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Prospects of Plant Tissue Culture in Orchid Propagation: A Review

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Abstract

Orchids (Family Orchidaceae) are ornamentally valuable and unique group of flowering plants. They are valued mostly for their long lasting and diverse morphology of flowers. Some orchids are highly prized due to their application in food, flavor and medicinal industry. Seed germination and vegetative propagation are the methods through which orchid plants regenerate in nature. Both these methods of regeneration are quite slow to fulfill orchid demand. Plant tissue culture technique has been used extensively in multiplication of orchids. Although seed germination produces heterogenous population but most of orchid *species* are multiplied by asymbiotic seed germination. Propagation through protocorm-like-bodies (PLBs) or direct from explant regeneration is quite more advantageous to multiply orchids. Artificial seed technology is another method used for orchid multiplication, transport and conservation of endangered orchid *species*. Culture of medicinal orchids for production of bioactive metabolites could be used as substitute for dependence on natural population.

Keywords: Orchids; Propagation; Protocom like bodies; Thidiazuron; Secondary metabolite; Synthetic seed.

Introduction

Orchids (Family Orchdaceae) are generally characterized with long lasting, diverse morphology and exquisite flowers that have been extensively used in floriculture since time immemorial. At present, in world's flower industry it account with an annual sales more than US$4 billion.[69] The family includes about 736 genera with more than 28,000 *species* which are mostly distributed in the wet tropics worldwide except few isolated islands, polar and desert regions.[14,69] India has rich orchid diversity and approximately 158 genera and 1331 *species* flourishing up to an elevation of 5000m are recorded.[54] In comparison to plants from other families the mycorrhizal specificity, pollinator specialization and very low germination of seeds restricts the distribution of orchid *species* narrowly and are quite more susceptible to habitat distribution.[70]

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The family is considered as most diverse family among the flowering plants.[15] They grow on all kinds of habitats, except the aquatic systems and on their growth habitat they may be characterized as terrestrial, epiphytic or saprophytic. Most of the orchids are epiphytic in nature, while the rest are terrestrial. Although orchids are grown mainly for ornamental applications but, some are employed as herbal medicines and food (tubers of *Cynorchis* and *Eulophia*) by many different cultures and tribes.[4] Vanillin obtained from *Vanila planifolia*, an

odourous constituent is a very useful commercial product. It is used for flavouring in baking, ice-creams, custards, pudding and in aromatherapy and perfume industry.[35] *Gastrodia elata* has medicinal application in treating tetanus, headaches, dizziness and epilepsy.[62] *Macodes, Ludisia, Anoectochilus* and *Goodyera* genera have attractive foliage and are known as jewel orchids. The wild population of orchids can multiply sexually by seed, and asexually by vegetative propagation. However, the vegetative propagation is very slow and only few seeds germinate in spite millions seed produced in each capsule. The seeds have simple seed coat structure and reduced embryos. The embryos are minute size generally varies in length from 0.05 to 6 mm, width 0.01 to 0.93 mm, and 0.3 to 14 µg in weight.[2] The seeds have varied suspensor morphology, lack cotyledons, endosperm and depicts availability of insufficient nutrient reserves needed for germination. The association of orchid seeds with appropriate fungus for germination is necessary to fulfill the essential physiochemical stimulus needed for seed germination and seedling development. Collection of wild population as a source of stock plant for various socio-economic trades instead of their commercial multiplication is a serious threat to several *species* of orchids. Low multiplication of wild population and extensive uses in floral and other industries has resulted in depletion of population of many *species* at an alarming rate and some of them are at the verge of extinction.[16,42] In recent past, in vitro culture methods of propagation have been used to reduce collection pressures on wild population. In addition to asymbiotic seed germination, orchids are regenerated through explants such as leaves, root rhizome, shoot meristem, axillary buds, pseudonode etc.[16] In this communication, we have reviewed the recent progress made in plant tissue culture for multiplication, conservation and genetic modification of orchids.

1. Asymbiotic Seed germination

In nature orchid seed germination is dependent on association with mycorrhizal fungus. Knudson[32] demonstrated that no mycorrhizal association is needed on in vitro germination of orchid seeds and by using simple nutrient medium along with sucrose Knudson pioneered the germination of several epiphytic orchids. Multiplication of orchids through in vitro seed germination technique have been developed for several *species* and are most commonly used method for multiplying different orchid *species* and hybrids representing diverse habits and habitats.[3,49] Under culture conditions germination of seeds are affected by several physico-chemical factors and it is requisite to optimize seed germination for each *species* of orchid. A number of media such as Murashige and Skoog,[43] Mitra et al.,[41] Kundson[33] and Gamborg et al.,[21] etc. have been used for seed germination and propagation through tissue culture. Growth regulators like auxins and cytokinins are added into medium for germination and growth of seedlings of several orchids.[54]

The organic additives such as peptone, banana extract, coconut water, casein hydryolysate, yeast extract etc. accelerate seed germination and protocorm formation.[29] Availability of natural source of carbohydrates, vitamins, amino acids, inorganic ions and phytohormones in such additives fulfill the nutritional requirement of germinating seeds which in wild are fulfilled by mycorrhizal association.[25,54]

Both immature and mature seeds can be germinated using tissue culture technique but germination of mature seeds is quite more difficult in comparison to immature seeds. Germination of seeds generally depends on their nutrient reserves. Several variations in reserve food composition between immature and mature seeds have been reported by researchers. In *Corallorhiza maculata* the immature seeds have starch as reserve food while mature seeds contain lipids.[27] This was suggested that during the process of seed maturation starch and other carbohydrates of immature seeds is converted into lipids of mature seeds. The lack of metabolic machinery in mature seeds is another hindrance in germination of mature seeds.[26] Dormancy of mature seeds and accumulation of germination inhibitors in the seed coat also restrict germination of mature seeds. The dormancy of seeds can be broken by low temperature treatment[27] or by using sucrose.[66] In the germination process, seeds first form protocorm and protocorm proliferates to form more protocorm or can differentiate root and shoot.

2. Multiplication from vegetative explants

Plant tissue culture techniques have been used for regeneration of orchids during last several years and a few *species* of orchids have been multiplied either through direct shoot regeneration[16,54,58] or from protocorm like bodies formation from culture of vegetative explants.[16,54] Orchid propagation through seed germination give rise to heterozygous plants, while micropropagation through various vegetative parts of the plants can control this problem, however multiplication of orchids through micropropagation is limited due to obstacle such

as low population of plants in nature, phenolics exudation, field transplantation, somaclonal variation etc. Of the various reports regeneration from the explants selected from plants growing in nature or greenhouse is very few in comparison from the explants selected from in vitro raised seedlings (Table 1). Explants such as apical meristem, node, pseudonode, shoot, leaf, leaf tip, rhizome etc. have been used for clonal multiplication. Different cytokinins such as BAP (N6 –benzylaminopurine), Kinetin, 2-iP (2-isopentyladenine), TDZ (Thidiazuron), either alone or in combination with auxin 2,4-D (2,4 dichlorophenoxy acetic acid), NAA (α-naphthalene acetic acid), IAA have been tested for shoot proliferation and PLBs formation (Table 1). In *Oncidium flexuosum* , TDZ was reported to be effective in direct PLB formation from leaf explants under dark while plants with well developed shoots and roots grew only after transfer of PLBs to under light and growth regulator free medium.[38] Roy et al.,[51] reported that low concentration (0.909 µM) of TDZ induced more PLBs from seedling derived pseudostem segments of *Cymbidium giganteum* while in high concentration, PLB multiplication increased but reduced plantlet and root development from the PLBs. They also suggested that in dual phase culture system where a thin layer of the liquid medium overlaid the semi-solid medium, low concentration of TDZ is most appropriate for inducing healthy plantlets from pseudostem segments of *Cymbidium giganteum*. In *Dendrobium longicornu* shoot formation from the nodal explants was observed in medium containing 15 µM BAP and 5 µM NAA while PLBs were developed when the medium contained BAP in combination with 2,4-D.[20] In some orchids light is also considered among the various factors that affect PLB induction. In *Phalaenopsis* absence of light induce PLB formation while differentiation of PLBs into plantlets was favored by light. However, most studies in *Phalaenopsis* describe presence of light for PLB regeneration. Induction of PLBs is also affected by the type of light. According to Mehraj et al.[39] with the use of blue and white light emitting diodes (LED) combined with trehalose as carbohydrate source or red and white LED with sucrose as carbohydrate is favorable for PLB regeneration. Hybridization is very useful technique for producing orchid cultivar with attractive morphology, color and fragrance of flowers. Mass multiplication of orchid hybrids can be made possible certainly by plant tissue culture technique.[12]

Table 1: Micropropagation through vegetative explants culture of some orchids.

Orchid	Explant	Medium and Growth Regulator(s)	Result/Response	Reference
Arundina graminifolia	Node explants derived from three-year-old field grown plants	½ MS + 6.97 µM kinetin (Kn), or 15% coconut water (CW) or 13.3 µM BA 1/2MS + 44.4 µM BA	sprouting of the axillary bud PLB from base of bud	(36)
Cattleya 'Almakee'	epidermal (=foliar) peel root explants from the greenhouse plants	Mitra with Kn (1mg L-1) + NAA (1 mgL-1)	PLB and somatic embryogenesis	(53)
Coelogyne cristata	segment of PLBs (3 mm2 in size)	½ MS with 1.0 mg l−1 NAA and 0.5 mg l−1 BA with Coconut Powder	Shoot formation	(44)
Cymbidium giganteum	Pseudostem from seedling	MS with 0.909 µM TDZ	PLB formation	[51]
Cymbidium goeringii	Rhizome explants from seedlings	MS with 20 µM 2,4-D and 2 µM TDZ MS with 2 µM NAA	Shoot formation Rooting of shoots	(46)
Dendrobium sp.	Leaf tip	MS + 0.5 mgl−1 NAA + 0.5 mg−1 BAP	Shoot initiation and plant formation	(24)
Dendrobium sp.	Leaf tip	MS + 10 mg−1 2, 4-D	PLB	(24)
Dendrobium lituiflorum	Axillary buds from 5-month-old in vitro grown plants	MS medium supplemented with 0.5 mg l−1 2,4-D	PLB	(17)

Doritaenopsis	Root tip from in vitro plantlets	modified MS +2.3 µM TDZ	PLB	(47)
Dendrobium chrysotoxum	Transverse sections (2 mm thickness) of stem-nodes from in vitro raised seedlings	semisolid and liquid Murashige and Skoog medium supplemented with BA 4.44 µM and KIN 4.65 µM	Shoot buds on cytokinin containing media or (PLBs) on NAA containing media both solid and liquid.	(31)
Geodorum densiflorum	Rhizome tips from in vitro seedlings	MS + 2 mg/l BAP	Shoot formation	(5)
Rhynchostylis retusa	Leaf	MS with 1.5 mg/l BAP and 1.5 mg/l Kinetin	PLB and somatic embryogenesis	(28)
Oncidium flexuosum	Leaf	modified MS medium with 1.5 µM TDZ under dark	PLB formation	(38)
Phalaenopsis amabilis cv. 'Cool Breeze'	thin sections of Inflorescence axis	NDM (New Dogashima Medium) medium 0,5 + mgl-1 NAA, 2% sucrose, 10% coconut water, 2 gl-1 peptone and 1gl-1 activated charcoal	PLB formation	(60)
Thunia marshalliana	Pseudonodes from plants grown in glasshouse	MS with 22.0 µM BAP	Shoot regeneration	(58)
Vanilla planifolia	Leaf from field growing plant	MS medium + 4.52µM 2,4-D + 2.22µM BAP	Shoot regeneration from callus	(30)
Vanilla planifolia	Shoot tip from plants growing in greenhouse	MS with 1.0mg/l BAP + 150ml coconut water	Shoot regeneration	(34)

Table 2: Production of Secondary metabolite from in vitro cultures of some medicinal orchids.

Orchid	Culture type	Secondary metabolite/activity	Reference
Ansellia africana	In vitro regenerated PLBs	phenolic acids exhibiting a high degree of antioxidant activity	(7)
Coelogyne ovalis	Leaves of micropropagated plantlets	phenolics, flavonoids and anthocyanins) and antioxidant activity	(59)
Dendrobium thyrsiflorum	Shoot	Antioxidant activity	(6)
Dendrobium aphyllum	In vitro plantlets	Antioxidant activity	(9)
Dendrobium fimbriatum	In vitro regenerated shoot, root and leaves	β-sitosterol	(48)
Dendrobium hybrid(Dendrobium enopi x Dendrobium Pink Lady)	LED illuminated Protocorm-Like Bodies	Flavonoid, Phenolics and anticancer activity	(67)
Habenaria edgeworthii	callus suspension cultures	Phenolics and antioxidant activity	(22)
Malaxis acuminata	In vitro regenerated leaf and shoot	Dietary fatty acids, α-hydroxy acids, phenolic acids, sterols, amino acids, sugars and glycosides	(10)

3. Production of Synthetic seed and in vitro conservation

Artificial seed technology is considered as an effective and extensively used method for propagation of plant *species* exhibiting reproductive barrier.[1,37,57] The difficulty in propagation of orchids through seeds can be replaced by using artificial seed technology. Transportation and delivery of propagules can be easily carried out through artificial seeds. Several orchid *species* such as *Cymbidium devonianum*,[18] *Cymbidium eburneum*, Cymbidium hookerianum,[23] *Geodorum densiflorum*,[19] *Paphiopedilum wardii*[68] *Dendrobium wardianum*,[52] Ansellia africana[8] etc. have been mass propagated successfully through artificial seed. Its application in conservation of desirable, rare and endangered orchids has been reported by several researchers. Plantlets of *Vanda coerulia* was obtained by encapsulation of six month old PLBs regenerated from leaf explants, the encapsulated PLBs was stored for 100 days at 4ºC.[55] Artificial

seeds of *Cymbidium aliofolium* was prepared by encapsulating protocorms with 4% (w/v) sodium alginate and 0.2 mol/l calcium chloride solution showed 100% germination and also the encapsulated protocorm could be stored for 28 days at 4ºC.[50] Reducing the nutrient strength in the encapsulated matrix and low temperature improves storage period of PLBs of *Cymbidium devonianum*.[18] In *Dendrobium nobile* incorporation of sucrose and mannitol (7.5 and 12 %) in the encapsulating matrix showed storage of encapsulated PLBs till 60 days.[42]

4. In vitro culture and secondary metabolite production

In addition to extensive use of orchids as ornamentals many orchids are used extensively as herbal medicines. A number of reports are available on medicinal uses of orchids. Some orchids such as *Dendrobium nobile*, *Gastrodia elata* and *Bletilla striata* are routinely used in Traditional Chinese Medicine.[45,56] The *genus Anoctochilu, Calanthe, Coelogyne, Cymbidium, Cypipedium, Dendrobium, Ephemerantha, Gymnadenia, Habenaria, Nevilia* and *Thunia* are common orchid genera which exhibit biosynthesis and accumulation of medicinally useful metabolites.[45] Phytochemicals such as alkaloids, bibenzyl derivatives, flavonoids, phenanthrenes etc. have been reported from orchids.[56] The populations of medicinal orchids are decreasing at alarming rate due to their exploitation in medicinal uses. The in vitro culture of plant cells, tissues and organs has been demonstrated as a pivotal source for production of useful structurally complex and high value natural biomolecules.[64] The use of plant tissue culture is a substitute for dependence on natural habitat as well as conventional cultivation of orchid plants for the production of bioactive secondary metabolites. Accumulation of secondary metabolites in in vitro cultures of orchids has been reported in some studies (Table 2). Factors such as type of explants, composition and type of culture medium, growth regulators and their concentration, concentration of carbon source, culture age etc. influence biosynthesis and accumulation of secondary metabolites in in vitro culture.[45,56] Though some studies have been done on secondary metabolite production from in vitro culture of orchids but still extensive research is needed with orchid culture to optimize factors that influence secondary metabolite production.

5. Genetic engineering and orchid improvement

Improvement of orchids for some major traits such as flower morphology, colour, fragrance, cut-flower longevity, resistance to diseases, stress etc. through transgenic technologies can be advantageous over the breeding of orchids through traditional methods. Modification of orchids for desirable character by using plant genetic engineering has been demonstrated in a few orchid species.[40] Longevity of flowers of *Dendrobium chrysotoxum* in vase life has been enhanced by *Agrobacterium* mediated transfer of an antisense ACC oxidase gene into protocorms and the ACC oxidase inactivated in regenerated transgenic plants.[11] The bright yellow of the wild type color of *Oncidium* 'Gower Ramsey' flower was changed to more attractive light and white-yellow by using *Agrobacterium tumefaciens* mediated transfer of RNA interference for β-ring carotene hydroxylase (BCH2) gene.[65] Shoot multiplication of *Phalaenopsis amabilis*[61] and *Dendrobium lasianthera*[63] has been enhanced by inserting Knat 1 (Knotted 1 like *Arabidopsis thaliana*) gene. *Phalaenopsis* orchid with enhanced resistance to Cymbidium Mosaic Virus (CymMV) and *Erwinia carotovora* was developed by double transformation of PLBs. Using Particle bombardment initially PLBs were transformed with CymMV coat protein cDNA (CP) then re-transformed by *Agrobacterium* mediated transfer of sweet pepper ferredoxin-like protein cDNA (Pflp).[13] Though remarkable advances have been made for production of transgenic orchids however, extensive studies remain to be done.

6. Conclusion and Future prospects

Orchids are considered as largest and most diverse group of angiosperms. Apart from their use in ornamental industry they are also used in medicinal and food industry and many orchid *species* are considered as endangered due to their extensive collection and several other anthropological activities. Vegetative and sexual multiplication process is extremely slow to meet its demand worldwide. There is serious need to multiply orchids through alternate method and some orchids so far have been multiplied through in vitro culture technique. Though, plant tissue culture technique has been used mostly for asymbiotic seed germination but multiplication of orchids through seeds generates heterozygous plants. Establishment of protocol for regeneration of orchids from various vegetative explants are required. Improvement in flower quality by strengthening of orchid biotechnology can advance floriculture industry. In vitro production of secondary metabolites from medicinal orchids is another aspect which is initiated in few orchid *species* and extensive emphasis should be given on metabolites engineering techniques to enhanced production and identification of secondary metabolite.

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