Review Article

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A Review on Asymbiotic Seed Germination in Orchids through Plant Tissue Culture

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Orchid seeds are very small, dust like in appearance, fusiform in shape, lacking endosperm and have undifferentiated embryo covered by transparent seed coat. Mycorrhizal association is required for seed germination of naturally growing orchids. In this symbiotic association, orchid species are dependent on mycorrhizal association for supply of mineral nutrients. In *in-vitro* condition, such demand of minerals may be compensated by external supply of sugar and mineral nutrients that are required for seed germination of orchid. Several orchids are responded by this asymbiotic seed culture and have commercial importance. Therefore, formulation of efficient *in-vitro* protocol is important for commercially important orchid species as well as endangered orchid species for conservation. This review paper is focused on various aspects of asymbiotic seed germination of orchids and the role of organic additives in successful seed germination.

Key words: Orchids, asymbiotic seed germination, media, *in-vitro* culture, organic additives.

1. Introduction

Plant tissue culture has a long history. History of plant tissue culture is based on reviews by Krikorian and Berquam[1], Gautheret [2], Bhojwani and Razdan [3], Gamborg [4], Dodds and Roberts [5], Trigiano and Gray [6], and Vasil [7]. Thousands of commercial orchids (family: Orchidaceae) are mainly grown *in-vitro* for their beautiful flowers and medicinal importance. It is estimated that about 28000 species of orchid belonging to 736 genera under family Orchidaceae occur worldwide [8 and 9]. This is the largest and highly evolved family of flowering plants [10]. Seventy percent of orchids are epiphytes which constitute around two-third of the world's epiphytic flora [11]. Of 1100 species, nearly 150 are economically important in India, representing one of the major orchid-rich regions [12]. Orchid has highly specialized pollination mechanism and also have small, thin and non-endospermic seeds. Symbiotic association with mycorrhizal fungi is required for orchid seed germination in their natural habitats [13]. Orchids are diverse with universal habitats and have extraordinary mechanism of adaptation to persist in adverse environmental conditions [13, 14, and 15]. Most of the time orchids charmed botanists, horticulturists, and evolutionary biologists. Propagation of orchids via seed germination has a long history. The large-scale production of orchids in nature through conventional horticultural methods is quite difficult due to their slow growth rate and poor rate of seed germination under natural conditions [16 and 17]. Moreover, growth of orchids is enhanced by specialized microclimatic condition and by the protective canopy of the plants in their natural habitats [18]. Demand of orchids has been commercially increasing day by day. As a result, the rapid propagation of orchids totally depends upon the development of *in-vitro* techniques [16 and 17]. Therefore, *in-vitro* mass propagation techniques are widely practiced for conservation and commercialization of orchid species. Asymbiotic seed germination protocol techniques were developed by Knudson and Knudson C medium formulated by him in 1946 [19] is still used today. This medium is also used for propagation of a variety of terrestrial and epiphytic orchid species. The aim of this review is to summarize the knowledge about asymbiotic seed culture of orchid and the common media that are used in orchid seed germination. Different additives are also used for enhancement of seed germination of orchids like coconut water, peptone, banana extract, potato extract, charcoal, yeast extract, case in hydrolysate etc.

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2. Orchid Seeds

Orchid seeds are extremely small (0.05–6.0 mm in length and 0.01–0.9 mm in diameter), very much light and produced in large numbers which vary from 50–400000 per capsule [20]. Generally, the embryos of orchid seeds remain undifferentiated in mature pods and endosperm development is suppressed [21]. When orchid seeds become mature, they contain lipidaceous food reserves which are present within the cells of embryos [22]. According to Knudson [23], analysis of *Cymbidium* seeds showed that they contain 32% lipid, 1% sugars, and no starch, but starch has been recorded in other orchid seed embryos [24]. Due to the limited food present in the orchid seeds, fungal endophytes are required for seed germination of orchid by facilitating nutrition uptake to enhance growth in natural condition [25]. It is believed that carbohydrate, nitrogen, minerals and vitamins are provided by fungus during germination of orchid [26]. As a result, *in-vitro* asymbiotic seed germination technique is very useful for germination of orchid seeds.

2.1 Immature Seed Germination

Several investigators reported that *in-vitro* asymbiotic seed germination is the alternative technique for propagating a large number of orchid species and hybrids using simple culture medium containing sucrose [27, 28, 29, 30 and 31]. Orchid seeds are capable to germinate prior to achieving maturity and this type of germination is called "ovule/embryo/green-pod" culture [32]. It has added new dimensions to conservation and commercialization of orchids. This type of techniques helps in i) production of virus free seedlings, ii) propagation of rare and endangered species, and iii) recovering progenies of desired mating types [33].

Immature seeds have better germination potential, due to presence of high moister content of testa cells and metabolically awakened embryos, lack of dormancy and/or inhibitory factors [34 and 35]. Mature seeds have the low osmolarity and water potential is a regulatory factor for protein accumulation in ripening seeds. During germination and developmental changes of mature seeds in culture medium, rehydration and mobilization of storage protein takes place [36]. It has been established that "Green-pod" culture technique has a great significance in orchid tissue culture. The nutritional requirements of the young embryos are complex as compared to the mature embryos [37]. Arditti et al. [28] reported that the seeds collected from half mature capsules exhibited a better germination response.

2.2 Mature Seed Germination

Seed germination rate decreased due to lack of appropriate metabolic machinery in mature seeds. Such seeds are capable of utilizing their own lipidaceous reserves [38]; accumulation of germination inhibitors in the seed coat; increase dormancy in the mature seed; and loss of viability [39]. Cold temperature also can break the dormancy of mature seeds in orchids [40].

In case of *Dactylorhizamaculata*, seed dormancy is responsible for the presence of abscisic acid content which is 15 times greater in mature seeds than the immature ones [41]. Due to simpler nutritional requirements, the epiphytic species germinate better than the terrestrial ones [28].

2.3 Media for Asymbiotic Seed Germination

Different culture media have been formulated during past several decades. Knudson (1922) succeeded in formulating medium for seed germination of orchid [42]. Presently asymbiotic seed germination is carried out in simple basal medium with sucrose and without growth regulators. Mature seed coat acts as a physical barrier for germination. To overcome this problem, different seed pretreatment is also required for enhancement of seed germination like sodium hypochlorite solution treatment. To encourage seed germination, some organic additives and plant growth regulators can be added like coconut water, peptone etc. [43].

Commonly used medium for seed germination of orchids are Knudson's C [19] which is frequently used till date. Vacin and Went medium [44] is used for more stable pH for orchid culture. Another common medium Murashige and Skoog's (MS) medium [45] used for seed germination and micropropagation of orchid. Lindemann et al. [46] formulated a medium for meristem culture of *Cattleya*. Mitra et al. [47] formulated a medium for the study of protocorm formation. Harvais [48 and 49] and Malmgren [50 and 51] have developed a medium for optimization of asymbiotic seed germination of *Cypripedium* species. Ichihashi and Yamshita [52] have devised a medium for *Bletilla* seed germination. VanWaes and Debergh [53] have optimized a medium for *in-vitro* germination of some Western European orchids. Hyponex medium is developed using Hyponex fertilizer powder with an N-P-K ratio of 6.5-6-19. This medium is used for seed germination and plantlet development studies [54].

Some commercial media formulations are available for maintenance of orchid plants. The companies are Sigma, Phyto Technology Laboratories, and Duchefa Biochemic. Media compositions are also available in websites.

2.4 Decontamination Agent of Seeds

Proper sterilization of seeds is most important for seed germination. Commonly used disinfectant solutions are sodium hypochlorite (0.5–5%), calcium hypochlorite (9–10%), and commercial bleach solutions (10–20%). Other disinfectants for short treatment are ethanol (70–90%), hydrogen peroxide (10%) and mercuric chloride (0.1–0.2%) [43]. Mercuric chloride at the concentration of 0.1% solution treated for 8 min shows a better result in the seed germination of different species of *Dendrobium*. Sometimes treatment by 70% ethanol for 30 seconds followed by treatment with 1.0% sodium hypochlorite for 10 minutes is also carried out. In some cases after the treatment of 70% ethanol, again treatment with 3% sodium hypochloride with 2–3 drops of tween 20 per 100 ml for 10 minutes gave a better result [55].

3. Common Additives

Growth of plant can be enhanced by addition of various organic supplements and plant extracts. Some commonly used additives are peptone, coconut water, yeast extract, beef extract, casein hydrolysate, banana homogenate, apple juice, extract of silkworm pupae, fish extract, and honey [56 and 57]. Different organic additives used for asymbiotic seed germination in different orchids is given in Table 1.

Coconut water (CW) is a colourless liquid endosperm. Coconut water contains soluble sugar which is a natural source of carbon, amino acids, phenols, fiber and vitamins. It also contains diphenyl urea which functions like cytokinin. CW promote cell division, help in organ differentiation in orchid culture. Some variable ions found in CW, such as potassium, phosphorus, calcium, magnesium, iron and manganese. CW also contains some water-soluble vitamins such as thiamin (B1), riboflavin (B2), niacin (B3), pantothenic acid (B5), pyridoxine (B6), myo-inositol and ascorbic acid (C) [8].

Yeast extract is used in germination and proliferation in many orchids. This is a good source of organic nitrogen, amino acid, vitamins, especially inositol and thiamin [55].

Casein hydrolysate (CH) is an amino acid complex which can help in seed germination and seedling growth of orchids. CH can be prepared by acid hydrolysis or enzymatic digestion of some natural product like milk products, plant and animal tissues, and microbial culture. Chitin is present in cell wall of fungi. This is a polymer which is most abundant on earth. Chitosan is used in agriculture for their antifungal properties, increase phytochemical production and decrease transpiration rates. It is also used in orchid seed germination. Activated charcoal has been used in different micropropagation systems including orchid seed germination.Potato extract is a rich source of nutrient. It contains starch, proteins, amino acids, vitamins and minerals. Potato extract shows positive effect on orchid growth [43].

Table 1: Different organic additives used for asymbiotic seed germination in different orchids

Organic supplements used	Plant names	References
Peptone	*Spiranthescernua* (L.) Rich.	[58]
	Goodyerabiflora (Lindl.) Hook. f.	[59]
	Paphiopedilum acmodontum M. W. Wood	[60]
	Phaius australis F. Muell	[60]
	Geodorumdensiflorum (Lam.) Schltr.	[61]
	Cymbidium macrorhizon Lindl.	[62]
	Spathoglottisplicata Blume	[60]
	Calopogontuberosus (L.) Britton, Sterns & Poggenb	[63]
	Dactylorhizamaculata (L.) Soo	[53]
	Vanda tessellata (Roxb.) Hook. ex G. Don	[64]
	Calanthe discolor Lindl.	[65]
Coconut water	*Anoectochilusformosanus* Hayata	[67]
	Calanthe hybrids	[67]
	Dendrobium kingianum Bidwill ex Lindl.	[68]
Banana homogenate	*Paphiopedilum ciliolare* (Rchb. f.) Stein	[69]
	Anoectochilusformosanus Hayata	[70]
	Cattleya lowrenceana Rchb. f.	[56]
	Hetaeria cristata Blume	[71]
	Phalaenopsis sp.	[72]
	Paphiopedilum ciliolare (Rchb. f.) Stein	[73]
	Dendrobium lituiflorum Lindl.	[74]
Casein hydrolysate	*Dactylorhizapurpurella* (T. Stephenson & T. A. Stephenson) Soo	[75]
	Aerides multiflora Roxb.	[76]
	Rhynchostylis retusa (L.) Blume	[76]
	Saccolabiumpapillosum Lindl.	[76]
	Vanda testacea (Lindl.) Rchb. f	[76]
	Bletiaurbana Dressler	[77]
	Eulophiacullenii (Wight) Blume	[78]
	Herminiumlanceum (Thunb. Ex Sw.) Vuikj	[79]
	Dactylorhizamaculata (L.) Soo	[53]
	Vanda stangeana Rchb. f.	[80]
Activated charcoal	*Cymbidium goeringii* (Rchb. f.) Rchb. f.	[81]
	Zygostates grandiflora (Lindl.) Mansf.	[82]

Banana extract that contains carbohydrates, minerals, amino acids, fatty acids, niacin, vitamins, cellulose, polyols, sterols, various phytohormones like IAA, GAs and cytokinins are widely used in seed germination of orchids [8].

4. Conclusion

Asymbiotic seed germination provides an effective way for mass production of orchids. Development of efficient protocol for seed germination and reintroduction in natural habitat is much important for conservation of rare and endangered orchid species. A better theoretical and practical understanding will give us better germination process which leads to the formation of protocorm. Orchid seed germination medium with organic supplements promotes the seed germination, protocorm formation and generate seedlings. These organic supplements provide natural source of carbohydrates, inorganic ions, amino acids, vitamins and phytohormones. Considering the decreasing population of rare orchid species, incorporation of this simple *in-vitro* technique can help to solve the demand of pharmaceutical and floriculture industry.

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