ARTÍCULO DE INVESTIGACIÓN

Acclimatization of *Phalaenopsis* and *Cattleya* obtained by micropropagation

Aclimatización de *Phalaenopsis* y *Cattleya* obtenidas por micropropagación

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Abstract

The quality of micropropagated plants relies on the acclimatization stage. This research intends to develop an efficient protocol to obtain the acclimatization of *Phalaenopsis* and *Cattleya*. Plants of *Phalaenopsis* obtained from protocorms were selected. They came from flowering stalks grown at modified Murashige and Skoog (MS) (1962) medium and classified by growth ranks and put into moss, mesquite wood shaving and perlite (1:1:1), into a humidity chamber. The protocorms were multiplied at MS from *Cattleya* sown in Knudson C (1946) medium; regenerated plants of 1-2 cm were selected, and implanted in humidity chamber on: moss, coal and perlite (1:1:1) MCP; mesquite wood shavings, coal and perlite (1:1:1) ACP; moss and perlite (1:1) MP; mesquite wood shaving and perlite (1:1) AP. The following results were obtained: *Phalanopsis*: a) Survival: 44% in R_0 and 100% in R_I and R_{II}. b) Number of leaves: R_I gave on average 1 more leaf than the range 0; c) Roots number and length: R_I and R_{II} gave on average 2 more roots than R_0, and there were no significant differences in length. d) Height: R_{II} presented greater growth than R_I and R_o. *Cattleya*: a) The survival in MCP was 0%, MP 16 %, ACP 32% and AP 80%. b) The height in MP was significantly superior to the ones in ACP and AP. Plants from both genera need to achieve a 2 to 4 cm growth rank *in vitro* to endure the greenhouse conditions. MAP was the best substrate in *Phalaenopsis* and moss-perlite in *Cattleya*.

Key words: *Orchidaceae*, substrates, *in vitro* culture.

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Resumen

La calidad final de las plantas producidas por micropropagación depende de la etapa de aclimatización. Se intenta desarrollar un protocolo eficiente para la aclimatización de *Phalaenopsis y Cattleya*. Se seleccionaron plantas de *Phalaenopsis,* obtenidas de protocormos provenientes de estacas florales cultivadas en Murashige y Skoog modificado (MS) (1962), por rangos de crecimiento e implantadas en musgo, viruta de algarrobo y perlita (1:1:1), en cámara húmeda. De siembras de *Cattleya* en medio de Knudson C (1951) se multiplicaron protocormos en MS; se seleccionaron plantas regeneradas de 1-2 cm, e implantadas en cámara húmeda en los sustratos: musgo, carbón y perlita (1:1:1) MCP; viruta de algarrobo, carbón y perlita (1:1:1) ACP; musgo y perlita (1:1) MP; viruta de algarrobo y perlita (1:1) AP. Se obtuvieron los siguientes resultados: en *Phalaenopsis*: a) Supervivencia: para R_0 de 44% y R_I y R_{II} del 100%; b) número de hojas: R_I generó en promedio 1 hoja más que el rango 0; c) número y longitud de raíces: R_I y R_{II} generaron en promedio dos raíces más que R_0, no detectándose diferencias significativas en longitud; d) altura: R_{II} presentó mayor crecimiento que R_I y R_0 En *Cattleya*: a) La supervivencia en MCP fue 0%, MP 16%, ACP 32% y AP 80%; b) La altura en MP resultó significativamente superior que en ACP y AP. Ambos géneros necesitan alcanzar un crecimiento de 2 a 4 cm *in vitro* para tolerar las condiciones de invernáculo. El mejor sustrato fue MAP en *Phalaenopsis*, y la mezcla musgo-perlita en *Cattleya*.

Palabras clave: *Orchidaceae*, sustratos, cultivo *in vitro.*

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Introduction

Micropropagation is a massal culture system developed under conditions of asepsis, high humidity and controlled lightness and temperature. Acclimatization is a critical stage in micropropagation. During this period the higher percentages of plant losses occur due to several reasons (Kozai, 1991; van Huylendroeck *et al.*, 1998). This is the reason why is necessary to obtain quality plantlets under *in vitro* conditions to ensure a high survival percentage and an appropriate growth under greenhouse conditions. In order to survive *ex vitro* a plantlet must achieve a growth stage with an appropriate sprout number, foliage area and radicular system, considering roots number and length.

Another aspect to be considered is that many plantlets do not survive the acclimatization stage when transferred to a septic substrate because of media contamination. The effects of temperature, humidity and lightness in the greenhouse and also the plantlets nutritional conditions should be taken into account as well.

Pospišilová (1999*)* stated that *in vitro* acclimatization is one of the key factors in producing healthy plantlets before they are transplanted to *ex vitro* conditions. According to Preece and Sutter (1991) acclimatization will allow the plant to reach a state of autotrophic growing (Teixeira da Silva *et al.*, 2005) in environments of lesser relative humidity, more light and septic substrates. Transferring plants from an almost ideal situation to a greenhouse or *ex vitro* situation presents a challenge for survival. Plants will move to a heterotrophic state to an autotrophic one, undergoing physiological and morphological changes as well as a greater exposition to the action of plagues and diseases. The phenotype is one of the characteristics being modified under *in vitro* conditions, i.e; stems are thinner, with lesser wax quantities, reduction of support mechanical tissues, greater content of cell water and heterotrophic growing (Denng and Donnelly, 1993). All these alterations make necessary to include an acclimatization stage within the micropropagation protocols for the plant to recover their morphological and physiological characteris-

tics. The conditions during this stage involve increased lightning, reduced humidity, termic variations, septicity, the right selection of substrate and an optimal growth stage in order to obtain an adequate survival percentage.

Cha-um *et al.*(2009) state that environmental conditions for *ex vitro* growth are quite different from those used for *in vitro* cultivation Plant growth retardants, i.e., uniconazole (UCZ), paclobutrazol (PBZ) triapenthenol (TPN), triadimefon (TDM) and hexaconazole (HCZ) have been reported as effective agents in reducing the size of plants, but retaining dark-green leaves and thick roots, which define them as healthy plantlets, and aiding antiwilting, leading to better survival and growth in *ex vitro* condition

The selection of a proper substrate with low septicity, high aeration, permeability and a correct acidity grade is a requisite to guarantee conditions of initiation and autotrophic growth. It is also necessary that the substrates keep these conditions for a long period without deteriorating to avoid compaction and lack of aeration and permeability.

A substrate is considered to be a solid and porous, natural or syntethic material, which combined or not, permits and adequate plants growth under controlled environment conditions (Abad, 1989). The substrate function is to provide mechanical support and to improve air and water absorption by the roots (Tortosa, 1990). The substrate may be related or not to the mineral nutrition management. Sanitation is a major issue so that the substrate should be obtained from inert or easy to disinfect material like earthworm humus, compost (Agramonte Peñalver *et al.*, 1998; Díaz *et al.*, 2004), tezontle, tezontle –vermiculite, tezontle-dicalite, pine bark and dark lava rock chippings (Avila-Díaz *et al.*, 2009).

The substrate choice is conditioned by the plant species, just as the case of the epiphytic orchids genera studied in this paper. The substrate requirements are: acidity, aeration and permeability. It is also necessary to know the appropriate environmental conditions for growing in greenhouse (Iriarte *et al.*, 2002). According to Northen (1990) *Phalaenopsis* demands temperatures between 15 ºC and 35 ºC, 10% solar light and 70% relative humidity (RH) while *Cattleya* needs temperatures between 10 ºC and 35 ºC, 30% solar light and 50% RH. In Argentina both genera are economically important because of their ornamental value as flowers and interior plants. A basic need to fulfill the requirements of orchid producers is to develop protocols allowing a high quality massal propagation and plants uniformity.

This research intends to develop an efficient protocol to obtain the acclimatization of *Phalaenopsis and Cattleya* and the objectives were: I) Achieving the adaptability of *Phalaenopsis* from *in vitro* to *ex vitro* conditions using a mixture of substrates and evaluating the effects of different growth ranks on survival and growth. II) Achieving the adaptability of *Cattleya* from *in vitro* to *ex vitro* conditions by using substrate combinations from different origins.

Materials and methods

Four substrates were used as cultivation media: A) Perlite (P): it is a substrate widely used in the preparation of compost from different cultures. It is ground inert volcanic lava expanded at 800ºC, thus facilitating sustained aeration and permeability conditions in any mixture. Their inherent properties of porosity and sanity are important when used at the acclimatization stage. B) Moss (*Meteoropsis onustum*) (M): vegetal substrate being able to absorbe water up to 20 times their own weight. It is acid (pH 5-6), creating optimal conditions for epiphytic orchids cultivation. The disadvantage is that after 2 to 4 months this characteristic vanishes so it has to be replaced after that period. C) Vegetal charcoal (C): it is an organic product obtained by burning wood from different plant species. It is stable in cultivation media but it is hydrophobic so it has to be submerged in water for at least 24 hours in order to eliminate

the air and gets to absorbed water. D) Mesquite wood shaving (A): it is a product obtained by manual or mechanical wood planing, widely used in carpentry. Their physical characteristics persist for a period of over a year since mesquite provides a hard wood. It has to be sifted before being used in order to separate the big shavings. An intermediate size is required to facilitate small plants cultivation.

The vegetal material comes from the following *in vitro* cultivated genera:

I) *Phalaenopsis:* the hybrids were cultivated from floral nodes (Arditti and Ernst, 1993) (Photo 1) incubated in Murashige and Skoog (MS) (1962) medium with salts and vitamins (50%); 6 benzyl-amino-purine, BAP, (10 mg/l); 1-naphtalen acetic acid, ANA, (1 mg/l); sucrose (3%), activated charcoal (0,2%) and agar (0,5%). Protocorms and plantlets were regenerated (Photo 2). The material was selected by growth ranks: R_0: 1 to less than 2 cm height and two 1-4 cm long roots; R_I: 2 to less than 3 cm height and three 1-6 cm long roots; R_{II}: 3 up to 4 cm height and three 1-8 cm long roots. The substrate composition was a mixture of moss, mesquite wood shavings and perlite (MAP) in 1:1:1 proportion (Photo 3). 75 plantlets (25 in each rank category) were implanted in three 40 x 60 cm plastic collective trays, at the end of springtime, and kept in humid chamber during the first two weeks. After that period intermittent ultra low volume (fog) irrigation system, 10% luminosity and 30% solar light (using half shadow or saran) was applied. The greenhouse conditions were: 10 °C (night) and 25 °C (day) during the winter; 20 °C (night) and 35 °C (day) during the summer.

II) *Cattleya:* the *Cattleya maxima x nobilior* hybrid was used, originated from seeds cultivated *in vitro* in Knudson C (1951) medium and multiplied from small and medium size protocorms in MS (1962) with salts and vitamins MS (50%), BAP (0,5 mg/l), ANA (0,1 mg/l), sucrose (3%) and agar (0,5%) (Photo 4). 1-2 cm long regenerated plants were selected and implanted, in humid chamber, on the following substrates: MCP: moss, charcoal and perlite (1:1:1) (Photo 5); ACP: mesquite wood shavings, charcoal and perlite (1:1:1) (Photo 6); MP: moss and perlite (1:1) (Photo 7); AP: mesquite wood shavings and perlite (1:1:1) (Photo 8). 100 plantlets were implanted in four 40 x 60 cm plastic collective trays, each one with a different substrate (25 plantlets per subtrate). The implanted plantlets had between 1 and 2 cm length with two roots, 1-4 cm long, corresponding to rank 0. No enough material corresponding to ranks I and II was achieved in order to carried out and experiment similar to the one in *Phalaenopsis*. Experimental environment conditions were the same as in *Phalaenopis* mentioned before.

In both genera, supplementary lighting was provided during the fall, winter and spring initiating from 6 AM to 11 PM i.e. a 17-hours photoperiod. The plants were fertilized every fifteen days by using a mixture of N:P:K (7:3,1:7,3) and ANA (40 mg/l). After three weeks of implantation the plantlets were watered with intermittent ultra low volume (fog) irrigation system. Water was biologically stabilized or chlorine free.

The following evaluations were carried out: I) *Phalaepnosis*: survival on four evaluation dates (25, 40, 55 and 85 days after implantation), and roots height, number and length after 60 and 90 days from implantation. II) *Cattleya*: survival was quantified at 35, 50, 75 and 105 days after implantation. Plant length was measured after 90 days from implantation.

The following substrate measurements were evaluated: pH, electric conductivity (EC) (1:10 dilution) and ashes by calcination (%) (Table 1). Analyses were conducted at the laboratory of Cátedra Edafología. FAZ-UNT.

The perlite chemical composition was also determined (%): Si O_2:74 -79; Al_2O_3: 13-17; K_2O: 0.5- 5; Na_2O: 2-5; CaO: 0,4-0,6; Fe_2O_3: 0,3-0,9 and MgO: 0,04-0,15.

The following statistical techniques were used: a) Survival: percentage of survived plants was calculated in relation to the initial im-

planted number, on every evaluation date; b) Treatments effect on plants height, root number and length: analysis of variance and Tukey's pairwise comparison test.

Graphics and analysis were run on R (R Development Core Team, 2010).

Results and discussion

Phalaenopsis and *Cattleya* adaptation is slow and difficult, however, no references were found concerning *in vitro* adaptation by using different origin substrates for these species. *Doritaenopsi*s post micropropagation acclimatization CAM orchids, was studied by Jeon *et al.* (2005) who evaluated the effect of light flow density on morphology, photosynthesis and growth. Teixeira da Silva *et al.*, (2005) worked on banana and *Cymbidium in vitro* acclimatization and they included the evaluation of growth parameters in *ex vitro* conditions. In the present paper the effect of light on acclimatization was not evaluated. A gradual plantlets adaptation to light was accomplished by using half-shadow as well as controlled watering and nutrition. The plantlets were fertilized every fifteen days by using a mixture of N:P:K (7:3,1:7,3) and ANA (40 mg/l).

Colombo *et al.* (2005) worked on the acclimatization of a *Cattleya* hybrid by using several vegetal substrates and two irrigation systems. They found that the coconut powder substrate and intermittent irrigation system were the most indicated for the acclimatization of the *Cattleya* chocolate drop (*C. guttata* x *L. tenebrosa*) orchid with a 90% survival. In this paper only the intermittent irrigation system was applied. Colombo *et al.* (2005) also found that moss showed the lowest survival (72%) in *Cattleya* when combined with a manual irrigation system

Torres, Laskowski and Sanabria (2006**)** evaluated *Cattleya jenmanii* Rolfe leaf epidermis anatomy, *in vitro* multiplication and acclimatization in orchidarium. They determined that, during the acclimatization stage, leaves from *in vitro* plants increased the stomata size and the thickness of anticlinal walls in typical cells in order to favor mechanical resistance and stiffness.

A few references were found on different origin substrates usage on micro propagated plants acclimatization (Agramonte Peñalver *et al.*, 1998). Díaz *et al.* (2004) used earthworm humus as a substrate for sugar cane micropropagated plants acclimatization. Shiau *et al.* (2002) worked on the establishment of *Anoectochilus formosanus Hayata ex vitro* plantlets, which achieved 90% survival after transferring the material to *ex vitro* conditions in coconut fiber closed recipients and then incubated in peat moss and vermiculite (1:1). Avila-Díaz *et al.* (2009) studied the survival and acclimatization of seedlings from *Laelia speciosa* using different potting mixtures.

Table 1. Substrates pH, electric conductivity and ashes (%)

	pH	EC	Ashes (%)
Mesquite	6.63	0.27	2,15
Moss	6.03	2.00	7,78
Perlite	6.83	0.00	99,2
Charcoal	7.69	0.05	2,61
Charcoal H_2O	7.34	1.20	_

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I)

In this research was intended to reproduce the natural conditions under which these epiphytes grow using permeable, aerated, durable, acid or neutral substrates so as to guarantee the *in vitro* material survival, establishment and growth under greenhouse conditions (table 1).

I) *Phalaenopsis*: Phalaenopsis: Christenson (2001) quoted by Lee *et al.* (2008) states that the genus Phalaenopsis (Orchidaceae) comprises about 63 species that have produced numerous attractive hybrids and cultivars. This genus presents the following characteristics: monopodial growth with indefinite growing apical meristem, fast growth, flowering from the second life year on and producing up to 9 leaves.

The survival of the material implanted on MAP was: 44% in R_0 and 100% in R_I and R_{II}. This result indicates that the plant needs to achieve certain *in vitro* growth rank to endure the external conditions in greenhouse.

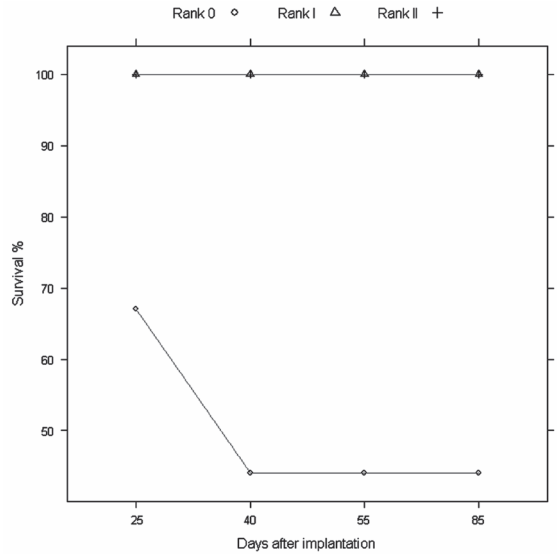

Figure 1. *Phalaenopsis* survival percentage according to growth ranks and days after implantation (year 2007).

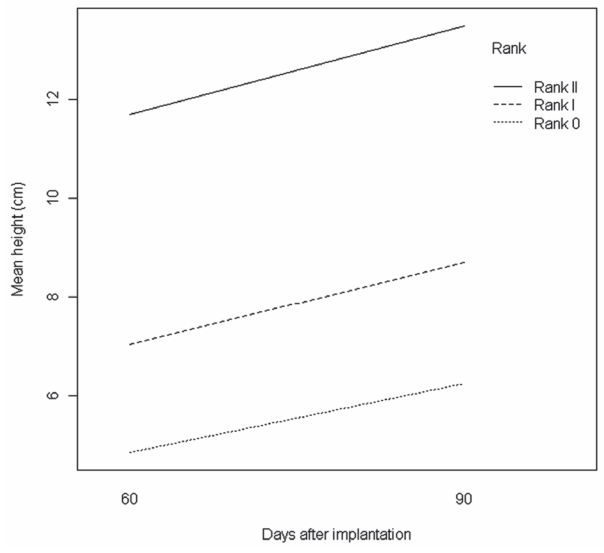

Figure 2. *Phalaenopsis* mean height (cm) according to growth rank and days after implantation on MAP (year 2007).

As far as growth concerns (figure 2), a differential behavior among ranks was observed. Plants in the ranks I and II achieved a greater mean height during the same time period (statistically different at $\alpha = 0.05$).

According to the resulst a critical threshold was observed in order to ensure the survival and posterior growth in *Phalaenopsis*. This threshold should be plantlets in R_I and $R_{II,}$ i.e. 2 to 4 cm height and three 1-8 cm root long. Shushan (1959) in Cattleya *mossiae* x *C. trianae* observed that the appropriate aerial development was 0.5 to 2 cm.

In relation to roots growth, during the first 60 days no plants with new roots were observed. However, in the second evaluation (90 days), the ranks I and II generated a significantly greater root number (α=0.05) than rank 0. The plants in the ranks I and II generated, on average, two more roots than the ones in rank 0 (figure 3). No difference was found in roots length when comparing the three ranks (figure 4).

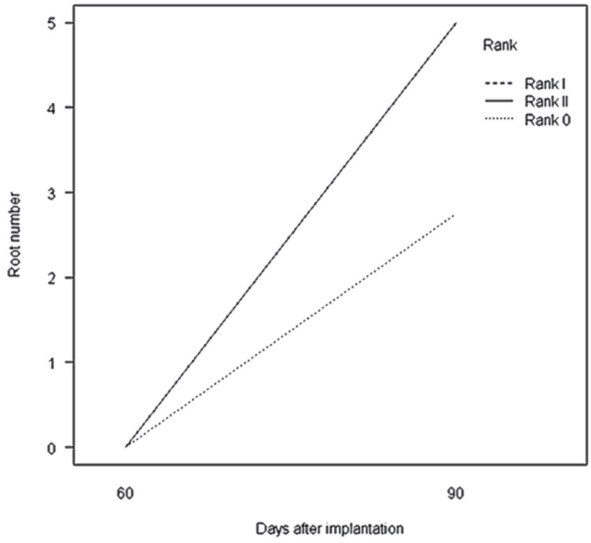

Figure 3. *Phalaenopsis* roots number according to rank and days after implantation on MAP (year 2007).

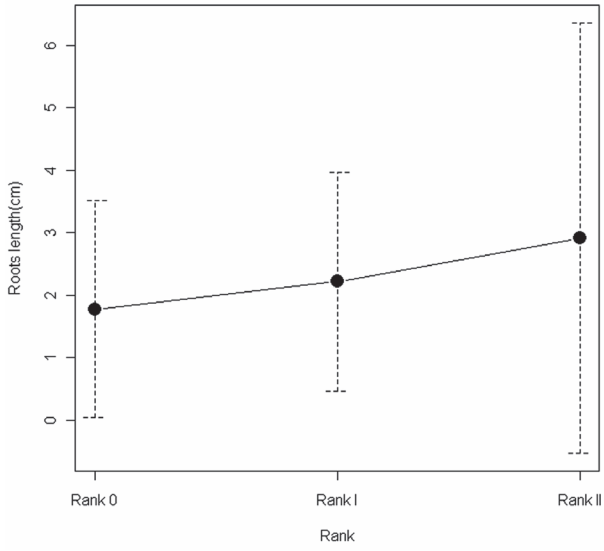

Figure 4. *Phalaenopsis* roots length mean and 95% confidence intervals after 90 days from implantation on MAP (year 2007).

The development of the root system (root number and length) is vital to anchor the plant and also to ensure water and nutrients absorption. This is coincident with the fact expressed before related to the existence of a minimum plantlet length threshold. Maene and Debergh (1983) found that 2.5 to 5 cm long micro shoots of *Cordilyne terminalis* rooted better while the ones shorter than 2.5 and larger than 6 cm plantlets showed a decreased percentage of roots. The results in this research showed that the best results in survival and growth were achieved with plants between 2 to 4 cm and 3 roots. Ávila- Díaz *et al.* (2009) transplanted *Laelia speciosa* plantlets of 5 cm in length to the greenhouse and a survival rate of 77.5% of was obtained.

The table 2 shows that the leaves number in R_I is, on average, one more unit than in R_0. This fact confirms the results from Preece and Sutter (1991) and Dietrich *et al.* (1992), quoted by Pospisilova *et al.* (1999), who stated that in many plant species leaves formed *in vitro* are not capable of keep growing under *ex vitro* conditions and that they are replaced by new ones. These authors did not study the growth rank effects in their research. A differential effect was found among ranks, particularly in R_I, in this experience. Sushan (1959) observed under greenhouse conditions in the primary hybrid *Cattleya* x *Trimos*, the formation of 6 to 7 leaves in 12 months and up to 9 leaves were found in 22 months.

Table 2. Tukey`s test for *Phalaenopsis* leaf number

Rank	Leaf average number
R_I	3,6 A
R_{II}	3,2 AB
R_0	2,6 B

Treatments with the same letter do not differ significantly (α= 0.05)

II) *Cattleya*: is an American tropical genus with pseudobulbs and sympodial growth (Font Quer, 1965). Its growth is slow reaching physiological maturity after seven years.

80% survival was obtained on AP substrate, 90 days after implantation; MCP showed the worst performance (0% at 90 days). The other two media were intermediate: 16% MP and 32% ACP (figure 5).

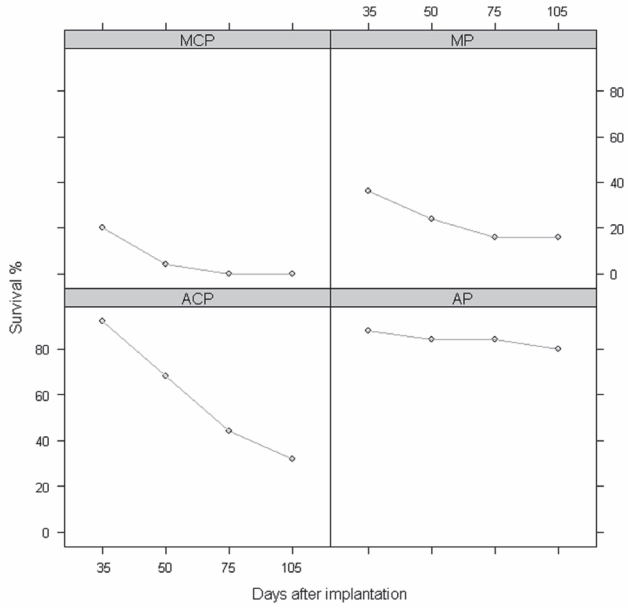

Figure 5. *Cattleya* survival percentage according to days after implantation on four substrates.

The figure 6 shows significant differences among average plants height. MP produced plants 1 cm larger on average. Shushan (1959) determined an aerial development of 0.5 to 2 cm in 12 months under greenhouse conditions. This size was the right one in the sense that the plantlet reached the proper roots number and length and foliage area to survive under greenhouse conditions. Ávila-Díaz *et al.* (2009) determined the relationship between survival and seedling size in different substrates and arrived to the conclusion that seedlings of 5cm in length had the highest frequency of survival (77.5%) whilst sizes of 2 and 1 cm long showed 5 to 0% survival respectively.

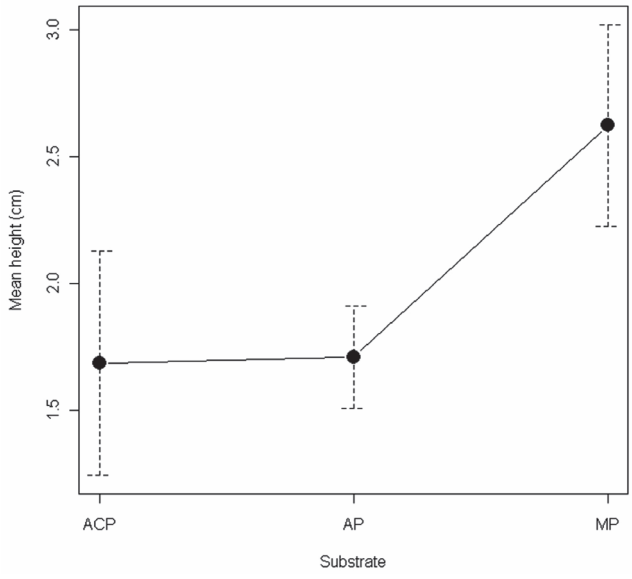

Figure 6. 95% confidence intervals for *Cattleya* plant height (year 2007) for three substrate mixtures.

In this research was found, for both genera, that the plantlets must achieve a 2-4 cm long growth. By reaching this size the plantlets also gets a proper number of sprouts, foliage area (leaves size and number) and roots number and length thus allowing for maximal survival under *ex vitro* conditions.

Conclusion

Plants from *Phalaenopsis* and *Cattleya* need to achieve from 2 to 4 cm growth rank *in vitro* in order to endure the external conditions in greenhouse. MAP cultivated *Phalaenopsis* got the better growth response when length was 2 to 4 cm with three roots and 1 to 8 cm long; these values being a critical survival threshold. The best substrate in *Cattleya* was the mixture of moss-perlite (MP)

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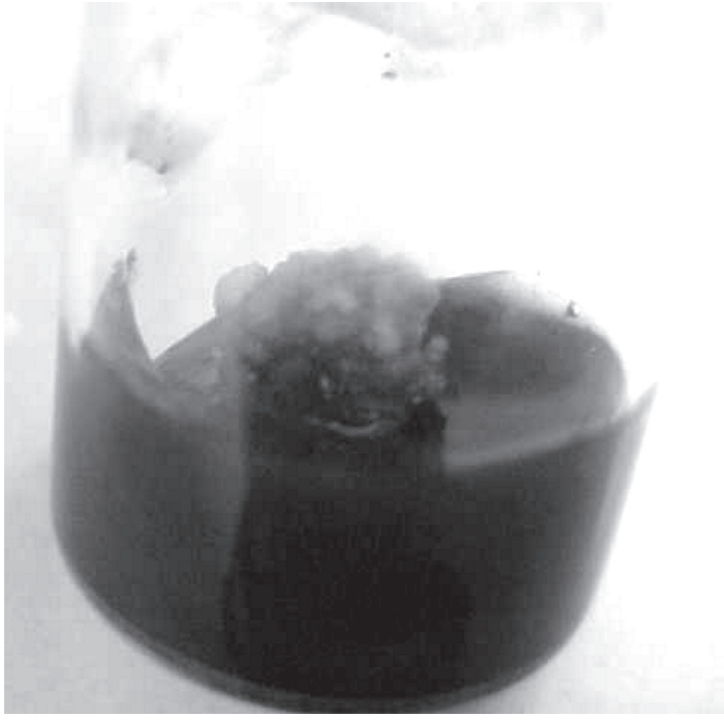

Photo 1. *Phalaenopsis* protocorms regenerated from floral nodes.

Photo 2. *Phalaenopsis in vitro* plantlets.

Photo 3. *Phalaenopsis* plantlets on MAP potting mixture.

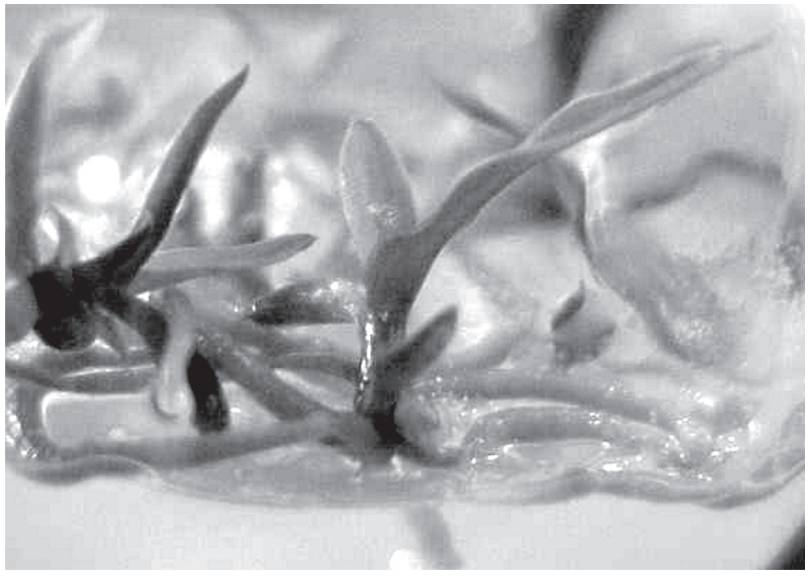

Photo 4. *In vitro Cattleya* plantlets.

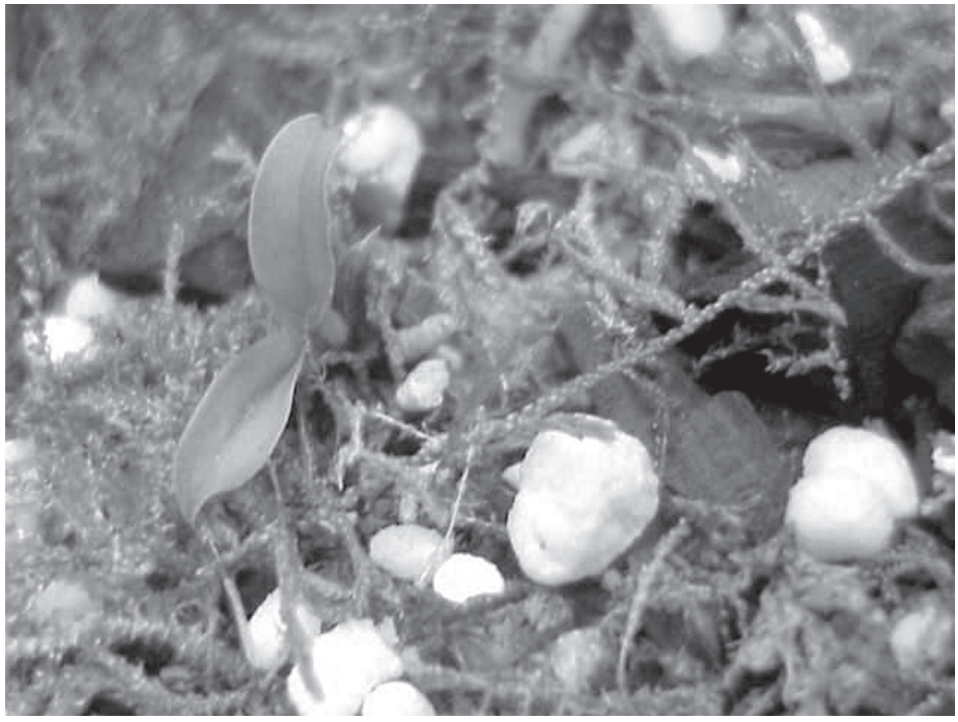

Photo 5. *Cattleya* plantlets on MCP potting mixture.

Photo 6. *Cattleya* plantlets on ACP potting mixture.

Photo 7. *Cattleya* plantlets on MP potting mixture.

Photo 8. *Cattleya* plantlets on AP potting mixture.