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Plant Growth Regulators Used For In Vitro Micropropagation Of Orchids: A Research Review

Jayarama Reddy, Niveshika*, Ajin Shaju, Aiswarya Jose, Ann Betty, Yarmichon H

Affiliation

Department of Botany, St. Joseph's College (Autonomous), Bengaluru-27, India.

Abstract

Orchids are nature's most exotic flowering plants which are distributed worldwide. They are not only important for their ornamental values but also for their medicinal values. In vitro micropropagation is a successful method for the cultivation of orchids. Different parts of orchids are used as explants like shoot tips, root tips, flowers stalk, leaves, nodal tips, rhizome etc. Explants like shoot, root, leaves and flowers of few orchid species were studied and shoot and root explants has shown the best result. Although micropropagation of orchid has shown vast development in the recent years, the widespread use of it is believed to be still minimal due to the issues like transplantation to field, phenolics exudation and various other reasons. To overcome a few of these issues plant growth regulators must be included in the media preparation. Growth hormones are chemicals that modify the growth of plants by increasing branching, bud initiation, altering fruit maturity etc. Hormones like auxin and cytokinin should be added in particular ratios. Even if there is a slight decrease or increase in the amount of hormones added it causes a huge damage in the desired yield. The most common auxins used in Orchid tissue culture media are naturally occurring auxins like Indoleacetic acid (IAA) and artificial auxins like Naphthaleneacetic acid (NAA). Cytokinins are also equally used in media preparation. BAP and kinetin are widely used for the explant in the orchid culture.

Keywords: Orchid, Explants, Micropropagation, Plant growth regulator, Auxin, Cytokinins

Introduction

In vitro tissue culture is an important tool of plant biotechnology that has discovered the ability of a single plant cell to form a complete plantlet. This ability is called totipotency. This concept was first proposed by Haberlandt in the year 1902 [1]. The production of orchid plants is growing worldwide. Among the floral plants and floriculture, orchids are outstanding in various ways, like diverse forms, shapes and colours [2]. Orchids are considered as ornamentals not only for their beauty but also for their long shelf life. At present orchids are one of the marketing industries in many countries like Australia, Malaysia, Thailand and many others. Some of the orchids are even considered as food and herbal medicines by many different tribes. It was very difficult in the beginning to cultivate orchids but as the technology developed now many varieties of orchids are easily cultivated. Plant tissue culture has helped both in situ conservation and ex-situ conservation of orchids [3]. Micropropagation can solve the loss of orchid gene pool and help to protect the gene Bank of orchid variety. The propagation using seeds leads to heterozygous plant production of orchids. From the past 50 years, tissue culture techniques using various explants have been extensively studied not only for rapid propagation but also for ex situ conservation. Different methods and procedures have been considered and various parts of the Orchid have been used including flowers stalk, shoot tips nodes, stems, buds, root tips and rhizome segments [4]. Additions of hormones in the media are a very essential step.The formation of a complete plant that depends upon the amount and the type of plant growth regulators used. The ingredients of tissue culture medium have significant effect on the development and growth of explants and seeds of orchids [5]. The two main plant growth regulators used in Orchid micropropagation is auxin and cytokinin [6]. Auxin mainly helps in ovule/ovary maturation, formation of vegetative structure, root induction and auxin have many more functions [7]. Both natural and artificial auxins are equally important for Orchid growth. Cytokinins are also important for induction of axillary shoots without intervening callus [8].

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Corresponding Author
Dr. Niveshika
Assistant professor
Department of Botany,
St. Jospeh's College, Bangalore
Email: niveshika87@gmail.com

Auxin inhibits leaf shedding, enhances the natural abscission of old leaves and fruits or flowers, initiates root development, assists cell division, balances and regulates xylem differentiation. Auxin has natural types like Indole butyric acid (IBA) and Indole-3-acetic acid (IAA) and synthetic auxins like 2,4-dichlorophenoxyacetic (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and Naphthalene acetic acid (NAA) are indispensable for tissue culture in general and orchid micropropagation in particular [9].

Cytokinin promotes cell division, stimulates lateral shoot growth, instigates cell division in mature tissues, promotes cell division, helps to disrupt bud and seed dormancy, delay the aging of flowers and leaves. Some of the commonly used cytokinins are 6- benzyl aminopurine (BAP), 6-furfurylaminopurine (Kinetin), N-Isopentenyl aminopurine (2IP), Thidiazuron (TDZ) 1-phenyl-3 (1,2,3-thiadiazol-5-yl) urea, 4-hydroxy-3-methyl-trans-2-butenyl aminopurine and zeatin 4-hydroxy-3 methyl-trans-2-butenyl aminopurine.

Gibberellin regulates cell elongation and determines plant height, most commonly used is GA3. Abscisic acid (ABA) inhibits cell division, promotes somatic embryogenesis and enhances somatic embryo quality. Hormones like gibberellin and ethylene are used in very minimal amounts but it also shows effective impact on the growth of orchids.

Along with these growth regulators addition of coconut water to MS medium has also influenced axillary bud induction of orchid [10].Temperature also plays a major role in development of the orchid crops. Short day or low temperature treatment can alter the levels of endogenous growth regulators [11].

Shoot culture

The most common method of micropropagation is through shoot culture. Shoot culture is widely used in orchid micropropagation. Usually in orchid shoot culture a clump of identical bodies called protocorm like bodies (PLBs) are formed which can in turn produce new plants. Plant growth regulators like auxin and cytokinin play a major role in different stages of shoot culture. Orchid species like *Papilionanthe teres*, *Nervilia aragoana*, *Bulbophyllum umbellatum*, *Cymbidium aloifolium* shoot apices are used for micropropagation [4].

Auxins and cytokines ratios influenced shoot growth and shoot length of *Paphiopedilum* var. *densissimum*, *P. insigne* and *P. bellatulum*. The addition of NAA and high kinetin on to the basal medium helps in the shoot differentiation [12]. The concentration of 5.5 mg/L 6-Benzyladenine (BA) with 0.5 mg/L Naphthalene acetic acid (NAA) showed the highest organogenesis of shoot. In some species all the PGR combinations showed the shoot organogenesis as in the P. armeniacum [13]. In the in vitro growth of orchid species *Coelogyne pandurata,* a black orchid hybrid the shoot appearance was shown on the addition of Naphthalene acetic acid (NAA) and some organic additives. The organic additives like bananas, coconut water, sweet potatoes and potatoes were used to speed up the shoot emergence. Proportions of auxin and cytokinin from coconut water NAA from sweet potato were used to produce the largest number of shoots with highest shoot length. Coconut water has such a great impotence in accelerating cell division since it is rich in auxin and thiamine. By increasing the NAA concentration up to 5ppm inhibits the shoot emergence in *Coelogyne pandurata*. The highest number of shoots was seen under the combination coconut water with 1ppm NAA concentration [5].

In the in vitro propagation of *Vanda tessellata*, maximum rate of shoot induction was seen under addition of 0.5 mg/L 6-Benzylaminopurine, benzyl adenine (BAP) and 0.5 mg/L Kinetin (KIN) to the Murashige and Skoog (MS) medium. Different concentrations of BAP, NAA, IAA(3-indoleacetic acid) and KIN were used for shoot induction. For multiple shoot induction 1.0 mg/L BAP + 1.0 mg/L NAA concentrations were used. During the micropropagation of *Vanda coerulea* combination of 30 µM BAP and I5 µM IAA in MS medium were given and thus showed maximum frequency of PLB formation. Protocorm multiplication is affected by auxin and cytokinin concentrations. Another concentration of 30 µM showed the best shoot formation, whereas when the auxin and cytokinin concentrations were enhanced, the rate of shoot formation was considerably decreased [14]. In the in vitro micropropagation of dancing dolls orchid *Oncidium* sp. BAP at concentration of 2.0 mg/L showed maximum percentage of shoot formation. Whereas at 0.5 mg/L showed the least percentage of shoot formation. BAP and NAA together at the concentration of 2.0 mg/L + 1.5 mg/L showed the maximum percentage of formation of shoots, however 4.0 mg/L + 2.5 mg/L concentration showed the least shoot formation [15].

In the in vitro micropropagation of *Cymbidium faberi* adventitious shoot formation took place with specific concentrations of N6-benzyladenine (BA), thidiazuron (TDZ) and TDZ + NAA. When the PLBs of the medium were supplemented with 45.5% TDZ, 2.5 times of increased shoot induction was observed. Strong and healthy shoot seedlings were observed under right concentrations of TDZ and NAA. How ever increasing the concentrations of TDZ to 3.0 mg/L shoot formation was inhibited [16]. In the mass micropropagation of *Dendrobium primulinum* Lindl., an epiphytic orchid with an average of 4.5 shoots per each explant was produced when the concentration was 1.5 mg/L BAP. At this concentration the longest shoot was also found. During this experiment also on increasing the BAP concentration the multiple shoot production was decreased. At the concentration of 1.5 mg/L BAP the least shoot formation was observed [8].

During the in vitro culture of flowering plants the highest shoot induction was observed under the concentration of 1mg/L BAP and 20 mg/L chitosan oligomers and polymers. Throughout the shoot multiplication BAP showed the best result than KIN, but on increasing the KIN concentration number of shoots formed were also high. The longest shoot was seen on using BAP at 1mg/L [17]. In vitro micropropagation of *Dendrobium nobile* also showed that various concentration of benzylaminopurine (BAP), kinetin (Kin) and coconut water (CW) plays a significant role in the production in both number and length of the shoot induction from the explants. The maximum shoot was observed under 2mg/L BAP solution also the length of the shoot were reduced under the same concentration. Both the number and length of the shoot can be promoted only till a specific increase of the BAP, Kin and CW concentrations, above the specified concentrations the shoot induction is inhibited [18]. Addition of coconut water and cytokinins on endangered orchid species showed axillary shoot induction on the MS media [10].

Table 1 showed that the variation in plant growth regulators can affect the shoot induction in different ways. Usually inhibition of shoot is found at higher concentrations. Also light quality with NAA and TDZ could affect shoot induction [19]. Plant and animal material can also be considered as a growth stimulating factor [20].

Flower Culture

Several agents are known to induce flowering in-vitro [21]. For the in-vitro micropropagation of orchids, flower culture is not widely practiced. The vegetative tissues such as root, shoot and leaves are usually used. It is found that even though these tissues produce embryos in large numbers the mother plant gets damaged after the exertion of these explants. In this scenario floral tissues such as flower-stalk tips, micro-inflorescence and buds can be used as explants [22]. For the mass propagation of Orchids of some genera like Phalaenopsis flower culture technique can be used [3]. For inducing embryogenesis the role of hormone is different in different explants [22]. Plant hormone, auxin and cytokinin have greater effect on the tissue culture technique. Different natural and synthetic plant growth regulators are used for the micropropagation.

From the flower buds of Paphiopedilum orchids, a successful method was adopted for micro propagation. Since these orchids are listed under endangered species, the micropropagation using flour culture helps in the conservation of this species. It was found that only flower buds which contain the basal tissues were able to produce the shoot. The bud size also has a significant role in shoot induction and plant regeneration along with the effect of plant growth regulators [23]. In the experiment done on the flowers stalk explants, it was observed that slight changes in the concentration of naphthalene acetic acid and TDZ-1-Phenyl-3-(1,2,3 thiadiazol-5 yl)- urea has greater effect on the in vitro morphogenesis of the flowers stalk derived callus [22].

Table.1: Effect of PGRs on shoot growth

Supplements	Concentration	Plant Name	Growth variations
Benzyladenine + Naphthalene acetic acid	5.5 mg + 0.5 mg	*Paphiopedilum var. densissimum*, *P. insigne* and *P. bellatulum*	High shoot growth
Naphthalene acetic acid	5 ppm	*Coelogyne pandurata*	Inhibition of shoot
Benzylaminopurine, benzyl adenine (BAP) and Kinetin	0.5 mg/L + 0.5 mg/L	*Vanda tessellata*	Shoot induction
BAP	2.0 mg/L	*Oncidium* sp.	Maximum shoot formation percentage
BAP	0.5 mg/L	*Oncidium* sp.	Minimum shoot formation percentage
TDZ	45.5%	*Cymbidium faberi*	Adventitious shoot formation
TDZ	3.0 mg/L	*Cymbidium faberi*	Shoot inhibition
BAP	1.5 mg/L	*Dendrobium primulinum*	Longest shoot
BAP	2 mg/L	*Dendrobium nobile*	Maximum shoot

In the study of micropropagation of Phalaenopsis and Doritaenopsis using the shooting of flower stalk buds, plant growth regulator established methods can be used [24]. Different concentration of BAP (Benzylaminopurine) and NAA shows variation in the rate of survival, formation of protocorm like bodies, their colour and the rate of shoot formation. It is identified that as the concentration of NAA and BAP increases the survival rate decreases except for a

few combinations. More number of protocorm like bodies was formed when 0.1 mg/L NAA and 20 mg/L BAP was used [24].

The effect of different cytokinin on the proliferation of protocol like body and the shoot formation is studied by Chen et al. When 0.28 u Zeatin-riboside was used, the highest proliferation rate was found. Higher number of protocorm-like bodies was found when kinetin at 1.39 um was used [25]. High concentration of synthetic cytokinin, Benzyladenine was found to be promoting sprouting of the flower bud. But leaf malformation was found when higher concentration is used. 2.5 ppm of Benzyladenine was found to be optimum concentration [26].

Table 2 showed the effect of different concentrations of cytokinin in the formation of protocorm-like bodies. It was observed in Table 2 that the best result on the number of PLBs per tube was obtained at 4.44 µM of TDZ. Best response on the PLB proliferation rate is at 0.28 µM of ZR and the highest number of PLBs with shoot per tube was obtained at 1.39 µM of Kinetin. The proliferation rate is calculated by dividing the final fresh weight by the initial fresh weight.

It was observed that flower buds of 1st and 2nd florets of Cymbidium goeringii show different sensitivity towards auxin and cytokinin. For first florets, auxin and cytokinin have only a limited role in the induction of rhizome development and the plantlet regeneration. But for the flower buds of the second floret, auxin and cytokinin is required [27].

Thus it is evident that the effect of auxin and cytokinin is different in different plants according to their concentration. Ethylene is also an important factor which helps in development of floral parts [28]. Their presence has a significant role in the regeneration of the plant.

Leaf culture

Leaf culture system was used to study the effects of growth regulators, efficient regeneration systems through direct somatic embryogenesis [29]. In the leaf culture different parts of the leaf like tips, petioles, various sections of the blade, base parenchyma etc are used. Younger leaves give better results than the old leaves. Leaves proved highly amenable for tissue culture. The abaxial surface is free from any division while the adaxial undergoes differentiation. Leaf segment culture are easier to obtain and not restricted to any season. Epidermal and mesophyll cell of leaf tip produce a cluster of somatic embryo segments taken from young leaves of orchids (*Oncidium*) in a defined medium supplement with low dosage of TDZ (0.3 mg/L). The stages of the maturity of the leaves affected the initial appearance of the foliar embryos [30]. Murashige-Skoog medium (MS), Heller's medium (HM), Knudson C medium (KC) are some of the culture mediums used with different concentrations of growth regulators.

Table.2: Effects of four cytokinins on PLB proliferation and shoot formation [17]

Plant hormone (Cytokinins)	Highest No. of PLBs per tube	Highest No. of PLB proliferation rate	Highest No. of PLBs with shoots per tube
TDZ	95.3 at 4.54 µM	11.6 at 4.54 µM	72.3 at 1.36 µM
BA	**117 at 4.44 µM**	11.7 at 4.44 µM	98.7 at 0.44 µM
ZR	97.3 at 28.46 µM	**30.3 at 0.28 µM**	53.7 at 0.85 µM
Kinetin	98.5 at 1.39 µM	8.4 at 4.65 µM	**154 at 1.39 µM**

The effect of growth regulator from leaf explant of *Phalaenopsis amabilis* and *Phalaenopsis nebula* result that ancymidol, auxin, polyamines inhibit direct embryo formation for both species and used of Cytokinin has genotypic effect on direct embryo induction. 13.321M BA and 4,921M 2IP are the most effective cytokinin in *P. amabilis* and *P. nebula* [6].

Leaf tissue culture of endangered *Renanthera imschootiana* show a successful regeneration of large number of phenotypically uniform plants from the basal part of the leaves.Method of regeneration of *syngonium podophyllum* results that no somatic embryo was form from leaf explants irrespective of growth regulator concentration or combinations used, no differentiation of callus thus no regeneration of plantlets, whereas somatic embryo formed on petiole explant directly on MS medium supplemented 2.0 mg/L TDZ with 0.2 mg/L NAA or 2.5 mg/L TDZ with 2.0 mg/L NAA or with 0.2 and 0.5 mg/L 2,4-D respectively. From a single petiole explant approximately 50-150 plantlets were regenerated. Up to 85% somatic embryos were able to germinate with the appearance of shoot and roots [31].

Leaf explants taken from two hybrids of *Paphiopedilum philippinense* regeneration capacities vary with cultivator genotypes. Leaf segment explant of hybrid PH59 formed shoots and have higher regeneration capacity on plant growth regulator- free medium but hybrid PH60 does not form shoots. From leaf segment explants of hybrid

PH59, 2,4-D plus TDZ both retarded shoot bud formation. In hybrid PH60, 2,4-D + TDZ promote to form shoot bud from leaf segment explants. In *Paphiopedilum* TDZ inhibits shoot proliferation and rooting. TDZ combined with 2,4-D induced the formation of calli in different genus of Orchidaceae[29].

The production of protocorm like body and survival of leaf thin section depend on concentration and types of cytokinins in the medium.TDZ was found to be more effective inducer of protocorms-like body than other cytokinin like zeatin and benzyl adenine from thin leaf section of *Doritaenopsis* [32]. Leaf explants of *Oncidium flexuosum*, the presence of Auxin, NAA and TDZ in combination with 2,4-D in culture medium inhibit the regeneration of protocorm like body [33].

Leaf tip cultured of *Epidendrum* form PLB on MS medium and *Laeliocattleya* form plantlets on H medium preceded by callus formation [34]. The embryo formation on leaf explants was promoted by cytokinins like 2IP, BAP, KIN, TDZ and zeatin but retarded by auxins IAA, NAA, IBA and TDZ [35].

Successful micropropagation using leaf explant depends on the factors like source of leaf, age, parts of the leaf taken, explant orientation, growth hormones and medium nutrient composition. Popular use of leaf explant mass scale cultivation of orchids in industries is restricted because of the time and costs involved in standardizing the above factors.

Conclusion

Extensive research is still needed to conclude the complete growth of the orchid species in different cultures. Due to their small range of population and size more care is recommended for their growth. The amount of growth hormones used should be in correct proportion in order to get a healthy and disease free plant.Further studies has to be followed to understand the actual functioning of the hormones in explants for, hair initiation,primary leaf formation, meristem production,primary root formation as these structures grow from a mass of undifferentiated cells, the protocorm. Few beautiful orchid species have reached the threatened conditions because of both natural and human activities.The best method to conserve orchids are through plant tissue culture where many different varieties of orchids can be cultivated and widely distributed. Tissue culture is one of the most suitable tools to reduce the pressure on natural distribution of orchids and their sustainable utilization.

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Conflict of Interest

No known conflict of interest.

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