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A Review on: *In Vitro* Cloning of Orchids

Dipika Sarmah[1*], Swathi Kolukunde[1], Monoj Sutradhar[2], Brijesh Kumar Singh[2], Tapas Mandal[1] and Nirmal Mandal[2]

[1]Department of Floriculture and Landscaping, [2]Department of Agricultural Biotechnology, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, West Bengal-741252, India
**Corresponding author*

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A B S T R A C T

Orchids are the most fascinatingly beautiful flowers and unique group of plants of nature. With their exotic shapes, hues and the added advantage of longevity, these flowers of rare beauty have become increasingly popular in the 21st century. They belong to the *Orchidaceae* family and consisting 600-800 genera and 25,000-35,000 species. Today growing orchid is more than just a hobby, it is an international business covering around 10% of the world floriculture trade. Especially, some of the exquisitely rare hybrids of orchid are among the top ten cut flowers. It has become possible by adopting *in vitro* tissue culture techniques for their rapid multiplication. Since, orchids are strictly out breeders, seed propagation results in unwanted heterozygous types. So, vegetative propagation techniques require accurate regeneration protocol for obtaining *in vitro* cultured true to type plants. In fact, the technical aspect of micropropagated orchids have improvised significantly in past few years. But the loop holes in micropropagation stems from the somaclonal variation, phenolic compound exudation explants, hardening and so on. We endeavour to include the major contribution based on orchid tissue culture starting from the pioneering work of Rotor, G. 1949 to Balilashaki *et al.*, 2014.

Introduction

''If nature ever showed her playfulness in the formation of flowers, this is visible in the most striking way among the orchids.''. Most of us would definitely echo this view of the seventeenth century German botanist Jakol Breyne (Davis *et al.*, 1983). Orchids are being praised as one of the most favourite cut flower and an ornamental plant all around the world for their exotic beauty and long lasting shelf life. Currently, a multimillion dollar orchid industry is booming in countries like Australia, Malaysia, Singapore, Thailand and several others. Currently, the orchids hold sixth position among the top ten cut flowers of the world and *Cymbidium* orchid in particular contributes 3% of the total cut flower production (De and Debnath, 2011).

Though orchids are grown primarily as ornamentals, some are employed as herbal medicines and food (tubers of *Cynorchis* and *Eulophia*) by many different cultures and tribes (Arditti, 1992). Some orchid species of the *Macodes*, *Ludisia*, *Anoectochilus* and *Goodyera*, genera are known as jewel orchids because of their beautiful foliage. Few orchids are also being used as spice, *e.g.* Vanilla.

The Indian orchid diversity consisting approximately 158 genera and 1331 species are thriving upto an elevation of 5000 m. Most of terrestrial orchids are grown in humus rich moist shady locations of North Western India whereas, hills of Western Ghats harbour most of the rare small flowered orchids. The North Eastern hilly regions of India are heaven for epiphytic orchids which can be found to grow upto an altitude of 2000 m from sea level (Chowdhery, 2009). Some of native Indian genera like *Arachnis*, *Cymbidium*, *Dendrobium*are, *Paphiopedilum*, *Vanda* are largely cultivated for cut flower production. *Cymbidium* is popularly grown in Arunachal Pradesh, Assam Darjeeling and Sikkim. However, tropical orchids are found in some parts Kerala and Tamil Nadu. These native Indian orchid species rule the market for high ornamental value and also for breeding purposes. In terms of export economy, winter and spring flowering orchids of temperate regions are more preferable because quality deterioration during transport is less during colder months December to May.

The status of orchid trade and industry

‘‘Orchid growing has not fully achieved the transaction from a hobby to an industry’’ James Shoemaker, 1957. But in twenty first century, it would be completely unfair to address orchid cultivation as a mere hobby, but it has flourished into an international industry business (Griesbach, 2002). Today orchids are dominating the cut flower and potted plant commerce due to its long lasting charm, high productivity, right seasonal blooming, convenient packing and transportation (De *et al.,* 2014). The largest exporters of potted orchids are Taiwan, Brazil, Italy, Thailand, Japan, New Zealand and UK whereas, United States stands first in import (Chugh *et al.,* 2009). Asia is the main source of orchid to enter the world. The economy of many ASEAN (Association of the South East Asian Nations) countries are substantially benefitted by industrial production of *Dendrobium*, *Phalaenopsis*, *Oncidium* and *Cymbidium* orchids (Hew, 1994; Laws, 1995). During 2005 in United States, potted orchids gained a total wholesale value of US$ 144 million (U.S. Department of Agriculture, 2006) and *Phalaenopsis* sp. earned 75% of all the orchids sold replacing *Cattleya* (most popular type of 1980s) Griesbach, 2002. Thailand, which is the world’s sixth largest exporter of cut flowers, earns US$ 30 million a year from orchid exports, and Singapore earns US$ 16 million a year (Reddy, 2008).

The value of fresh cut orchids and buds trade during 2007-2012 is concise in table 1. In 2012, the total transaction of the global orchid trade reached US $ 504 million with more than 40 exporting and 60 importing countries around the world.

The top most orchid exporting countries are Netherlands (39.67%), Thailand (28.41%), Taiwan (10%), Singapore (10%) and New Zealand (6%) whereas, importing countries are mainly Japan (30%), UK (12%), Italy (10%), France (7%) and the USA (6%). Among the total cut flower orchid species of world, 85% are *Dendrobium*, 15% are *Phalaenopsis* and *Cymbidium* (Cheamuangphan *et al.,* 2013).

According to Chugh *et al.,* (2009), the annual flower production of India is around 1000 tonnes with a miniscule international market share of 0.01%. Even though since the last few years orchids have made their presence felt in the Indian cut flower trade, orchid cultivation and commerce in India is still at a budding stage. According to the Indian tissue culture market survey by Biotech Consortium India Limited (Department of Biotechnology) and Small Farmers’ Agri-Business

Consortium, 2005, *Dendrobium* sp. (as cut flower) and *Vanilla* (for spice) are the most important plants suitable for tissue culture propagation in India. Varied agroclimatic zones, cheap labour, ever growing high end consumer markets make it a highly profitable proposition to grow orchids in India (Singh *et al.,* 2008). So far, the north-eastern hill region and few parts of Kerala and Karnataka are the only Indian regions which are being used for commercial orchid production. In reality, the cut flower orchid business continuously gets hampered by lack of control in airports, huge quantities of diseased and rejected cut flowers, even toxic dye treated flowers get dumped in Indian cities for biosafety suspicion (De, 2008).

Trends in growth of some important commercial orchids

Cymbidium spp.

The semi-terrestrial orchid *Cymbidium* is originated from tropical and subtropical Asia. But, they are mainly grown in winter and spring blooming seasons and in cooler climates at high elevations. The most popular *Cymbidium* orchid growing countries are Philippines, Borneo islands, Japan, Malaysia, China, North Australia and North Eastern India. They are highly prized as cut flowers, potted plants, hanging baskets, herbal medicines and as genetic resources. It has been considered as top commercial orchids in Europe since many years. They fetch the highest price in the international markets of Singapore, Japan and the Dutch market. In India, the hills of Sikkim, Darjeeling and Arunachal Pradesh with cooler nights and monsoonal rain of summer set the most favourable climate for ideal cultivation of *Cymbidium*. Hybrid *Cymbidium* orchids (more than 350) are commercially grown in around 25 ha of land in Sikkim with 5 lakhs spikes of annual production. East Sikkim has already been declared as Agri Export Zone exclusively for production of *Cymbidium* orchids (De *et al.,* 2014). There is a huge export potential of orchid export industry from north eastern hill region especially Sikkim with improvement in supporting industries like packaging, cold storage and transportation which may also generate better employment opportunity in those underprivileged states

Dendrobium spp.

Dendrobiums come under one of the most common type of orchids which are accepted in both cut flower and potted plant form around the world. These mesmerizing orchids are known for their vast range of flower colour, size, shape and floriferousness. The everlasting orchids are also available throughout the year. The most popular potted *Dendrobium* growing regions in the United States are Hawaii, California and Florida. The wholesale value of sales for this commodity in Hawaii has been found for several decades. In the Netherlands, production of potted orchids is now 40 to 50 million units with *Dendrobium* increasing in popularity. Imports from Thailand, the world's largest exporter of tropical cut orchids and second largest supplier to the EU, accounted for 22% of supplies to the EU. Thailand holds a particularly strong position in *Dendrobium* orchids (De *et al.,* 2014).

Phalaenopsis spp.

Phalaenopsis is the second most important cut flower and potted plant orchid of the world. It is gaining popularity due to ease in cultural practices and diverse flower colour, shape, size and delicacy. It is commercially grown in Germany, Japan, Netherlands, Taiwan and United States. In USA, around 13,500,000 *Phalaenopsis* were sold during 2005 and currently it shares 75% of the

purchased orchids. The export value of *Phalaenopsis* from Taiwan to the United States increased from $8 million in 2005 to $ 13 million in 2006 (De *et al.*, 2014).

In vitro clonal propagation of orchids

The concept of i*n vitro* culture was first proposed by Haberlandt (1902) and explicitly verified for the first time by Steward *et al.*, (1958). In this technique, the totipotency of plant is exploited to multiply the plants from their meristematic cells in controlled *invitro* condition. It can also be called as tissue culture, cell culture or organ culture under *in vitro* condition (Debergh and Read, 1991). Large scale proliferation of healthy and disease free quality propagating material can be obtained with this technique. It has also been proved to be economically viable option for efficient biodiversity and gene pool conservation.

The orchids are highly heterozygous and their vegetative propagation through division, splitting of shoots and kiekis are very slow which yields only a meagre number of plants even after five to six years. Although orchid seeds are produced in large number, *i.e.*, two to three millions per capsule, as they lack endosperm, they germinate very poorly in nature.

The propagation and cultivation of orchids was revolutionized by the discovery of Knudson (1992), showed that orchid seeds can germinate on a relatively simple medium containing sucrose. This became the standard procedure for germinating orchid seeds. The earliest report of using tissue culture techniques in the clonal propagation of orchid was that of Rotor (1949). Observing that plantlets could develop from buds of *Phalaenopsis*, he cultured flower stalk nodes *in vitro* and obtained some plantlets. The tremendous development made in orchid industry through the wide applications of tissue culture techniques owes its credit to Morel (1960) who was the first to magnificently initiate Cymbidium meristem tip culture. The orchids are the symbol of the first successful mass micropropagated floriculture crop.

Factor influencing *in vitro* cloning

Explant

Explant sterilization

Explant sterilization is most important for *in vitro* propagation. Bacterial and fungal infections can be eliminated by proper immersion time of explants in the sterilization agents (Yildiz and Er, 2002). Maximum survival percentage of cultures of *Dendrobium nobile* var. Emma white with limited necrosis and infection was observed when sterilization of axillary buds was done for 8 min with 10% (v/v) (Asghar *et al.*, 2011).

Balilashaki *et al.*, (2014) carried out a study to identify the best method for sterilization of nodes of *Phalaenopsis amabilis* cv. Cool 'Breeze' with different concentration of sodium hypochlorite and they found that best assembly for sterilization is about 7% sodium hypochlorite.

Type of explant

Rotor, 1949 first demonstrated that *Phalaenopsis* plantlets development from the buds of inflorescence stalks through micropropagation. The Morel, 1960 revolutionized commercial propagation of orchids by shoot meristem culture techniques. However, as the techniques entails the sacrifice of the mother plant or its entire new organ growth, organ culture (leaves, roots, inflorescence stalks) is the fast emerging as an

ideal method for cloning elite orchids (Arditti, 1977).

Meristem/ shoot tip culture for *in vitro* propagation

The technique of apical meristem culture for virus-free production of dahlias was first published by Morel and Martin (1952) and later on Morel, (1960) did the same for production of Cymbidium. However, Wimber (1963) was the true pioneer to establish the detailed protocol for *in vitro* production of Cymbidium using meristem culture as starting material.

Steward and Mapes (1971) gave an account of the growth and development of aseptically cultured cells and tissues showing that Cymbidium plants could be indefinitely multiplied as cell cultures which gave rise to PLBs. Infact, the time expenditure for transformation of free cells into abundant protocorms were comparatively very long (about 9 months). But, each of these obtained protocorms was capable of giving rise to a healthy plant. Since then, shoot tips are effectively being used for the induction of PLBs and shoot buds of many orchids (Table 2).

Vanda coerulea was rapidly multiplied by shoot tip culture technique and clonally propagated in forest segments of the Western Ghats (Seeni and Latha, 2000). Kalimuthu *et al.,* (2006) reported initiation, multiplication, elongation and rooting of *Vanilla* using MS + 1 mg/L BAP + 150 mg/L CW media under *invitro* condition.

Success in callus cultures in which the callus can be maintained for a prolonged period through subsequent subculture has been limited to a few orchids (Ishii *et al.,* 1998; Chang and Chang, 1998; Roy and Banerjee, 2003). It is because of the uncertainties related to callus induction, necrosis, restricted growth and proliferation (Roy *et al.,* 2007). Roy and Banerjee (2003) reported embryogenic callus induction from shoot tip *explant* of *Dendrobium fimbriatum Lindl*. var. *Oculatum* in modified KC medium + 0.5 mg/L NAA + 1 mg/L BAP). However, Roy *et al.,* (2007) observed the same response of *D. chrysotoxum* in modified MS medium + 2 mM TDZ/BAP.

It is a matter of fact that only two (*Vanda* and *Vanilla*) among all of the shoot tip culture adopted orchids are monopodial orchids which have a single upright shoot with indeterminate growth and absence of rhizomes (Arditti, 1992). So, growth and development retardation of mother plant is common in shoot tip culture of monopodial orchid. Therefore, it is not always considered to be economical (Philip and Nainar, 1986). However, sympodial orchids like *Dendrobium*, *Cymbidium*, *Arundina*, *Phaius* and *Anoectochilus* may be more adaptive towards shoot tip culture.

Leaf segment culture

Leaf segment explants are advantageous over other type of explants, since they are easy to obtain without sacrificing the mother plant and available around the year. Wimber (1965) first used *Cymbidium* leaf tissue culture for PLBs production. Seeni and Latha, 1992 successfully regenerated endangered *Renanthera imschootiana* Rolfe (Red Vanda) from leaf tissue culture. The direct adventive shoot bud formation was observed in the bases of the leaves. But there was no such response from chlorophyllous distal parts of the leaves of both flowering and *in vitro* grown plants. The mature leaves of *Vanda coerulea* (Blue Vanda) also could not regenerate shoot buds or PLBs (Seeni and Latha, 2000). The influence of tissue juvenility in terms of donor leaf size on the regeneration competence (frequency of response and number and nature of

regenerants) was also reported in Vanda Kasem's Delight 'Tom Boykin' (Vij *et al.*, 1994) and *Vanda coerulea* Griff. (Vij and Aggarwal, 2003) foliar cultures. Since, there was variation in the response of juvenile and mature leaves under same *in vitro* conditions, it clearly reflected the effect of explant source and physiological age (Murthy and Pyati, 2001).

Chen and Chang, 2001 reported deleterious effect on embryo formation from *Oncidium* leaf explants by auxins (IAA, IBA, NAA and 2, 4-D). However, they found promotive effect of cytokinins like 2iP, zeatin, Kin, BAP and TDZ in embryogenesis. They also found somatic embryogenesis promotion by ancymidol, paclobutrazol and demotion by GA3 and cycocel (Chen and Chang, 2003a). They again studied with auxin transport inhibitors (TIBA and quercetin) and auxin antagonist (PCIB). They found somatic embryogenesis from leaf tip *explant* retardation by IAA and 2, 4-D (Chen and Chang, 2004).

Ethylene precursor (ACC 1-amino cyclo propane-1-carboxylic acid) was shown to have a dose dependent response with lower concentrations (5 mM and 10 mM) significantly retarding and higher concentrations (20-50 mM) enhancing direct embryo formation. Ethylene inhibitors $AgNO_3$ and $CoCl_2$ retarded embryo formation suggesting that ethylene may be necessary for direct somatic embryogenesis (Chen and Chang, 2003b). The frequency of embryogenesis and the average number of embryos per embryo-producing explant were both reported to be affected by explant orientation. Chen and Chang (2002) observed higher somatic embryogenesis response with adaxial side up orientation than abaxial side up orientation. The use of leaf explants for effective micropropagation relies on factors such culture composition, growth regulators, leaf source (*in vitro/in vivo*), to be used portion, orientation and age. These above factors standardization time and cost requirement strictly restricts mass scale industrial micropropagation of orchids using leaf *explants* (Table 3).

Inflorescence axis and flower bud culture

Rotor (1949) first initiated the use of orchid inflorescence segment as explant in *Phalaenopsis* cultures using flower stalks *in vitro*. This technique is advantageous over others, since there is no requirement of sacrificing the whole plant to obtain the *explant*.

Moth orchid Phalaenopsis is monopodial, which mostly was mass propagated by this technique. This popular orchid genus is very difficult to propagate vegetatively. Out of the several techniques that have been developed for *in vitro* propagation of this popular orchid, most involve culturing the dormant buds present at the basal part of the inflorescence (Griesbach, 1983).

The *in vitro* cultured flower stalk buds have three modes of growth: dormant, vegetative and reproductive in most of the orchids. BA (4.40 mg/L) and NAA (1 mg/L) added to the MS medium promoted the large number of shoots derived from the inflorescence stalk node of *Phalaenopsis* (Balilashaki *et al.*, 2014). Except *Phalaenopsis*, inflorescence segment generated micropropagation of few other orchids is given table 4.

The regeneration response of orchid inflorescence explants also depends on the explant age. The younger source obtained explants of *Dendrobium* Miss Hawaii, *Phalaenopsis* Capitola, *Oncidium* Gower Ramsey, *Ascofinetia* and *Ponerorchis graminifolia* Rchb. f. have been responding well under *invitro* condition (Nuraini and Shaib, 1992 and Mitsukuri *et al.*, 2009).

Culture medium

The composition of the culture media affects the induction, regeneration, number and form of *Phalaenopsis* regenerants. Kosir *et al.*, (2004) obtained *Phalaenopsis* sp. direct shoot regeneration without callus formation from flower stalk's dormant buds on different media composition. They found rapid micropropagation of a large number of vegetative shoots without roots 160 days after inoculation in media supplemented with 2 mg/L of 6-benzylaminopurine (BAP) and 0.5 mg/L of α –naphthalene acetic acid (NAA). However, media supplemented with 4.41 mg/L BAP and 1 mg/L NAA ceased the regenerants to elongate. The highest multiplication of regenerants was observed in media containing 2 mg/L BAP and lower nitrogen concentration.

Sinha and Jahan, 2011 reported maximum *Phalaenopsis amabilis* PLBs production after 12 weeks of culture in media containing ½MS, 2% (w/v) sucrose, 2 g/L peptone, 10% (v/v) coconut water, 1 g/L activated charcoal, 6-BA @ 2.0 mg/L and NAA @ 0.5 mg/L.

Chen *et al.,* (2002) cultured leaf explants of *Paphiopedilum phiIippinense* hybrid's (hybrid PH59 and PH60) in modified MS medium (half strength macro and full strength micro elements) free of plant growth regulator in darkness. They observed adventitious shoot formation from wound regions of leaf within 1 month of culture.

Aktar *et al.,* (2008) reported ½ MS medium to be best suitable for *in vitro* regeneration of *Dendrobium* orchid. They also observed that different kinds of media composition with organic additives significantly influence the *explant* growth, development and regeneration.

Carbon source

The carbon sources of culture medium supplement the low CO_2 concentration, light energy deficiency and osmoregulation maintenance prevalent under *in vitro* conditions.

Rittirat *et al.,* (2012) used wounded protocorm segment explants of *Phalaenopsis cornu-cervi* (Breda) Blume and Rchbf. in ½ MS medium supplemented with 0.1 mg/L NAA and 0.1 mg/L TDZ for PLB production. The obtained PLBs produced best quality plantlets (without browning or necrotic tissues) within 5 months of culture in New Dogashima (ND) medium supplemented with 0.2% AC and 4% sucrose. There was 100% plantlet survival and those were transplanted into pots containing sphagnum moss, placed in a net house with about 60% shading and 80% relative humidity.

Table.1 Value of fresh cut orchids and buds global trade (2007-2012) (Unit: Million US$)

Year	2007	2008	2009	2010	2011	2012
Import	233,734,023	252,647,645	232,568,129	251,445,523	265,702,077	267,196,847
Export	230,470,421	238,702,950	217,781,745	227,389,789	244,996,271	237,543,797
Total	464,204,444	491,350,595	450,349,874	478,835,312	510,698,348	504,740,644

(Source: De *et al.,* 2014)

Table.2 Micropropagation of some orchids using shoot tip explants

Orchid species	Medium composition	Regenerants (PLB/shoot bud)	Source of explant (*in vitro*/*in vivo*)	References
Anacamptis pyramidalis (L.) Rich.	MS+ (NAA/IBA/IAA; 0.5–1 mg/L) + CW	PLBs	NA	Morel (1970)
Anoectochilus formosanus Hay.	Hyponex medium + 1mgdm^{-3}BAP/L-2mgdm^{-3}	Shoot buds	*In vivo*	Ket*et al.,* (2004)
Arundina bambusifolia Lindl.	Raghavan and Torrey's (1964)medium N and N medium	Shoots	*In vitro*	Nagaraju and Parthasarathy (1995)
Cymbidium aloifolium (L.) Sw.	VW+ 5.0 mg/L NAA	PLBs	*In vitro*	Devi *et al.,* (1997)
Cymbidium atropurpureum (Lindley) Rolfe.	MS+ 2.5 mg/L BAP	PLBs	NA	Subramanium and Taha (2003)
D. wardianum R. Warner	VW+ 1 mg/L BAP + 1.5 mg/L NAA	PLBs	*In vivo*	Sharma and Tandon (1992)
*Dendrobium*cv. Sonia	½ MS+ 1 mg/L BAP + 7.5%CW	Shoot buds	*In vivo*	Sheela*et al.,* (2004
Dendrobium Joannie Ostenhault	VW+ 15% CW	PLBs	-	Sharon and Vasundhara (1990)
Phaiustankervilleae (Banks ex Aiton)Blume	Raghavan and Torrey's (1964)basal medium	PLBs	*In vitro*	Nagaraju and Parthasarathy (1995)
*Vanilla planifolia*Andr.	MS+ 1 mg/L BAP + 150 ml/L CW	Shoots	*In vivo*	Kalimuthu*et al.,* (2006)

(Source: Chugh, *et al.,* 2009)

Table.3 Micropropagation of some orchids using leaf explants.

Orchid species	Medium composition	Regenerants (PLB/shoot bud)	Source of explant (*in vitro*/*in vivo*)	References
Aerides crispum L.	MS+ 2.0mM BAP	PLBs	*In vitro*	Sheelavanthmath*et al.*, (2005)
Aerides multiflora Roxb.	MPR + 2 mg/L BAP + 0.5 mg/L NAA	PLBs	*In vitro*	Vij*et al.*, (2004a,b)
Dendrobium Cheingmai Pink	½ MS+ 18.16mM TDZ	Somatic embryos	*In vitro*	Chung *et al.*, (2005)
Dendrobium hybrids (Sonia 17 and 28)	MS + 44.4mM BAP	PLBs	*In vitro*	Martin and Madassery (2006)
*Microperapallida*Lindl.	½ MS+ 2 mg/L NAA+ 2 mg/L BAP	PLBs	*In vitro*	Bhadra and Hossain (2004)
Phalaenopsis Little Steve	½ MS+ 4.54mM TDZ	Somatic embryos	*In vitro*	Kuo*et al.*, (2005)
Vanilla planifolia Andr.	MS+ 4.52mM 2,4-D + 2.22mM BAP MS+ 4.52mM 2,4-D + 2.22mM BAP	Callus Shoots from the callus	*In vivo*	Janarthanam and Seshadri (2008)

(Source: Chugh, *et al.*, 2009)

Table.4 Micropropagation of some orchids using inflorescence explants (Obtained from *in vivo* source)

Orchid species	Medium composition	Regenerants (PLB/shoot bud)	References
Aranda Deborah (*Arachis hookeriana* (Rcbh. f.) Rchb. f. × *Vanda lamellate* Lindl.)	KC+ 1 mg/L BAP +CW	PLBs	Goh and Wong (1990)
Epidendrum radicans Pav. Lindl.	½ MS+ 0.1 mg/L TDZ	PLBs/shoot buds	Chen *et al.*, (2002)
Oncidium Sweet Sugar	½ MS+ 5 mg/L BAP + 5 mg/L NAA	PLBs	Chen and Chang (2000)
Ponerorchis graminifolia Rchb. f.	½ MS+ 4.44mM BAP + 0.54mM NAA	Shoot buds	Mitsukuri*et al.*, (2009)

(Source: Chugh, *et al.*, 2009)

Lim and Chong, 2014 identified autotrophic, mix autotrophic-heterotrophic and heterotrophic growth in *Phalaenopsis deliciosawas* seedlings with 20 g/L optimum sucrose concentration. This concentration is recommended when raising *P. deliciosa* seedlings to retain photosynthetic activity for better survival during acclimatization.

Novotna *et al.,* (2007) found that best seedling development of *Dactylorhiza* species with glucose and sucrose at concentration of 10 g dm^{-3} each. Later the improvement of shoot growth rate and shoot length was enhanced by cytokinins N_6-(2-isopentenyl) adenine or N_6-benzyladenine and their combination with auxin indole butyric acid (IBA).

Jawan *et al.,* (2010) found that *Vanda dearei* protocroms prefer sucrose as compared to fructose and glucose. The media containing 1/2 MS, 2% (w/v) or 4% (w/v) sucrose is best for seedling formation.

Baque *et al.,* (2011) reported 15 g/L sucrose concentration to be most suitable for 'Bukduseong' × 'Hyesung', while 15 and 30 g/L sucrose were regarded as an optimal concentration for *in vitro* growth of the plantlets of 'Chunkwang' × 'Hyesung' hybrid. They also observed increasing plantlets abnormality and root growth with higher sucrose concentration (60 g/L).

Plant growth regulators

According to Krikorian (1982) the success of an *in vitro* system is directly influenced by the correct growth regulator used and its optimum concentration. The commonly used plant growth regulators belong to the auxin and cytokinin groups. Tokuhara and Mii (1993) reported that the combination and appropriate concentration of hormones α-naphthalene acetic acid (NAA), 6-benzylamino purine (BAP), macro and micro elements in the culture medium are major factors for commercial *Phalaenopsis* micropropagation.

Niknejad *et al.,* (2011) documented *Phalaenopsis gigantean* micropropagation protocol with PLB and leaf explant. They observed calli and PLBs development within 6 weeks of culture in New Dogashima Medium (NDM) supplemented with TDZ @ of 0.5 mg/L NAA @ of 0.1 mg/L.

Habiba *et al.,* (2014) investigated *in vitro* organogenesis of *Epidendrum* 'Rouge Star No.8' PLBs with different types of cytokinins. They used three types of cytokinins including kinetin (Kin), 6-benzylaminopurine (BA) and 2-isopentenyl adenine (2ip) which was evaluated with various concentrations of 0, 0.1, 1 and 10 mg/L in modified MS medium. They reported that three cytokinins at all concentrations were found significantly to enhance organogenesis of PLBs in *Epidendrum* 'Rouge Star No. 8' orchid except 10 mg/L of BA treatment when compared with control and 0.1mg/L kinetin gave highest number of PLB. In case of shoot formation, they observed highest number of shoots per explant at 1mg/L BA but the shoot formation rate (66.7%) was highest at 1mg/L kinetin. Results showed that kinetin enhanced root induction when compared with 2ip and BA. MS medium supplemented with BA (4.40 mg/L) and NAA (1.0 mg/L) promoted maximum number of shoot formation of *Phalaenopsis amabilis* at (Balilashaki *et al.,* 2014).

The *Dendrobium transparens* L. immature embryos from 120 days old capsule were germinated on ½ MS supplemented with 1 mg/L NAA + 2 mg/L BAP and same hormonal combination showed highest number of multiple shoot while rooting with 1 mg/L IAA (Suntibala and Kisore, 2009).

Asghar *et al.,* (2011) reported that 2 mg/L BAP produced maximum number of shoots, while 1.5 mg/L of Kin exhibited the highest shoot length of *Dendrobium nobile* var. Emma white. IBA (2 mg/L) increased the rooting percentage (97.5%) more efficiently than NAA. However, necrotic yellow shoots were observed treatments with higher concentrations of BAP, Kin (3.0 mg/L) and CW (300 ml).

Pant and Thapa (2012) reported rapid *in vitro* micropropagation of *Dendrobium primulinum* Lindl, an endangered epiphytic orchid. They used small shoot tip explants (0.3 to 0.5mm) which were previously obtained from *in vitro* grown seedlings. They observed that BAP (1.5 mg/L) + NAA (0.5 mg/L) supplemented MS medium to be most effective for the shoot multiplication and IAA was found to be effective hormone for rooting in comparison to IBA and NAA.

Kumari *et al.,* (2013) developed a protocol for *in vitro* propagation of commercially important cut flower orchid *Dendrobium Sonia* (Fa. *Orchidaceae*) 'Earsakul' with stem nodal explants. They found ½ MS and 4 mg/L BA to be best suitable for early bud break. However, they concluded 2.0 mg/L kinetin+ 0.1 mg/L NAA in shooting media and 0.5 mg/L NAA in rooting media for earliest growth and development.

Devi *et al.,* (2013) adopted different explants for rapid clonal propagation of *Aerides odorata* Lour. They used ½ MS, thidiazuron (TDZ), 6-benzylaminopurine (BAP) and leaf base as explant. They observed 1.0 mg/L TDZ medium to produce PLBs at the leaf base. Highest frequency of calli induction from leaf base explants after 60 days of culture was observed in media containing 2 mg/L (NAA) and direct shoot regeneration with NAA (2 mg/L) and BAP (4 mg/L). The media containing ½ MS and 0.5 mg/L NAA generated highest frequency of root induction. The results also highlighted exogenous auxin dependent embryonic callus proliferation.

Verma *et al.,* (2011) reported that TDZ was the most effective for shoot multiplication of *Digitalis lamarckii* Ivan. via direct organogenesis than BAP, zeatin and kinetin. Pawar *et al.,* (2012) proved that 0.2 mg/L IAA + 2.0 mg/L zeatin supplemented MS medium is the best regeneration efficient medium tomato (*Solanum lycopersicum* L.).

Medium supplements

There are several organic additives like banana extract (BE), carrot juice, coconut water (CW), peptone, potato extract, tomato juice etc. which are reported have beneficial effects on seedling growth in many orchid species like *Aranda* Deborah (Goh and Wong, 1990), *Vanda coerulea* (Seeni and Latha, 2000), *Vanda spathulata* (Decruse *et al.,* 2003), *Dendrobium tosaense* (Lo *et al.,* 2004). Shivkumar *et al.,* (2005) first suggested the use of peptone for enhancing plant tissues growth and development.

Murdad *et al.,* (2010) determined the effects of three types of sugars as the carbon source and potato homogenate (PH) on *in vitro* growth and development *in vitro* derived *Phalaenopsis gigantean* protocorms. They protocorms developed well within 150 days of culture on media containing either fructose or PH. However, on the media containing both sugar and PH, the protocorms were stunted yellow, pale with lower growth during the culture period. Surprisingly, both of the leaf size and root length of *P. gigantea* seedlings were significantly enhanced when the media only contained sugar.

Sinha and Jahan (2011) documented *Phalaenopsis amabilis* cv. Golden horizon's *in vitro* mass clonal propagation. They cultured mature plant derived young leaf explants on ½ MS supplemented with α-

naphthalene acetic acid (0.5 mg/L), N6-benzyladenine (2.0 mg/L), 10% (v/v) coconut water, 2% (w/v) sucrose, 1 g/L activated charcoal and 2 g/L peptone.

They observed that leafy shoots rooted on the previously mentioned media without growth supplements, where 100% explants were developed into plantlets with roots within 8 weeks.

CW is cost effectively employed for the micropropagation of imperative species of orchids due to its endless benefits (Peixe *et al.,* 2007). It is a natural growth promoter which contains higher levels of zeatin, zeatin ribosides, 1,3-diphenylurea (contains cytokinin like activity), auxins, nitrogenous compounds, inorganic elements, organic acids, sugars and their alcohols, peptides, vitamins, amino acids and many other unknown components in its composition (Tokuhara and Mii, 2001; Nasib *et al.*, 2008).George *et al.,* (2008) demonstrated that physiologically active substances presents in CW promote the cell divisions which further enhance shoot multiplication. Baque *et al.,* (2011) demonstrated effective plantlets growth enhancement in 'Bukduseong' × 'Hyesung' and 'Chunkwang' × 'Hyesung' hybrids of *Calanthe* with 50 m/L coconut water application.

Kaur and Bhutani (2012) were successful to regenerate and multiply *Cymbidium pendulum* using protocorms explant under *invitro* condition in M medium (Mitra medium) supplemented with banana homogenate, coconut water and peptone. The primary protocorm segments first regenerated into secondary protocorms and differentiated into shoots without any callus stage in between.

They observed regeneration frequency to be significantly higher in organic growth supplement enriched (banana homogenate @ 50 g/L, 10%coconut water and peptone @ 2 g/L) medium than control. Vijaykumar *et al.,* (2012) reported higher rate of germination, more number of PLBs, shoots and root formation of *Dendrobium aggregatum* cultured in MS medium supplemented with 1.5 mg/L BAP, 3% sucrose and 15% coconut water (CW).

Shekarriz *et al.,* (2014) reported that modified KC medium fortified with 2 g/L peptone without CW showed highest survival percentage of seed of *Phalaenopsis* hybrid 'Manchester' and germination percentage was observed in 15% (v/v), 2 g/L peptone and modified VW medium. They suggested that *Phalaenopsis* seeds cultured on a ½ MS medium containing CW and peptone can be used for clone propagation.

Anti-contaminants

During micropropagation the release of phenolic substances is a very common phenomenon and often adversely affects the response of the explants. Browning or blackening of cultured explants is cause only after the wounding. This activity promoted the oxidation of phenolic substances under the control of polyphenol oxidase. Tanaka and Sakanishi (1977) reported poor regeneration in *Phalaenopsis* tissue culture because of phenolic compound exudation.

Good growth and development of *Phalaenopsis* plantlets *in vitro* were obtained when culture media were supplemented with 0.2% (w/v) AC (Hinnen *et al.,* 1989; Ernst, 1994). Activated charcoal reduce the phenolic exudates, the beneficial effects of AC could be due to positive stimulation of many development processes (Van Winkle and Pullman, 2006) and phenolic compounds absorbtion. Rittirat *et al.,* 2012 reported *Phalenopsis cornu-cerve* (Breda) Blume Rchb f. best growth in ND medium supplemented with 4% sucrose and 0.2% AC without callus browning or necrotic tissues.

Hardening of *in vitro* grown plant

Orchids are grown in a special media, since root needs plenty of air around them in all times. The media used should provide support to the plant, supply water and nutrients to the roots and enough air for the roots to breath. Puchooa (2004) suggested that high humidity (about 90%) around the plantlets for two weeks was essential for hardening of the orchid plantlets. He found 84% survival rate of *Dendrobium* plantlets transferred to the green house into baskets containing wood charcoal as wood charcoal provides good drainage and adequate aeration to the roots, which is of primordial importance in the culture of orchids.

The rooted plantlet of *Dendrobium microbulbon* when transferred to a thermocol cups containing a mixture of sand, soil, brick pieces and charcoal pieces (1:1:4:4) showed 60% survival rate and the same plantlets grown directly on the tree trunk showed 40% survival rate after six weeks of their removal from *in vitro* conditions (Sharma *et al.,* 2006). Suntibala and Kishore (2009) successfully acclimatized *Dendrobium tranparens* L plantlets in a potting mixture of brick and charcoal (2:1) and more than 90% of transplanted plants survived under greenhouse condition.

Pant and Thapa (2012) observed that the potting mixture containing coco peat and sphagnum moss in the ratio of 2:1 can sustain the hardening of *in vitro* rooted plantlets with 70% of plantlets survival. However, they found maximum 66.67% survival of *Dendrobium* Sonia 'Earsakul' in hardening mixture charcoal and brick pieces (@1:1 proportion), transplanted in green house condition.

This review provides an overview of what the authors consider to be the most significant literature in orchid micropropagation (historical aspect – Rotor, G. 1949 to Balilashaki *et al.,* 2014). It is a matter of fact that orchids are aesthetically gorgeous gifts of nature and these beauties are tremendously efficient in holding their flowering phase for a long time unlike any other cut flower present on earth. Fortunately, the commercial demand of these rare breeds has been increasing day by day which may ensure their conservation in coming years. The advanced tissue culture techniques with clonal *in vitro* micropropagation sounds potential for industrial production of high quality orchid plant material which may earn appreciable level of revenue in international market and strengthen the national economy.

A huge number of research has been done on several kinds of orchid species *in vitro* micropropagation which are commercially demanded.

But all the protocols developed so far may not be successful on industrial level in terms of economic point of view. Hence, we have attempted to put together most of the available literature of *in vitro* cloning of orchid using shoot tip, leaf, inflorescence axis and flower bud as explants. This may be helpful in adopting the most suitable protocol for its respective orchid species and also improvising the currently available method for *in vitro* mass propagation.

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