + Potato extract (100 g/l) + BA (2.0 mg/L) + NAA (2.0 mg/L) | Shoot proliferation | Chen *et al.*, 2014 |
| 11 | *D. macrostachyum* | Immature seeds | VW + BAP (0.5 mg/L) + NAA (0.5 mg/L) | PLBs and Multiple plantlets | Mary and Divakar, 2015 |
| 12 | *D. nobile* | Seeds | OMM + NAA (1.0 mg/L) + CW (25%) | PLBs and plantlets | Ranju Tamang, 2017 |
| 13 | *D. lasianthera* | Seeds | VW + Peptone (3.0 g/l) | Protocorms and seedlings | Utami *et al.*, 2017 |
| 14 | *D. aggregatum* | Immature seeds | MS + BAP (1.5 mg/L) + CW (15%) | PLBs | Vijayakumar *et al.*, 2012 |
| 15 | *D. chrysanthum* | Mature seeds | MS | PLBs | Hajong *et al.*, 2010 |
| 16 | *D. thyrsiflorum* | Mature seeds | Mitra + Kin (2.0 mg/L) | MPLBs | Tikendra, 2018 |
| 17 | *D. hookerianum* | Seeds | MS | Protocorms | Paul *et al.*, 2011 |
| 18 | *D. longicornu* | Mature seeds | MS + IAA (0.057 µM) | Protocorms | Dohling *et al.*, 2008 |
| 19 | *D. densiflorum* | Immature seeds | MS + NAA (3.0 µM) + BA (3.0 µM) | PLBs | Pongener and Deb, 2016 |

of many orchids as a source of $N_2$ is well established. The source of $N_2$ in the MS medium is ammonium nitrate. The presence of it may explain the high germination rate because $NH_4^+$ is readily assimilated during the initial stages of development and greatly influences growth and differentiation. The optimum seed germination percentages in most Dendrobium species were recorded in the MS medium. The suitability of MS medium for seed germination, protocorm formation and seedling development in many *Dendrobes* could be attributed to the fact that the MS medium is rich in macro and micronutrients. The nitrogen present in MS medium greatly influences the growth and differentiation of cells. Nitrogen in the form of ammonium nitrate in the MS medium could have been the most suitable source for seed germination and seedling development in *D. longicornu* and *D. formosum* (Dohling *et al.*, 2008).

The high germination percentage of seeds of *D. chrysanthum* on $B_5$ medium was due to the presence of nitrogen in the form of potassium nitrate and the poor response in the K.C. medium have been due to the lower amount of nutrients and vitamins, which were not sufficient for complete development of the seedlings (Hajong *et al.*, 2010). The ½ MS medium containing Zeatin (1.0 mg/L) proved the best medium for seed germination and spherule formation in *D. ovatum* (Shetty *et al.*, 2015). Adhikari and Pant (2019), reported that the ½ MS basal medium was ideal for the spherule formation in *D. primulinum*. However, the presence of NAA (0.5 mg/L) or a combination of BAP (0.5 mg/L) + NAA (0.5 mg/L) expressed a synergistic effect to enhance the protocorm formation and seedling development. Similarly, in the *D. sonia*-28, where the MS medium fortified with BAP (4.44 or 8.88 µM) with NAA (8.88 µM) resulted in an increased PLB growth rate compared to when added separately to the nutrient medium (Julkiflee *et al.*, 2014). Nongdam and Tikendra (2014) cultured unripe green capsule-derived seeds of *D. chrysotoxum* on Mitra *et al.* medium supplemented with A.C. (0.4%), BAP (2.0 mg/L) and IAA (2.0 mg/L) produced highest seed germination and developed into complete plantlets. The mature seeds of *D. aqueum* were inoculated onto twenty basal media for seed germination studies.

Among the media tested, ½ MS medium observed the highest percentage of germination and even seedling development. However, in K.P., W and S, Lindell, Curtis, Fast and Mitra media, there was no development of protocorms due to insufficient/ deficient nutrients to produce chlorophyll and so no further development (Parthibhan *et al.*, 2012). Barman *et al.* (2020) showed that the nitrogen source affects seed germination. Different vitamins like pyridoxine, thiamine and nicotinic acid in MS medium may also promote the germination rate. Protocorms supplemented with BAP (2.0µM) and NAA (0.5µM) yielded the highest number of shoots and roots, suggesting the synergistic effect of cytokinin and auxin vary on shoot and root formation. Both full and half-strength Prasad and Mitra medium and the hormonal combinations of BAP (3.0 mg/L) and NAA (1.0 mg/L) showed excellent growth for high-frequency germination of mature seeds in *D. macrostachyum* (Parveen *et al.*, 2016). Based on different results by many investigators, it is clear that MS and ½ MS media are the most commonly used basal culture media for *Dendrobium* seed germination. While K.C., $N_6$, and V.W. media are the second most used formulations. It was revealed in a meta-analysis of the *Dendrobium* asymbiotic seed germination literature that 37 *Dendrobium* genotypes (including hybrids) were used in the study. As abiotic factors 25±2 $^oC$ as temperature, a 12-h photoperiod and 30-42 µ mol $m^{-2}s^{-1}$ white fluorescent light intensity was reported as the most favorable conditions for successful germination and further development (Teixeira da Silva *et al.*, 2015). The role of PGRs in asymbiotic orchid germination is uncertain, and responses to growth regulators are often species-specific. A major obstacle to understanding the role of exogenous and endogenous PGRs in promoting/inhibiting germinating orchid seeds may be the small size of the seeds and possible low levels of PGRs in the embryo. Investigating the concentrations of endogenous PGRs in orchid seeds and when PGRs are active in germinating would greatly enhance the current knowledge of how PGRs affect the germination of orchid seeds (Kauth *et al.*, 2008).

**Effect of complex organic additives on asymbiotic seed germination:** Several complex organic growth supplements including Peptone (P), Yeast Extract (Y.E.), Casein Hydrolysate (C.H.), Cane Juice (C.J.), Potato Homogenate (P.H.), Banana Homogenate (B.H.), Tomato Juice (T.J.), Coconut Water (C.W.) *etc.* are routinely employed to enrich the nutrient media for orchid seed germination. The growth-promoting effect of these supplements has been attributed to their organic nitrogenous compounds (amino acids and amides), minor elements and vitamin constituents. Steward and Simmonds (1954) reported that substances that stimulate cell divisions in carrot cells are present in the formative layers of banana fruit. It has often been used as an organic additive in, *in vitro* cultures because of its high levels of potassium, manganese, calcium, sodium, iron, zinc, thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, ascorbic acid, folic acid, and natural growth regulators such as zeatin, gibberellins and IAA. It is also rich in carbohydrates, supplying energy to heterotrophic plants during the early stages of *in vitro* cultivation (Mohapatra *et al.*, 2010). Islam *et al.* (2015), investigated the effect of B.H. concentrations (0, 2.5, 5, 10 and 20%) on the growth and development of PLBs in *Dendrobium* species and showed ½ MS medium supplemented with B.H. (10%) was the most suitable for PLB regeneration. The ½ MS medium with B.H. stimulated the formation of the highest root numbers in *Dendrobium* hybrids (Hapsoro *et al.*, 2018). Nambiar *et al.* (2012), studied the effect of homogenate of various local bananas on seed germination in *D. alya pink*. The seeds cultured on ½ MS medium supplemented with 5, 10, 20 and 30% B.H. were not effective in enhancing the growth of the PLBs, PLBs under the treatment of 20 and 30% of B.H. failed to survive due to necrosis. Although B.H. has been widely used in plant tissue culture, adding this organic additive was not a good option to improve PLB proliferation.

C.W. is the most common and popular complex organic additive used for orchid cultivation *in vitro*. The use of C.W. in plant tissue culture was first attempted in 1941 by Van Overbeak (1941) in the *in vitro* development of *Datura stamonium* embryos. C.W. contains enormous nutrients, minerals, carbohydrates, amino acids, vitamins *etc.* (Tan *et al.*, 2014). Klaocheed *et al.* (2021) showed that the MS medium supplemented with C.W. (15%) enhanced the highest shooting percentage and number of shoots per explant in *D. crumenatum*. The MS medium with the addition of 3% sucrose, BAP (1.5 mg/L) and C.W.

(15%) favored the higher rate of germination in *D. aggregatum* (Vijayakumar *et al.*, 2012). The simple medium that consists of Growmore 10-55-10, a foliar fertilizer supplemented with C.W. (10%) gave 100% seed germination and dark green protocorms, facilitated the best plantlet growth and induced the highest root growth in *D. antennatum* (Nugroho *et al.*, 2018). Ranju Tamang (2017), investigated the effect of C.W. in *D. nobile* on orchid maintenance medium (OMM) supplemented with NAA (1.0 mg/L), BAP (4.0mg/L) and C.W. (25%) gave highest percentage of germination and in ½ OMM with NAA (1.0mg/L) and C.W. (10%) regenerated maximum percentage of plantlets. V.W. medium supplemented with BAP (1.5 mg/L), NAA (1.5 mg/L) and C.W. (50 ml) with A.C. (500 mg/L) was found to be the most suitable medium for *in vitro* rooting of *D. macrostychum* (Mary and Diwakar, 2015). Kaur *et al.* (2015), reported that Mitra *et al.* medium supplemented with C.W. (200 mL/L) was the most suitable for enhancing protocorm multiplication in *D. nobile*. Gurudeva (2019), investigated that C.W. (200 mL/L) and Peptone (4.0 g/L) in the modified White's medium enhanced the percentage of seed germination in *D. ovatum*. Harahap *et al.* (2020), observed that the different parameters like, the number of buds, number of leaves and number of roots in the treatment with various concentrations of BAP and C.W. with the basal MS medium for the *Dendrobium* species showed that the highest number of buds, leaves and roots obtained from the medium containing BAP (1ppm) with C.W. (5%). Teixeira da Silva *et al.* (2015), concluded that natural organic complex such as C.W. is very commonly used as an organic supplement in, *in vitro* propagation of *Dendrobium* species, usually at concentrations of 10-20%.

Peptone (P) is also an organic supplement to the orchid culture's media. Various investigations have shown that P supplement enhances seed germination, protocorm formation and seedling development. P's stimulatory effect may be because it contains amino acids, proteins, nitrogen and vitamins like biotin, pyridoxine and thymine, increasing growth and development. When P (2.0 g/L) was added to the V.W. medium, the germination of seeds (100%) in *D. lasianthera* was stimulated. Without the P, only (57.6%) of germination was recorded (Utami *et al.*, 2017). Adding P (2.0 g/L) to the K.C. medium was suitable for shoot induction, protocorms and root formation in *D. lowii* (Jualang *et al.*, 2016). Tharapan *et al.* (2014) showed that Potato extract with P in the Hyponex (H) medium proved beneficial for the healthy shoot growth of *D. Judy Ritz* seedlings and *D. discolor* protocorms. Hossain *et al.* (2013), reported that the mature seeds of *D. aphyllum* germinated on Phytomax medium when P (2.0 g/L) was added, remarkably enhanced germination percentage and subsequent development of protocorms. The optimum germination (58.03%) of *D. phalaenopsis* was documented on V.W. medium supplemented with C.W. (100 mL/L). When P (2.0 mg/L) and tomato extract (100 mL/L) were added to the medium significantly increased the germination percentage (94.42%), the growth rate of embryos, protocorms and seedlings (Setiari *et al.*, 2016). Lin *et al.* (2020) showed that ½ MS medium, pH 5.7, NAA (1.0 mg/L) + B.A. (1.0 mg/L), temperature 23±2 ºC and light intensity of 1000 lux conditions stimulated the seed germination, protocorm proliferation and differentiations in *D. cariniferum,* addition of P (1.5 g/L) into the medium effectively promoted rooting.

***In vitro* regeneration of *Dendrobiums* through explants:** Orchids are out breeders, their propagation using seeds to produce heterozygous plants is in practice. Hence protocols providing regeneration from various vegetative parts of the mature plants or *in vitro* grown plants are essential. The choice of an explant is the most crucial factor in establishing micropropagation protocols for many plants, including orchids. The explants most commonly used in micropropagation are derived from *in vitro*-grown seedlings or greenhouse-grown plants and include shoot tips, axillary buds, nodal segments, protocorms, leaves, young stems, pseudobulbs, inflorescence stalks, root tips, thin cell layers, *etc.* (Table 2).

**Shoot tips/meristem:** Using the shoot tip culture technique has rapidly multiplied many orchids. Shoot tips from both *in vitro* and mature plants successfully utilize for clonal propagation. Shoot tips have effectively induced multiple shoots and PLBs of many *Dendrobiums.* Cultured shoots of about 0.4 cm from the PLBs of *Dendrobium "Gradiata 31"* on ½ MS medium containing TDZ (1.0 mg/L) and B.A. (0.5 mg/L), when C.W. (15%) was added to the medium optimal growth and proliferation of PLBs (Winarto and Teixeira da Silva, 2015). Mamun *et al.* (2018), reported that the shoot tips of *D. red bull* cultured on MS medium fortified with the combination of BAP (3.0 mg/L) and NAA (1.0 mg/L) produced the maximum number of multiple shoots. The shoot explants of D. *aurantiacum* were cultured on MS medium fortified with 2,4-D (10.0 mg/L) initiated callus development. Then the proliferated callus was transferred to MS medium with the supplement of BAP (0.5 mg/L) and NAA (0.5 mg/L), resulting in the development of somatic embryos (Ma *et al.*, 2020). Reda Refish *et al.* (2016), investigated that the shoot explants of *D. candidum* cultured on MS medium supplemented with 2,4-D (1.0 mg/L) with Kinetin (0.5 mg/L), resulted in 100% callus induction. Qian *et al.* (2014), induced the callus from the shoot tips of D. *officinate* on MS medium fortified with B.A. (0.5 mg/L) and NAA (0.1 mg/L). This callus on subculture on hormone-free medium developed into PLBs. These PLBs were transferred onto MS medium containing NAA (1.0 mg/L) developed shoots. Similarly, Pant and Thapa (2012) showed that the shoot tips of *D. primulinum* when cultured on MS medium fortified with NAA (0.5 mg/L) and BAP (1.5 mg/L), produced multiple shoots and were effective for the shoot multiplication. Best rooting was observed in IAA (0.5 mg/L).

**Protocorm-like bodies (PLBs):** Attempts were made to micropropagate *Dendrobiums* through protocorm culture, which provides a useful way to reestablish plants in the wild for germplasm preservation and commercial propagation. Hence the PLBs have emerged as effective donor structures for micropropagation of orchids. The protocorm explants are highly meristematic cells with leafy shoot initials connected with vascular tissues. Due to the effect of PGRs, multiple numbers of new shoots were developed with the extending vascular connection from the explants. When the protocorms of *D. aqueum* were cultured on ½ MS medium fortified with NAA (3.0 mg/L) gave maximum shoots, these cultured on IBA (5.0 mg/L) produced a maximum number of roots per shoot (Parthibhan *et al.*, 2015). Ashok Pyati (2020), induced the callus from the injured PLBs of *D. barbatulum* on ½ MS fortified with BAP (3.23 µM). This callus on subculture onto the same medium

Table 2. *In vitro* Propagation of *Dendrobium* species by using various explants

| Sl. No. | Species | Explant | Media composition | Results | References |
|---|---|---|---|---|---|
| 01 | *D. huoshanense* | Leaf and Root | ½ MS+2,4-D (1.0 mg/L) + TDZ (1.0 mg/L) | Callus | Lee and Chen, 2014 |
| 02 | *D. sonia-28* | PLBs | ½ MS + Sucrose (10.0 g/l) | Increased growth rate of PLBs | Julkiflee *et al.*, 2014 |
| 03 | *D. candidum* | TLC (node) | ½ MS + NAA (1.2 mg/L) + 6-BA (1.2 mg/L) | Shoot regeneration | Zhao *et al.*, 2007 |
| 04 | *D. chrysotoxum* | Nodes | Liquid MS + NAA (5.37 µM) | PLBs | Kaur, 2017 |
| 05 | *D. palpebrae* | Pseudobulb | MS + NAA (1.0 mg/L) + BAP (2.0 mg/L) | Multiple shoot buds | Bhowmik and Rahman, 2020 |
| 06 | *D. aphyllum* | Nodes | PM + IAA (1.5 mg/L) + BAP (2.5 mg/L) | Multiple shoot buds | Bhattacharjee and Hossain, 2015 |
| 07 | *D. chrysanthum* | Nodal segments | MS + BAP (5 µM) + TDZ (5 µM) | Shoot buds | Hajong *et al.*, 2013 |
| 08 | *D. bensoniae* | Nodes | MS + BA (1.0 mg/L) + IBA (1.5 mg/L) | Shoot regeneration | Riva *et al.*, 2016 |
| 09 | *D. nobile* | Nodal explants | MS + Meta topolin (1.0 mg/L) + Putrescine (0.8 mg/L) | Shoot proliferation | Bhattacharyya *et al.*, 2016 |
| 10 | *D. thyrsiflorum* | Nodal explants | MS + TDZ (3.0 mg/L) | PLBs | Bhattacharyya *et al.*, 2015 |
| 11 | *D. chryseum* | Protocorms | MS + Kn (2.0 mg/L) + CW (10%) | Shoot multiplication | Maharjan *et al.*, 2020 |
| 12 | *D. crumenatum* | PLBs | MS + CW (15%) | Multiple shoots | Klaocheed *et al.*, 2021 |
| 13 | *D. barbatulum* | PLBs | ½ MS + BAP (3.23 µM) | Callus mediated regeneration | Ashok N. Pyati, 2020 |
| 14 | *D. aqueum* | Protocorms | ½ MS + NAA (3.0 mg/L) | Shoots | Parthibhan *et al.*, 2015 |
| 15 | *D. primulinum* | Shoot tips | MS + BAP (1.5 mg/L) + NAA (0.5 mg/L) | Shoot multiplication | Pant and Thapa, 2012 |
| 16 | *D. red bull* | Shoot tips | MS + BAP (3.0 mg/L) + NAA (1.0 mg/L) | Multiple shoots | Mamun *et al.*, 2018 |
| 17 | *D. hybrids sonia 17 and 28* | Foliar explants | ½ MS + BA (44.4 µM) | PLBs induction | Martin and Madassary, 2006 |
| 18 | *D. longicornu* | Axillary buds | MS + NAA (30 µM) | Multiple shoots | Dohling *et al.*, 2012 |
| 19 | *D. candidum* | Protocorms | ½ MS + BAP (8.8 µM) | Callus mediated regeneration | Zhao *et al.*, 2007 |
| 20 | *D. nanum* | Rhizome buds | MS + NAA (2.0 µM) + Kn (1.2 µM) | Callus mediated regeneration | Maridass *et al.*, 2010 |
| 21 | *D. transparens* | Pseudobulb | ½ MS + BAP (2.0 mg/L) + NAA (1.0 mg/L) | Multiple shoots | Sunitibala and Kishor, 2009 |
| 22 | *D. aurantiacum* | Shoots | MS + 2,4-D (10. mg/L) and MS + BAP (0.5 mg/L) + NAA (0.5mg/L) | Callus mediated somatic embryogenesis | Ma *et al.*, 2020 |
| 23 | *D. fimbriatum* | Nodal segment | MS + BAP (17.76 µM) + NAA (17.76 µM) | Shoots regeneration | Paul *et al.*, 2017 |
| 24 | *D. aqueum* | Stem TLC | ½ MS + Zea (0.5 mg $dm^{-3}$) and ½ MS + BA (0.5 mg $dm^{-3}$) + 2iP (1.5 mg $dm^{-3}$) + Kin (1.5 mg $dm^{-3}$) | Embryogenic callus and Somatic embryogenesis | Parthibhan *et al.*, 2018 |
| 25 | *D. hybrid sonia* | Shoot tips and TLC | Liquid MS + NAA ( 0.5 mg/L) + BAP (1.0 mg/L) and Putrescine (1.0 µM) | Callus and PLBs | Mandal *et al.*,2020 |

developed into primary and secondary PLBs later differentiated into plantlets. Maharjan *et al.* (2020), reported that the synergistic effect of BAP (0.5 mg/L) with C.W. (10%) in ½ MS medium produced the highest number of shoots from the protocorms of *D. chryseum* and the maximum number of roots were recorded on the same medium supplemented with Kn (0.5 mg/L). *In vitro* shoot regeneration from the protocorms of *D. crumenatum*, documented in MS medium supplemented with C.W. (15%) (Klaocheed *et al.*, 2021). Khosravi *et al.* (2008) cultured the PLBs of *D. serdang beauty* to induce the calli on MS medium containing IBA (1.5 mg/L). Kn treatment (1.0 mg/L) was the best for plantlet regeneration. The calli, PLBs and shoots were developed on longitudinally bisected PLBs of *D. spectabile* cultured on V.W. and ½ MS media supplemented with B.A. (0.5 mg/L) + NAA (0.5 mg/L). The optimum calli (44.40%) and PLBs (55.60%) developed on basal V.W. and ½ MS media, respectively (Soetopo and Purnamaningsih, 2012).

**Leaf segments:** Young leaves and leaf tips were successfully cultured *in vitro* to propagate some *Dendrobiums*. Using leaf explants for effective micropropagation relies on factors such as culture composition, growth regulators, *in vitro*/in vivo leaf source to be used portion, orientation and age. The above factor's standardization time and cost requirement strictly restrict mass-scale industrial micropropagation of orchids using leaf explants (Sarmah *et al.*, 2017; Mondal and Banerjee, 2017; Chugh *et al.*, 2009). In *Dendrobium* species, culturing leaf explants is very difficult; most of the early culturing attempts failed. Since then, the regeneration potential of leaf explants from *Dendrobiums* has been recorded by few investigators. Martin and Madassery (2006) used the leaf explants from *in vitro* grown shoots of *Dendrobium* cultivars *Sonia 17* and *28* cultured on ½ MS medium containing B.A. (44.4 µM) was most efficient for the induction of direct shoots in both the hybrids. A young leaf segment of *D. sonia earsakul* was divided into apical and basal parts, cultured on ½ MS medium with TDZ alone or combined with NAA. The basal leaf segments had more somatic embryos than the apical leaf segments. As for the effect of explant orientation on somatic embryo formation, placing the dorsal surface of the leaf segment gave a greater number of somatic embryos higher than placing the ventral surface on the medium (Juntada *et al.*, 2014). Goswami *et al.* (2015) showed that the leaf tip explants of *Dendrobium* species were placed on an MS medium containing a high concentration of 2, 4-D (10 mg/L), and developed maximum PLBs. The maximum number of shoots from PLBs was observed in the MS medium containing NAA (0.5 mg/L) + BAP (0.5 mg/L). The in vivo grown three years old *D. queen sonia* hybrid leaf explants were inoculated on V.W. medium supplemented with NAA (1.0 mg/L) + BAP (1.0 mg/L) developed maximum numbers of green PLBs. These differentiated into plantlets in V.W. medium

supplemented with 2,4-D (1.0 mg/L) + C.W. (150 ml) + A.C. (1.0%) with banana pulp (100 mg/L) (Jayarama Reddy, 2016). Bhattacharjee and Hossain (2015) inoculated the leaf explants from *in vitro* seedlings of *D. aphyllum* on MS and PM media along with various PGRs. The leaf segments did not respond to any of the media combinations used.

**Stem nodal segments and pseudobulbs:** Micropropagation of *Dendrobiums* through tissue culture using nodal stem segments has become an effective technique to reproduce/propagate, commercialize, conserve and save many species from extinction. Nodal segments of *in vitro*-grown seedlings of *D. chrysanthum* inoculated onto the MS medium-fortified TDZ (5.0 µM) and BAP (5.0 µM) developed highest number of shoots. These on subculturing with NAA (10.0 µM) resulted in root development (Hajong *et al.*, 2013). Nodal and inter-nodal segments of *in vitro* grown *D. aphyllum* seedlings cultured on MS and PM media supplemented with BAP (1.5 mg/L) and Picloram (1.5 mg/L) induced green and compact callus (Battacharjee and Hossain, 2015). The maximum number of multiple shoot buds (MSB) was produced in PM added with IAA (1.5 mg/L) and BAP (2.5 mg/L). Bhattacharya *et al.* (2015) induced direct and indirect shoot organogenesis in *D. thyrsiflorum*, when the nodal segments cultured on MS medium supplemented with TDZ (3.0 mg/L) exhibited the highest regeneration of adventitious shoot buds. Similarly, the nodal explants excised from *in vivo* grown *D. nobile* plants cultured on MS medium supplemented with BAP, formed PLBs. Furthermore, among the best-treated PGRs meta-topolin (1.0 mg/L) proved beneficial in inducing the highest frequency of shoot buds and primary and secondary PLBs (Bhattacharya *et al.*, 2016). Riva *et al.* (2016), reported that the shoot nodes of *D. bensoniae* cultured for rapid multiplication on MS medium fortified with B.A. (2.0 mg/L) was the most effective in inducing the shoots. Stem nodes of 22 weeks old *in vitro* grown plantlets of *D. chrysotoxum* were cultured on liquid and solid MS media. The liquid cultures were more efficient in inducing neo-formations than semi-solid medium. The MS liquid agitated medium supplemented with NAA (5.37 µM) caused PLBs formation on nodal explants. When these PLBs were cultured on an agarised MS medium supplemented with NAA and B.H. (50 g/L), developed into robust plantlets (Kaur, 2017). The nodal explants of *D. fimbriatum* were cultured on MS medium with the combination of PGRs BAP (17.76 µM) and NAA (17.76 µM) developed shoot buds. This corroboration suggests the synergistic effect of auxin in amalgamation with cytokinin promotes shoot proliferation (Paul *et al.*, 2017). Bhowmik and Rahman (2020) reported that the *in vitro*-grown pseudobulb segment of *D. palpebrae* cultured on MS medium supplemented with NAA (1.0 mg/L) and BAP (2.0 mg/L) formed into multiple shoot buds. These MSBs were cultured on the liquid MS medium fortified with Picloram (0.5 mg/L) and BAP (1.0 mg/L) and developed shoot primordia-like structures (SPSs) at the base of tiny seedlings, and these were used for mass scale production of seedlings.

**Thin cell layers (TLCs):** Thin cell layers, transverse as well as longitudinal sections of plant tissues such as shoots, stem nodes, pseudobulbs and PLBs have been successfully used as explants for regeneration in a few orchids as well as other plant species. TLCs have been successfully used for PLBs and callus induction in many orchids, including *Dendrobiums*. The advantage of the TLC system is to produce high-frequency organ regeneration and reduces the time interval required to generate plantlets. This technique is also for mass propagation of ornamental plants, conventional cash crops, and difficult-to-propagate species for research and commercial purposes. This protocol requires special attention to selecting appropriate explants, technical skills and careful handling of explants (Teixeira da Silva, 2013). The *in vitro* grown seedlings stem of *D. candidum* were transversely sliced and used as tTLC explants cultured on MS medium having macro-nutrients at half strength added with B.A. (1.5mg/L) induced shoots. The combinations B.A. (1.2 mg/L) and NAA (1.2 mg/L) showed the highest frequency of shoot regeneration (Zhao *et al.*, 2007). Parthibhan *et al.* (2018) reported that the stem TLC of *D. aqueum* was cultured on ½ MS medium supplemented with Zeatin (0.5 mg $dm^{-3}$), induced green compact embryogenic callus (E.C.), but it became necrotic. The TLCs cultured in the presence of 2iP (1.5 mg $dm^{-3}$) and Kn (1.5 mg $dm^{-3}$) continued to proliferate well and developed globular somatic embryos (S.E.s). Mandal *et al.* (2020), showed that the TLCs of *D. hybrid Sonia*, were cultured on ½ MS medium supplemented with the combinations of BAP (0.5 mg/L) and NAA (0.5 mg/L) induced callus and PLBs were developed when the TLCs were cultured on ½ MS fortified with NAA (0.5 mg/L) + BAP (1.0 mg/L). The TCL method revealed that, in the long run it will become a very effective system for generating various responses, particularly in callus formation and direct PLB formation in orchids.

***Ex vitro* hardening/acclimatization:** Micropropagation has been largely used for the rapid multiplication of disease-free plants and to conserve RET plant species *in vitro*. But plants may get damaged during transferring *in vitro* plantlets/seedlings to ex-vitro conditions. Many micro-propagated plants do not survive when transferred from *in vitro* to greenhouse or field environments with substantially lower relative humidity, higher light and septic environments than *in vitro* conditions. The transplantation stage continues to be a major bottleneck in the micropropagation of orchids. The percentage of plant loss depends on hardening substrates, environmental factors and plant species. Thus, acclimatization of *in vitro* plants to a greenhouse environment is an important stage in obtaining quality plants of orchids for commercial cultivation (Lakshanthi and Seran, 2019). Plant mortality is high in transfers of *in vitro* plantlets to ex-vitro conditions because the cultured plants under *in vitro* conditions have weak root systems and poorly developed cuticles. The plantlets/seedlings are vulnerable to serious damage and loss at this stage. Hence it is necessary to select a suitable growing medium in the acclimatization stage and provide optimal environmental conditions to obtain high survival rates of *in vitro* plantlets under ex vitro conditions. The potting medium having high water holding capacity and moderate aeration is best for acclimatization of *in vitro* grown *Dendrobiums*. Researchers use different combinations of potting media, which include coconut husk, charcoal, brick pieces, chip stones, leaf litter, cocopeat, *sphagnum* moss, vermiculite, decayed wood, bark pieces, leaf mold, pine bark, tree fern, *etc.,* in different combinations for hardening of *in vitro* raised plantlets. The survival rate was higher when plantlets were passed through hardening and acclimatization stages before transfer than direct transfer of the plantlets to the field. More than 85% of survival rates can be achieved in natural conditions when plantlets are passed through a pre-acclimatization

phase of nearly 1 to 2 months. In most of the available literature on the hardening of *in vitro* grown plants requirement of special media and plenty of air around root is emphasised. In the pre-acclimatization phase, high humidity (about 80-90%) around the plantlets for two weeks was essential for hardening.

Recent climatic changes and anthropological activities result in even more declination of most orchids. Apart from their use in the ornamental industry, they are also used in the medicinal and food industries. Their natural multiplication rate, such as through vegetative and sexual reproduction, is extremely slow. There is a serious need to multiply orchids through alternative methods. Orchids are out-breeders. Their propagation through seeds leads to the production of heterozygous plants. Hence the protocols providing regeneration from various vegetative parts of mature/*in vitro* grown plants are essential. The major advantage of clonal propagation is that the plantlets produced are usually identical to their clones (parents). During the last few decades, plant tissue culture techniques have been used for rapid and large-scale propagation and conservation. Different protocols have been developed for the large-scale propagation of orchid species through *in vitro* culture of various vegetative parts, including leaf, axillary buds, roots, shoot tips, nodes, inter-nodes, pseudobulbs, rhizome segments and TLCs. Successful micropropagation using different explants depends on factors like medium nutrient composition, orientation, and most importantly, the age of the explants. The protocols developed in the laboratories can be standardized and transferred to industries and organizations involved in the conservation and commercialization of orchids.

The present review on *Dendrobiums* was undertaken to learn about the effect of different PGRs and growth adjuvants on asymbiotic seed germination effect of explants on *in vitro* and TCL cultures. Plant tissue culture is the applied science for the conservation and preservation of orchid germplasm.

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