Article

Outbreak induction from floral stem orchid *Phalaenopsis* spp. (Blume) *in vitro*

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Abstract

The induction of outbreaks *in vitro* is a strategy of the vegetable micropropagation used to increase the production of orchids, in comparison with the traditional crop that requires a long period of time to obtain a substantial population for commercialization. The objective of this study was to induce *in vitro* outbreak formation from the flower outbreaks of the orchid *Phalaenopsis* spp., using an organogenesis route. Four mineral salt formulations (MS 100%, MS 50%, Knudson and NPK Fertilizer 18-9-18/15-30-15) and four varieties of *Phalaenopsis* spp. to determine the viability of explants. Two growth regulators (6.2-benzylaminopurine BAP 20.2 µM alone and combined with naphthalenacetic acid ANA 5.37 µM) were evaluated to determine outbreak induction. The results indicate that oxidation in explants depends on the mineral salts of the culture media, as well as the variety of orchid; outbreak induction depends on the growth regulator used. BAP/ANA regulators produced 2.4 outbreaks per outbreak against 2 in BAP. The MS 50% medium proved to be the best option to maintain the viability of the flower outbreaks.

Keywords: *Phalaenopsis*, micropropagation, organogenesis.

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Introduction

The Orchidaceae family have been known for thousands of years and since then they have had great value for humans. At present they are appreciated around the world as ornamental plants due to their flowers, which is the most striking part which presents a great variety of shapes, colors and aromas, these characteristics give them a high commercial demand to be used in all kinds of floral arrangements or for sale as an indoor plant.

Orchids are plants that belong to the Orchidaceae family, the largest in the plant kingdom with around 27 000 species registered and distributed almost worldwide (Campbell, 2013). Despite this great variety, only a few genera are used commercially in the ornamental market. One of the most appreciated genus is *Phalaenopsis*, commonly known as butterfly orchid, which has a great diversity in color and size of its flowers, which leads to a great demand in the ornamental market.

However, its traditional propagation is complicated and requires a long period of time, to obtain a little grandfather the mother plant must be at least one or two years old; in addition, their sexual reproduction has been affected by the sterility of some of their hybrids related to extensive hybridization work in this genus (Tirado *et al*., 2005). Due to the aforementioned factors, the need arises to make use of new technologies for their propagation.

The cultivation of plant tissues is a technique used for the commercial production of orchids and represents a solution to reduce the regeneration time and increase the populations for trade, since it allows to obtain a large number of plants, either from germination of seeds or some other type of explant, through *in vitro* propagation techniques with which the organogenic and embryogenic capacity can be determined, which are specific to each species.

Some genera produced by this technique are *Phalaenopsis*, *Oncidium*, *Cymbidium*, *Dendrobium* and *Paphiopedium*, (Chang and Chang, 1998; Flores *et al*., 2008). The main reason for using micropropagation in orchids is because it allows to obtain somatic clones from which whole plants with uniform characteristics are regenerated.

Frequently used explants are segments of floral wands, leaf segments, mature seeds, rhizomes, and bodies similar to PLBs probes (Chang and Chang, 1998; Nayak *et al*., 2002; Feria *et al*., 2007; Arditti, 2008; Salazar *et al*., 2013). For the *in vitro* propagation of orchids several culture media have been used such as Murashige and Skoog MS (Murashige and Skoog, 1962), B5 (Gamborg *et al*., 1968), New Medium Dogashima NMD (Tokuhara and Mii, 1993), Vacin and Went VW (Vacin and Went, 1949), Knudson C KC (Knudson, 1946) and the Hyponex medium (Nishimura, 1982), to which vitamins, growth regulators, antioxidant agents, among other compounds are commonly added, depending on the micropropagation stage.

Several methods of micropropagation have been developed by direct and indirect morphogenesis in this genus such as clonal propagation through leaf cultivation, flower outbreak cultivation, as well as induction of embryogenic callus and suspension cell culture. The main topics to be evaluated are the survival rate, the induction of protocorm and the formation of seedlings, seeking a high frequency of regeneration with the highest homogeneity between plants.

For the micropropagation different ways of multiplication can be followed, one of them is direct organogenesis, commonly used in *Phalaenopsis* (Park *et al*., 2002). This technique consists in stimulating the formation of multiple vegetative outbreaks per outbreak, which are de *novo* formed according to the morphogenetic potential of adventitious meristems.

For this, the culture media must be complemented with growth regulators in a relationship that stimulates the explant to produce such outbreaks; this relationship varies according to the species or hybrid with which it is working. The polar structures that are formed will generate seedlings that can be used as a source of more explants for micropropagation, or they can be brought to acclimatization (Krikorian, 1991; Merino, 2014). One of the successful protocols that allows to obtain uniform flowers is to stimulate the formation of leaves from the nodes of floral stems to later induce the formation of PLBs (Tanaka, 1992).

However, despite the protocols developed for *in vitro* culture for *Phalaenopsis*, not all genotypes respond in the same way under identical culture conditions, so they must be adjusted to the response of each hybrid (Park, *et al*., 2002). However, each author frequently claims to provide the best method of micropropagation to obtain a mass production for commercial purposes, so it is difficult to determine through a literature review which method is really the best since they all vary both components of the culture medium and explants and even environmental factors.

Therefore, in addition to the fact that there is no extensive research in this regard, it is necessary to provide results of experiments carried out in Mexico and adapt them to the needs and resources. Although, this technique is used for the commercial production of orchids in other countries, in Mexico it is a growth opportunity for small and large orchid producers, since there are only a few companies that have applied this technology in their facilities but without achieving cover the market demand, so the large orchid producers in the country are still forced to import seedlings and small producers are gradually increasing their populations.

It is necessary for the ornamental market of orchids of our country, to promote the production of seedlings in national territory to do so, generate new jobs, reduce dependence abroad, and boost the development of Mexican floriculture.

In the ornamental orchid industry, there are few countries that cover global demand, Mexico can enter this market, since it has the appropriate climates for the development of various species, despite this there are few greenhouses dedicated to the cultivation of orchids in the country. Thus, a great effort has been made, through *in vitro* culture, to ensure the propagation and conservation of endemic species, but there are few results for species with commercial potential.

Since orchids have an organogenic potential in flower outbreaks, it was considered to take advantage of this potential to obtain a clonal induction through an appropriate hormonal balance in the culture medium. So it was necessary: aseptically establish the butterfly orchid explants under *in vitro* conditions; determine the appropriate culture medium to keep the established explants of *Phalaenopsis* viable and define the hormonal balance that allows the induction of vegetative outbreaks in the flower outbreaks of this orchid.

Materials and methods

The study was carried out in the Plant Biotechnology Laboratory of FAUANL in Marin, NL. The floral escapes of four varieties of orchids *Phalaenopsis* spp. were used as an explant source, grown in greenhouse conditions for one year corresponding to *P*. *amabilis*, *P*. Kaleidoscope, *P*. Taisuco x Sogo, *P*. Taipei Gold (Chang and Veilleux, 2009) (Figure 1). The mother plants were kept for a month under controlled conditions of light and temperature, with constant irrigation and fertilization with applications of 15-30-15 NPK (nitrogen-phosphorus-potassium).

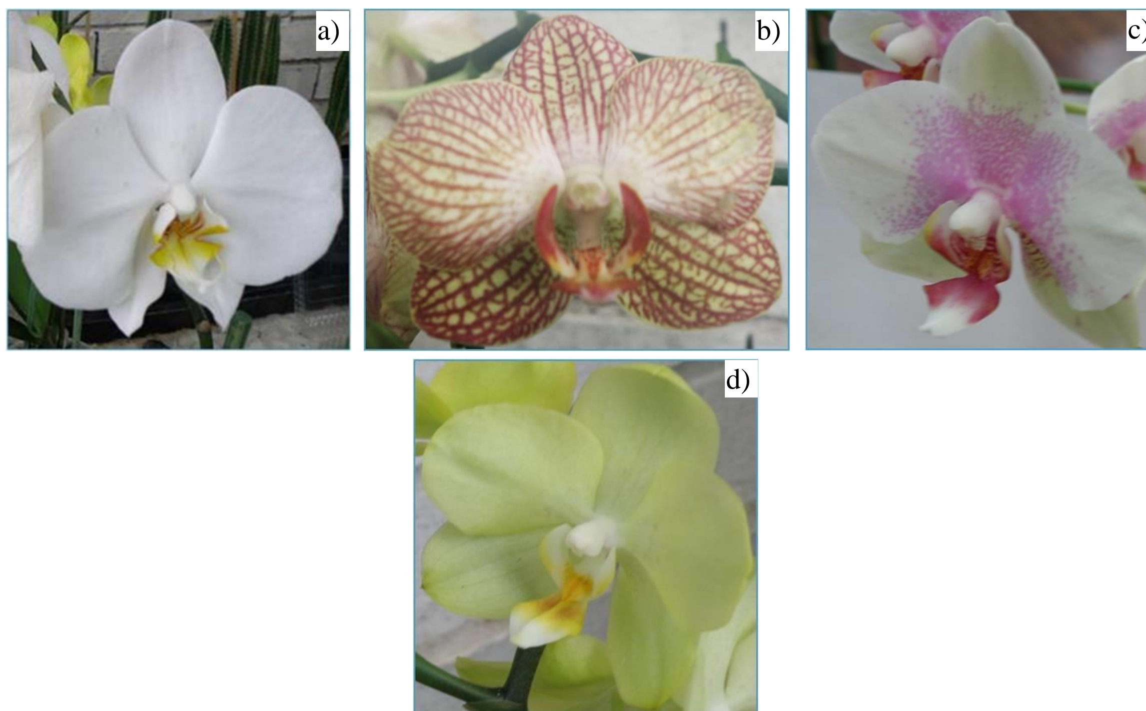

Figure 1. a) white orchid (*P. amabilis*); b) striped orchid (*P. Kaleidoscope*); c) pink orchid (*P. Taisuco* x *Sogo*); and d) yellow orchid (*P. Taipei Goid*).

The disinfection started with the washing of the floral wands in a soapy solution for which a soft bristle toothbrush was used to avoid damaging the plant tissue, the bracts were carefully removed, and a rinse was performed with plenty of drinking water. The rods were then immersed for 15 min in distilled water with 15 drops L^{-1} of a 0.32% colloidal silver solution. Under these conditions they were transferred to the laminar flow hood and under aseptic conditions they were cut into segments of approximately 1 cm in length so that each yolk remained in the center of the plant segment, except for the apical outbreaks that 1 cm in length it was below the apex, all the floral knots were used.

They were immersed in 70% ethanol for two minutes, and then transferred to a 1% NaClO solution with two drops of tween 20 per 100 mL of solution, remaining for 10 min. Concluded the time three rinses were made with sterile double-distilled water. In case of tissue damage at the edges of the explant, it was removed with a scalpel on a Petri dish. Finally, explants were established in the culture media of each treatment.

Two experiments were performed, one on mineral salts and one on organogenesis. To determine the most viable mineral salts, four culture media were evaluated: MS 50% of their salts, complete MS of salts, medium with Knudson C salts, and a mixture of two Fertilizers 18-09-18/15-30- 15 NPK, 1 g each. To evaluate the organogenic capacity, *P. amabilis* explants were used and two treatments were established in MS 50% medium with different growth regulators: the first was only 20 µM 6-Benzylaminopurine (BAP) and the second consisted of a combination of 6-Benzylaminopurine (BAP) 20 µM and naphthalenacetic acid (ANA) 5.37 µM.

Culture media were supplemented with 100 mg L^{-1} of myoinositol, 0.1 mg L^{-1} of thiamine, 0.5 mg L^{-1} of nicotinic acid, 0.5 mg L^{-1} of pyridoxine, 2.0 mg L^{-1} of glycine, 30 g L^{-1} sucrose, 10 mg L^{-1} citric acid, 8 g L^{-1} Agargel and the pH was adjusted to 5.8 ±0.02 using NaOH or 1N HCl, autoclaved at 121 °C at 1.5 atmospheres of pressure during 15 min. Experimental *in vitro* outbreaks or units were maintained for 16 weeks under controlled incubation conditions under a light intensity of 24 - 27 µM m^{-2} s^{-1} with photoperiod 16 light hours and eight dark at a temperature of 24 °C ±2 °C.

Both experiments are established with a completely random statistical design which is expressed as $Y_{ij}= \mu + T_i + E_{ij}$. The mineral salts experiment maintained a 4 x 4 factorial arrangement, where factor A is the culture media and factor B is the varieties; the oxidation percentage variable was evaluated, and the data were analyzed by non-parametric statistics with the chi-square method (X^2).

In the multiplication experiment the variables of percentage of induction and number of outbreaks were evaluated; the data were analyzed by X^2 and analysis of variance respectively. The statistical program Statistical Package for the Social Sciences (SPSS) was used for data analysis.

Results and discussion

After eight days of establishment, 75% survival was obtained from the disinfection method used in the explants of the first experiment. Regarding the culture media, great variability was observed in the oxidation of the explants, considering that an oxidized explant is one that loses its green coloration and changes to shades of yellow to light brown, with or without necrosis, since a tissue that does not express the green color is indicative of the absence of chloroplasts, basic organelles to generate enough energy for cellular functions (Taiz and Zeiger, 2006).

Brown secretions were observed in the culture medium (Figure 1), characteristic of phenolic compounds that cannot be dispersed continuously affect the explant cells, which contributes to the oxidation process, for this reason it was decided to perform subcultures to medium New every three weeks.

Figure 2. Oxidation of explants. Left: outbreak presenting oxidation; center: outbreak that is considered viable; right: phenolic compounds released into the culture medium.

When evaluating the results with the chi-square statistic, it was observed that oxidation in the explants depends on the culture medium used (Table 1).

Table 1. Percentage oxidation of explants by culture.

Culture medium	Oxidation percentage[a]
MS 50%	11%
Knudson C	67%
MS 100%	75%
Fertilizer	100%

[a]X^2= 16.714 with $p < 0.05$.

It is important to highlight that MS 100%, although it is a very complete medium because it contains macro and micro elements, is a medium with a high amount of salts and as Krikorian (1991) mentions, this can be sustainable for the growth of certain crops that they require high concentrations of ions, under the assumption that all known and necessary microelements for whole plants are also for cultured cells and tissues; however, orchids maintain a low mineral requirement because they have evolved in some aspects that help them survive in means of poor availability of water and nutrients, such as their thick leaves, roots with sails, pseudobulbs, among others (Gottschalk, 2012).

In contrast, the MS 50% was more favorable for maintaining the viability of the explants, since it maintained the same macro and micro elements, but at a reduced concentration. Knudson's medium maintains the same 1:2 ratio of ammonium: nitrate to that of MS, but differs in the nitrogen source, and that is why it is often used to assess growth in response to nitrogen nutrition (González *et al*., 2012); however, it does not contain the microelements that provide MS salts, such as zinc, sodium and copper, which are known to accumulate in roots or leaves in *Phalaenopsis* crops in hydroponics or in sphagnum moss (Trelka *et al*., 2010).

The medium made with fertilizers maintains a high salt concentration and does not supply microelements. Various investigations such as those of Park *et al*. (2002); Gow *et al*. (2008), in which they study different responses *in vitro* in *Phalaenopsis*, have been carried out with predilection by the MS 50% as a baseline, however, detailed studies of the requirements of microelements in *Phalaenopsis* and in many other orchids are necessary. Regarding the variety, it was observed that it influences the viability of the explants (Table 2).

Table 2. Percent oxidation of explants by *Phalaenopsis* variety.

Varieties	Oxidation percentage[a]
Phalaenopsis amibilis	25%
Phalaenopsis Taipei Gold	67%
Phalaenopsis Kaleidoscope	78%
Phalaenopsis Taisuco x Sogo	100%

[a]X^2= 13.810 with $p< 0.05$.

Previously, Brandelli and Lopes (2005) have demonstrated the relationship between peach darkening (*Prunus persica*) and the activity of the enzyme polyphenol oxidase (PPO). This enzyme is responsible for catalyzing the reaction between the phenolic compounds produced by the explants and the molecular oxygen that generates quinones, very reactive substances that inhibit growth, cause damage or even tissue death (He *et al*., 2009).

Scalzo *et al*. (2005) showed differences in the content of phenols between different genotypes of strawberry (*Fragaria* x *ananassa*), apricot (*Prunus armeniaca* L.) and peach (*Prunus persica* L.) and Pequeño *et al*. (2015) relates the phenol content and the activity of the PPO with the genotype in *Jatropha curcas in vitro*.

In this study there was a great variability in the oxidation between varieties, so the activity of the PPO could be evaluated under different antioxidant treatments. Although *P. amabilis* maintains greater adaptability to the establishment *in vitro* than the other hybrids, other factors that have not been evaluated in this experiment should be considered, such as the physiological state of the mother plants, fertilization regime, irrigation, light intensity, humidity, among others, as in the one where they were in the greenhouse of their origin, so that the stress caused by the alteration of these and other factors, added to the transfer, resulted in the accumulation of stress in the explant donor plants of the four varieties, which is remarkable both in the dryness of its roots, and wilting of the tip of the leaves.

George *et al*. (2008) mentions that an adequate choice of plant material can have an important effect on the success of tissue culture *in vitro*. That is why, to reduce stress, the mother plants were maintained for a month before the experiment in acclimatization conditions, with controlled light and temperature, as well as frequent irrigation and fertilization. However, if the slow characteristic growth of several orchid genera is considered (Cumo, 2013), it is defined that the acclimatization time was not enough for the other varieties, so it is suggested to extend the acclimatization time to two months, after the end of flowering, because at this time the plant has gone through the energy expenditure of flower production and a new period of vegetative growth begins.

The quality of the donor plants is a critical factor that will subsequently affect the yield of the *in vitro* culture, so it is advisable to standardize the growth conditions as much as possible (Preece, 2008). Also, the use of antioxidant agents during establishment such as ascorbic acid, or other agents such as activated carbon, which are incorporated into the culture medium to control the oxidation process (Ibarra *et al*., 2016) is recommended. In the experiment on the induction of outbreaks it is observed that the response of the explants depends on the regulator used (Table 3).

Table 3. Percentage of explant induction and number of outbreaks per explant according to the growth regulator used in *P. amabilis*.

Growth regulator	Induction percentage[1]	Number outbreaks
BAP	5%	2a
BAP/ANA	33%	2.4a

BAP= 6-Benzylaminopurine. ANA= naphthalenacetic acid. [1]X^2= 16,714 with $p< 0.05$. a= difference of means by Tukey.

Also, the influence of auxins and cytokinins is evidenced in the results of induction since a small amount of auxin can potentiate the effect of cytokinin, as described by Krikorian (1991). Despite these statements and since several auxins appear to have different sites of action, it would be convenient to test more extensive combinations and evaluate different auxins with different cytokinins.

The properties of the explant also govern in vitro growth, as it is well established that different genotypes may not respond in the same way even when they are in the same culture medium, which is why innumerable empirical studies have been necessary to optimize means for different species and even for cultivars of the same species since the regeneration capacity is specific for each species (Salgado *et al*., 2007; Preece, 2008).

The results obtained in the multiplication are not very competitive for a large-scale production that is sustainable for domestic producers, since authors such as Košir *et al*. (2004); Gow *et al*. (2008) have managed to obtain by direct organogenesis up to 8 and 15 outbreaks per explant respectively (Figure 3).

Again, although the cause can be multifactorial, the physiological state of the donor plants is considered to be the most influential since it significantly influences the morphogenetic capacity of the explant, in addition to the nutritional and hormonal requirements differ in the tissues grown from plants at different physiological ages (Villalobos and Torpe, 1991).

Another important factor is the choice of flower outbreaks in the flower stick, in this study all flower knots were used, however, dedifferentiation in the terminal outbreaks was not achieved (Figure 4). Jiménez and Guevara (1996) mention that the intermediate outbreaks are the most appropriate and those that will be more likely to resist the aseptic establishment process and produce an outbreak because they concentrate a greater amount of endogenous regulators, also consider that the first basal node does not have an axillary outbreak while the terminal outbreaks have a high degree of differentiation towards the reproductive state, so the intermediate outbreaks are the most suitable for inducing vegetative growth.

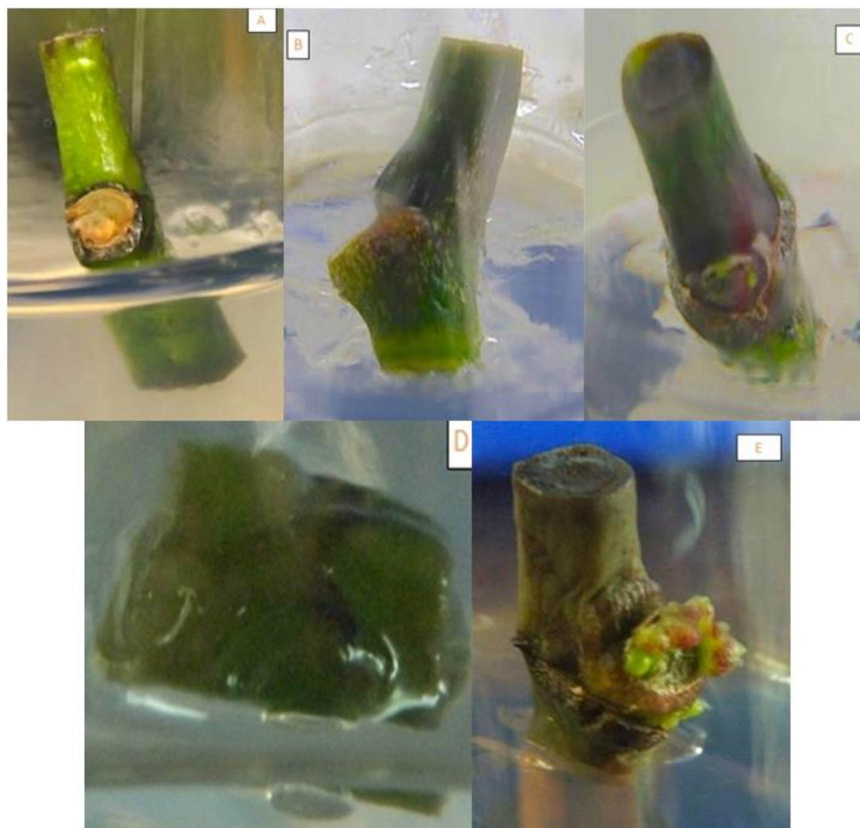

Figure 3. Induction of outbreak on flower outbreaks of the *Phalaenopsis* orchid. A) sleeping outbreak at 2 weeks; B) broadening of the lower part of the sleeping outbreak at 4 weeks; C) outbreak formation in axillary outbreak at 6 weeks; D) outbreak formation in axillary outbreak at 10 weeks; E) vegetative outbreak developed in sleeping outbreak at 14 weeks. A, B and C correspond to the treatment with BAP, D and E correspond to the treatment with BAP/ANA.

Coinciding, Park *et al*. (2002) recommend using between the second and fourth knot of the floral stem from the base and always containing a differentiated lateral outbreak. On the contrary, Košir *et al*. (2004) obtained a rapid micropropagation by using knots with sleeping outbreaks (Figure 4).

Figure 4. Established flower outbreaks. Left: sleeping outbreak of the upper end of the floral wand; center: active outbreaks of the center of the floral wand; right: apical outbreak of the floral wand.

Although other physical factors such as light should also be analyzed, it is only by rigorous characterization of the biological material that the range and magnitude of the variation can be estimated and understood. It must be considered that when using micropropagation, hormonal regulation will depend not only on the genotype of the chosen species, but also on the physical stimuli of the environment, so the response will be affected by the concentration and proportion of each hormone used.

Conclusions

With respect to the first experiment of the butterfly orchid, it is concluded that: a) the mineral salts used in the culture media, as well as the *Phalaenopsis* orchid variety significantly influence the oxidation of the explants; and b) the greatest viability of explants was achieved in the MS 50% medium.

With respect to the second experiment, it can be affirmed that the organogenesis in the *P. amabilis* orchid is obtained when its explants are established in the appropriate culture medium and with a correct hormonal balance, therefore, attention to factors such as combination and interaction of the growth regulators and their concentrations during the culture of their tissues, allowed the *in vitro* clonal multiplication of this orchid.

It is also concluded that: a) The hormonal balances used, either BAP 20 µM or BAP 20 µM/ANA 5.37 µM, in medium MS 50%, allow the induction of outbreaks in the flower outbreaks of this orchid; and b) However, the combination of both regulators significantly increases induction. Therefore, it is more appropriate to combine the regulators to obtain a greater number of explants with response with a good number of outbreaks.

It is convenient to evaluate different concentrations and combinations of both growth regulators, as well as to evaluate different auxins with different cytokinins knowing the effect of the former on the latter.

The quality of the explants influences morphogenesis, the younger the tissue, the micropropagation will have a greater chance of being successful, so the plant material is selected in optimal physiological and sanitation conditions, without forgetting the hormonal balance that is also associated with the state of development of plant material.

The quality of the donor plants is a critical factor that will subsequently affect the yield of the *in vitro* culture, so it is convenient to standardize the growth conditions prior to aseptic establishment, to ensure the quality of the explants, the main aspects that should be consider are light, temperature, irrigation, fertilization and humidity, in addition to making constant reviews to eliminate pests or diseases. Maintaining these conditions in a favorable manner, it can be observed that the mother plants are vigorous and healthy.

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