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# Plant growth regulators optimization for *in vitro* cultivation of the orchid *Guarianthe skinneri* (Bateman) Dressier & W.E.Higgins

## Optimización de reguladores de crecimiento para el cultivo *in vitro* de la orquídea *Guarianthe skinneri* (Bateman) Dressier & W.E.Higgins

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**ABSTRACT**

An *in vitro* culture procedure was developed to induce shoots and roots of *Guarianthe skinneri* (Orchidaceae) plantlets regenerated from seed-derived protocorms on a Murashige and Skoog (MS) medium supplemented with 6-benzyladenine (BA), indole-3-acetic acid (IAA), α-naftalenacetic acid (NAA) and gibberellic acid ($GA_3$). A maximum of 10.6 shoots was obtained with 16.1 μM NAA, 17.1 μM IAA, 6.3 x $10^{-9}$ μM $GA_3$ and 0.0023 μM BA. A maximum of 4.0 roots on each shoot with 5.4 μM NAA, 17.1 μM IAA, 0.001 μM $AG_3$ and 4.6 x $10^{-9}$ μM BA. Maximum shoot and root length was obtained with a minimum of $GA_3$, but a maximum IAA. $GA_3$ was the main factor controlling shoot and root induction and elongation.

**Keywords:** 6-benzyladenine, indole-3-acetic acid, α-naftalenacetic acid, gibberellic acid, shoot induction, rooting.

**RESUMEN**

Se desarrolló un procedimiento para el cultivo *in vitro* para inducir brotes y raíces en las plántulas de *Guarianthe skinneri* (Orchidaceae) regeneradas a partir de protocormos derivados de semillas en un medio de Murashige & Skoog (MS) complementado con 6-benziladenina (BA), ácido indol-3-acético (AIA), ácido α-naftalenacético (ANA) y ácido giberélico ($GA_3$). Se obtuvo un máximo de 10,6 brotes con 16,1 μM de ANA, 17,1 μM de AIA, 6,3 x $10^{-9}$ μM de $GA_3$ y 0,0023 μM de BA. Un máximo de 4,0 raíces en cada brote se obtuvo con 5,4 μM de ANA, 17,1 μM de AIA, 0,001 μM de $AG_3$ y 4,6 x $10^{-9}$ μM de BA. La longitud máxima de brotes y raíces se obtuvo con la más baja concentración de $GA_3$, y la mayor concentración de AIA. El $GA_3$ fue el factor principal que controló la inducción y la elongación de los brotes y raíces.

**Palabras clave:** 6-benciladenina, ácido indol-3-acético, ácido α-naftalenacético, ácido giberélico, inducción de brotes, enraizamiento.

## INTRODUCTION

*Guarianthe skinneri* (Bateman) Dressier & W.E.Higgins belongs to Orchidaceae. The ornamental use of this species has increased its demand, but its natural habitat is being destroyed by deforestation so it is now recognized as "threatened" under name *Cattleya skinneri* within mexican law NOM-059-ECOL-2001 (Diario Oficial, 2002). Micropropagation might be a useful technique to cultivate *G. skinneri*. It offers the possibility to produce thousands of plants of the desired clone. The development of an efficient micropropagation protocol can play a significant role in the

commercial cultivation of vulnerable plant species, thereby conserving them in their natural habitat (Amoo *et al*. 2009). Encouraging results have been obtained with other species recognized as vulnerable using micropropagation (Bopana & Saxena 2008). In micropropagation, growth regulators are very important. Gibberellic acid ($GA_3$) has a positive effect on root formation of loblolly pine (*Pinus taeda* L.) when added with indole butyric acid (IBA) and butyric acid (BA) (Tang 2001). Auxiliary shoots of loblolly pine were sub-cultured twice at a 4-week interval on woody plant basal medium (WPM) supplemented with BA, $GA_3$, or $GA_3$ + BA to improve shoot growth. A maximum of 93% shoot growth was obtained on WPM supplemented with 2.2 µM BA (Park *et al*. 2008). Micropropagation of lentils (*Lens culinaris* Medik.) was optimized on Murashige Skoog (MS) media (Murashige & Skoog 1962) to regenerate shoots *in vitro* from nodal segments. The number of shoots per explant, the number of nodes per shoot and shoot length was most affected by the concentration of $GA_3$ and 6-benzyladenine (BA), with only small interaction effects between them (Ahmad *et al*. 1997). Shoots of *Acacia mangium* were elongated on MS medium containing 0.045 µM thidiazuron (TDZ) supplemented with 7.22 µM $GA_3$ (Deyu & Hong 2001). For *in vitro* propagation of *Oroxylum indicum*, a forest tree, the best medium for proliferation was MS medium with 8.87µM 6-BA and 2.85µM indole-3-acetic acid (IAA). However, incorporation of 1.44 µM $GA_3$ was necessary to increase shoots elongation (Naomita & Ravishankar 2004). The study reported here was done to determine the optimum concentrations of 6-benzyladenine (BA), indole-3-acetic acid (IAA), α-naftalenacetic acid (NAA) and gibberellic acid ($GA_3$) to increase shoot and roots numbers in plantlets of *Guarianthe skinneri* grown *in vitro*.

## MATERIALS AND METHODS

### Capsules disinfection and cultivation

*G. skinneri* capsules were collected from the 'Cañon del Sumidero' national park 16º47´56.5´´ north latitude 93º05´28.5´´ west latitude at 966 masl in Chiapas (Mexico). Five capsules were washed with water and commercial soap for 5 min and washed 3 times with sterile distilled water. Capsules were disinfected in 70% (v/v) ethanol for 5 min, immersed in 10% (m/v) aqueous calcium hypochlorite solution for 10 min and washed three times with sterile distilled water (Fig. 1 a). Hundred and fifty assays tubes (25 $cm^3$) containing 20 $cm^3$ MS medium supplemented with sucrose 30 g $dm^{-3}$, myo-inositol 0.1 g $dm^{-3}$, $NaHPO_4$ 0.05 g $dm^{-3}$ and solidified with 2.5 g $dm^{-3}$ phytagel, were covered with plastic. The tubes with medium were sterilized at 1.5 kg $cm^{-2}$ for 15 min and two disinfected seeds were placed in each tube and incubated under cool white fluorescent light (50 µmol $m^{-2}$ $s^{-1}$) and 16/8 photoperiod at 25-27ºC. Seeds germinated after four weeks culturing and zygotic protocorms were obtained after another two weeks (Fig 1 b). Shoots developed two weeks later and each shoot was transferred to 150 $cm^3$ bottles containing 30 $cm^3$ of the same medium (Fig. 1c, d).

An orthogonal experimental design of $L_9$ ($3^4$) in triplicate was used to investigate the effects of $GA_3$, BA, NAA and IAA, on number of shoots and roots, and leaf and principal root length (Table I) (Ross 1989). The symbol $L_a$ ($b^c$) is used to represent the orthogonal array where 'a' is the number of experimental runs, 'b' the number of levels for each factor or variable and 'c' the number of factors investigated. Independent variables were different concentrations of GA (0, 2.9 and 8.7 µM), BA (0, 2.5 and 4.9 µM), NAA (0, 5.4 and 16.1 µM) and IAA (0, 5.7 and 17.1 µM). Bottles were incubated under cool white fluorescent light (50 µmol $m^{-2}$ $s^{-1}$) and 16/8 photoperiod at 25-27 ºC for two months. Number of shoots and roots, and length of shoots and roots of each plant were determined.

The plantlets obtained from each treatment with well-developed shoots and roots were transferred to pots containing a mixture of peat moss and agrolite for hardening at 22±2°C under diffuse light (16/8-h photoperiod). Potted plantlets were covered with polyethylene membranes to ensure high humidity and watered daily with liquid 1/2-MS medium free of sucrose. After 3 wk, the membranes were removed and the plantlets were irrigated with tap water. The plantlets were acclimatized for 1 wk in the laboratory conditions and then transferred to a greenhouse (Fig. 1 d).

### Statistical analyses

The Statistica (2000) software was used to analyze data obtained with the $L_9$ ($3^4$) orthogonal array using a confidence limit of 5%. The linear and quadratic values of all factors and the interactions between them were tested. The percent contribution was calculated to determine the portion of the total variation observed in an experiment attributable to each significant factor and/or interaction (Ross 1989). The percent contribution is a function of the sums of squares for each significant factor and indicates the relative power of each and/or interaction to reduce variation. If the factor and/or interaction levels are controlled, then the total variation can be reduced by the amount indicated by the percent contribution.

Characteristics of the plantlets were subjected to a one-way analysis of variance (ANOVA) to test for significant differences. The latter analyses were performed using SAS statistical package (SAS 1989).

## RESULTS AND DISCUSSION

### Shoots number

The number of shoots varied from 5.0 in treatment 8 to

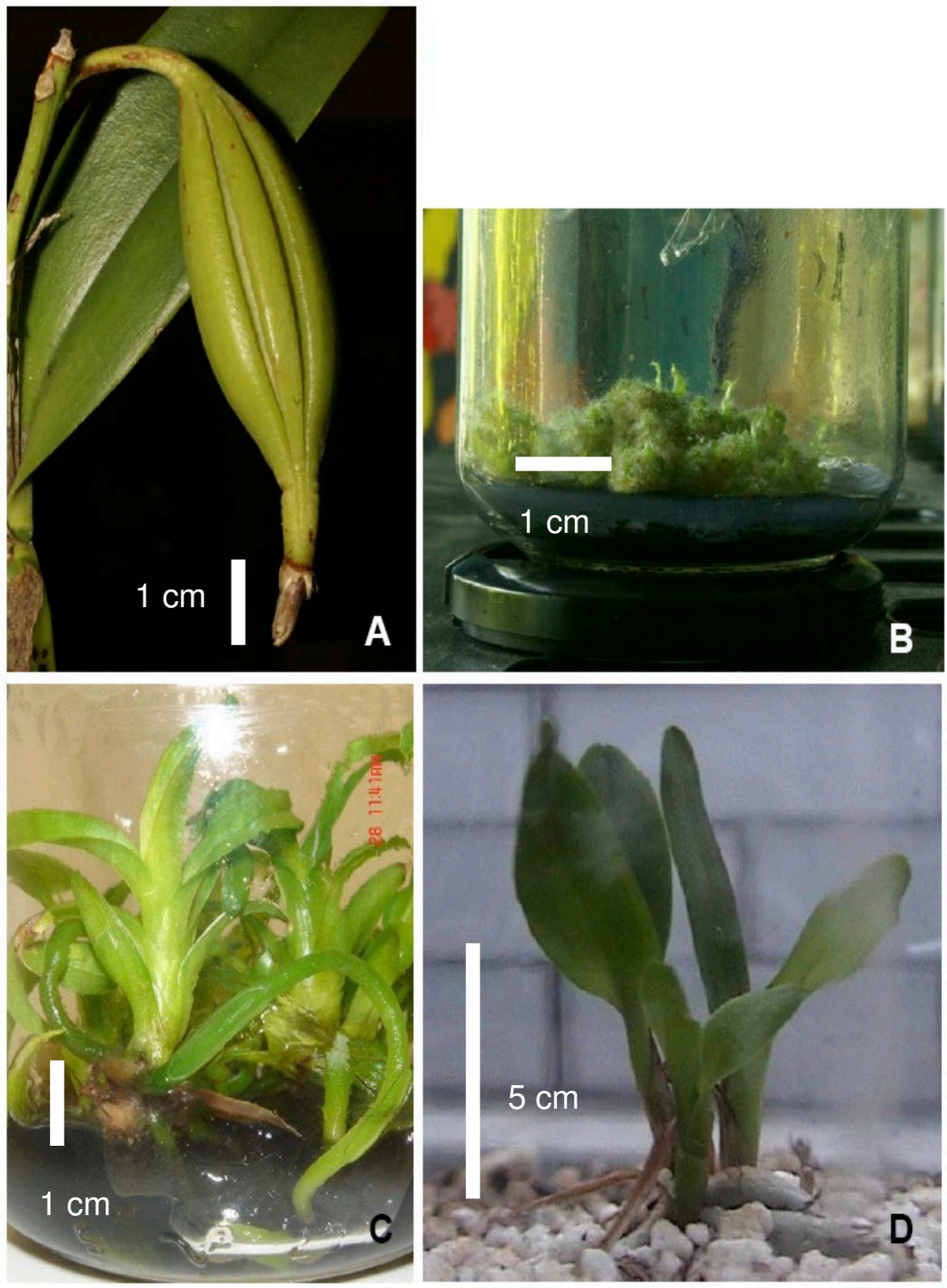


FIGURE 1. Micropropagation of *Guarianthe skinneri.* (a) Capsules, (b) germination and growth of protocorms, (c) protocorms differentiation and (d) plantlets.

FIGURA 1. Micropropagación de *Guarianthe skinneri.* (a) Cápsulas, (b) germinación y crecimiento de los protocormos, (c) diferenciación de los protocormos y (d) plántulas.

10.2 in treatment 3 (Table I). The most important factor for shoot proliferation was gibberellic acid and it explained 87% of the variation found, IAA 7% and BA and NAA 3% (Table II). A NAA concentration of 16.1 µM and 17.1 µM IAA in the MS medium resulted in a maximum number of shoots, but with a $GA_3$ concentration of only $6.3 \times 10^{-9}$ µM and 0.0023 µM BA. These concentrations induced 3.7 more shoots in *G. skinneri* plantlets when compared to the overall mean (Table III). The model derived from the experimental data explained 83% of the variability (Table IV).

The physiological effect of $GA_3$ and positive effect on plant growth is well-known as it increases the concentrations of soluble carbohydrates during seed germination and plant growth (Bialecka & Kepcznski 2007). $GA_3$ is therefore used in the cultivation of vegetables and fruits with plant tissue cultures (Isogai *et al*. 2008). $GA_3$ stimulated the germination of *Amaranthus caudatus* L. and the plantlets contained larger concentrations of glucose and maltose (Bialecka & Kepcznski 2007). However, the optimum concentrations required in this study were low compared to those reported in other studies. It might be that the used MS medium was different from the one used in other studies or the effect of $GA_3$ was transient and only a small 'pulse' was required to obtain the best results (Naor *et al*. 2008).

### Number of roots

The number of roots varied from 1.0 in treatment 8 to 3.7 in treatment 3 (Table I). The most important factor for root proliferation was $GA_3$ and it explained 45% of the variation, while IAA explained 30%, NAA 18% and BA 6% (Table II). A NAA concentration of 5.4 µM and IAA of 17.1 µM in the MS medium resulted in a maximum number of shoots, but with a $GA_3$ concentration of only 0.001 µM and $4.6\ 10^{-9}$ µM BA. These concentrations induced 0.6 more roots in *G. skinneri* plantlets when compared to the overall mean (Table III). The model derived from the experimental data explained 79% of the variability (Table III).

These results are important because a limited root formation is a major obstacle in micropropagation and conventional propagation, and conditions during *in vitro* rooting might have an important effect on performance after transfer *ex vitro* (De Klerk 2002). Contributions of $GA_3$ and IAA were the most important. Soon after the discovery of IAA its rhizogenic activity was reported (De Klerk *et al*. 1999). However, the complementary effect of IAA and $GA_3$ on root induction has not been reported yet. Hormonal action should be sequential because many researchers recognize that rooting is not a single process, but a developmental process consisting of distinct steps, each with its own requirements (De Klerk *et al*. 1999).

Table I. Orthogonal experimental design $L_9$ ($3^4$) done in triplicate to investigate the effect of different concentrations of gibberellic acid ($GA_3$), 6-bencyl adenine (BA), α-naftalenacetic acid (NAA) and indole acetic acid (IAA) on shoot and root number and length in plantlets of *Guarianthe skinneri* grown *in vitro*.

Tabla I. Diseño experimental ortogonal $L_9$ ($3^4$) realizado por triplicado para investigar el efecto de diferentes concentraciones de ácido giberélico ($GA_3$), 6-bencil adenina (BA), ácido α-naftalenacético (ANA) y ácido indol acético (AIA) sobre el número y longitud de los brotes y raíces en plántulas de *Guarianthe skinneri* cultivadas *in vitro*.

| | $GA_3$ | BA | NAA | IAA | Shoots | Roots | Shoot length | Root length |
|---|---|---|---|---|---|---|---|---|
| Treatment | (µmol) | | | | (per plant) | | (cm) | |
| 1 | 0 | 0 | 0 | 0 | 9.1 ba | 2.0 cb | 1.3 ba | 0.4 bc |
| 2 | 0 | 2.5 | 5.4 | 5.7 | 8.4 b | 2.2 b | 1.1 b | 0.5 b |
| 3 | 0 | 4.9 | 16.1 | 17.1 | 10.2 a | 3.7 a | 1.3 a | 0.7 ab |
| 4 | 2.9 | 0 | 5.4 | 17.1 | 7.7 bc | 3.7 a | 1.3 ba | 0.9 a |
| 5 | 2.9 | 2.5 | 16.1 | 0 | 6.4 dc | 1.9 cb | 0.9 c | 0.4 bc |
| 6 | 2.9 | 4.9 | 0 | 5.7 | 5.6 d | 1.5 cd | 0.9 c | 0.4 bc |
| 7 | 8.7 | 0 | 16.1 | 5.7 | 5.4 d | 1.1 d | 0.8 c | 0.2 c |
| 8 | 8.7 | 2.5 | 5.4 | 17.1 | 5.0 d | 1.0 d | 0.8 c | 0.2 c |
| 9 | 8.7 | 4.9 | 0 | 0 | 5.0 d | 1.4 d | 0.8 c | 0.2 c |
| LSD | | | | | 1.5 | 0.6 | 0.1 | 0.2 |

[a] Values with a different letter within columns are significantly different ($P<0.05$) / Valores con diferentes letras dentro de las columnas son diferentes significativamente ($P<0,05$).
[b] LSD: Least Significant Difference ($P<0.05$) / Diferencia mínima significativa ($P<0,05$).

Table II. ANOVA analysis using all factors with a significant (P<0.05) effect on shoot and root number and length in plantlets of *Guarianthe skinneri* grown *in vitro*.

Tabla II. Análisis ANOVA usando todos los factores con efecto significativo (P<0,05) sobre el número y longitud de los brotes y raíces en plántulas de *Guarianthe skinneri* cultivada *in vitro*.

| Factor | Sum of squares | DF | Coefficient of Variance | Percentage |
|---|---|---|---|---|
| Shoots number | | | | |
| Gibberellic acid ($GA_3$) | 26.07 | 2 | 13.04 | 86.86 |
| 6-bencyl adenine (BA) | 0.97 | 2 | 0.48 | 3.22 |
| A-naftalenacetic acid (NAA) | 0.82 | 2 | 0.41 | 2.73 |
| Indole acetic acid (IAA) | 2.16 | 2 | 1.08 | 7.19 |
| Total | 30.02 | 8 | | 100.0 |
| Roots number | | | | |
| Gibberellic acid ($GA_3$) | 3.78 | 2 | 1.89 | 44.87 |
| 6-bencyl adenine (BA) | 0.57 | 2 | 0.28 | 6.79 |
| A-naftalenacetic acid (NAA) | 1.51 | 2 | 0.76 | 17.98 |
| Indole acetic acid (IAA) | 2.55 | 2 | 1.27 | 30.36 |
| Total | 8.41 | 8 | | 100.00 |
| Shoot length | | | | |
| Gibberellic acid ($GA_3$) | 0.28 | 2 | 0.14 | 73.68 |
| 6-bencyl adenine (BA) | 0.04 | 2 | 0.02 | 10.53 |
| A-naftalenacetic acid (NAA) | 0.01 | 2 | 0.005 | 2.63 |
| Indole acetic acid (IAA) | 0.05 | 2 | 0.025 | 16.00 |
| Total | 0.38 | 8 | | 100.00 |
| Root length | | | | |
| Gibberellic acid ($GA_3$) | 0.24 | 2 | 0.12 | 55.81 |
| 6-bencyl adenine (BA) | 0.04 | 2 | 0.02 | 9.30 |
| A-naftalenacetic acid (NAA) | 0.05 | 2 | 0.03 | 11.63 |
| Indole acetic acid (IAA) | 0.10 | 2 | 0.05 | 23.26 |
| Total | 0.43 | 8 | | 100.00 |

Shoot length

Shoot length varied from 0.8 cm in treatment 7, 8 and 9 to 1.3 in treatments 3 and 4 (Table I). The most important factor for shoot growth was $GA_3$ and it explained 74% of the variation found, IAA 13%, BA 10% and NAA 3% (Table II). The overall mean shoot length was 1.0 cm and increased with 0.5 cm when grown in MS medium supplemented with $4.6 \times 10^{-9}$ µM $GA_3$, 0.0043 µM BA, $1.2 \times 10^{-5}$ µM NAA and 14.92 µM IAA (Table III). The model derived from the experimental data explained 82% of the variability (Table IV).

$GA_3$ is best known for its role in elongation of axial organs, such as stems, petioles and inflorescences (DeMason 2005). The effects of $GA_3$ on shoot elongation are well documented for several plants cultivated *in vitro,* e.g. *Cephaelis ipecacuanha* (Isogai *et al*. 2008) and *Acacia sinuate* (Vengadesan *et al*. 2002). The effects of $GA_3$ on shoot elongation are due to the increase in soluble carbohydrates induced by $GA_3$ and available for metabolic processes (Bialecka & Kepcznski 2007).

TABLE III. Optimal concentration and contribution of gibberellic acid ($GA_3$), 6-bencyl adenine (BA), α-naftalenacetic acid (NAA) and indole acetic acid (IAA) on shoot and root number and length in plantlets of *Guarianthe skinneri* grown *in vitro*.

TABLA III. Concentración optima y contribución del ácido giberélico ($GA_3$), 6-bencil adenina (BA), ácido α-naftalenacético (ANA) y ácido indolacético (AIA) sobre el número y longitud de los brotes y raíces en plántulas de *Guarianthe skinneri* cultivada *in vitro*.

| | SHOOTS NUMBER | | ROOTS NUMBER | | SHOOT LENGTH | | ROOT LENGTH | |
|---|---|---|---|---|---|---|---|---|
| FACTOR | concentration | contribution | concentration | contribution | concentration | contribution | concentration | contribution |
| | —μM— | | —μM— | | —μM— | | —μM— | |
| $GA_3$ | $6.3 \times 10^{-9}$ | 2.25 | 0.001 | 0.58 | $4.6 \times 10^{-9}$ | 0.22 | 0.031 | 0.12 |
| BA | 0.0023 | 0.42 | $4.6 \times 10^{-9}$ | 0.21 | 0.0043 | 0.08 | $7.0 \times 10^{-8}$ | 0.07 |
| NAA | 16.1 | 0.33 | 16.1 | 0.36 | $1.2 \times 10^{-5}$ | 0.05 | 16.1 | 0.09 |
| IAA | 17.1 | 0.65 | 17.1 | 0.75 | 14.9 | 0.10 | 17.0 | 0.15 |
| Total | | 10.6 | | 3.95 | | 1.47 | | 0.86 |
| Overall mean | | 6.99 | | 2.06 | | 1.03 | | 0.43 |
| Improvement | | 3.66 | | 1.89 | | 0.45 | | 0.43 |

TABLE IV. Regression models of responses and their significance for shoot number, root number, shoot length and root length in *Guarianthe skinneri* micropropagation ($P<0.05$).

TABLA IV. Modelos de regresión de las respuestas y su significancia para el número de brotes y de raíces y de la longitud de los brotes y raíces en la micropropagación de *Guarianthe skinneri* ($P<0,05$).

| FACTOR | MODEL | $R^2$ |
|---|---|---|
| Shoot number | shoot number = 8.09 - 0.40*$GA_3$ – 0.09*BA + 0.03*NAA + 0.06*IAA | 82.94 |
| Root number | root number = 1.87 - 0.21*$GA_3$ - 0.07*BA + 0.02*NAA + 0.09*IAA | 78.78 |
| Shoot length | shoot length = 1.21 - 0.05*$GA_3$ - 0.03*BA - 0.0008*NAA + 0.009*IAA | 82.39 |
| Root length | root length = 0.48 - 0.04*GA3 - 0.014*BA + 0.004*NAA + 0.016*IAA | 74.04 |

$R^2$: correlation coefficient, gibberellic acid ($GA_3$), 6-bencyl adenine (BA), α-naftalenacetic acid (NAA) and indole acetic acid (IAA) / coeficiente de correlación, Ac. Giberélico ($GA_3$), 6-bencil adenina (BA), Ac. α-naftalenacético (NAA) y Ac. Indolacético (IAA).

Root length

Root length varied from 0.2 cm in treatment 7, 8 and 9 to 0.9 in treatment 4 (Table I). The most important factor for root growth was $GA_3$ and it explained 55% of the variation found, IAA 24%, BA 8% and NAA 13% (Table II). The overall mean root length was 0.4 cm and increased with 0.4 cm when grown in MS medium supplemented with 0.031µM $GA_3$, 7.0 x $10^{-8}$ µM BA, 16.1 µM NAA and 17.02 µM IAA (Table III). The model derived from experimental data explained 74% of the variability (Table IV).

These results are due to gibberellic acid ($GA_3$) is a growth factor that promotes elongation, due to the increase in the rate of cell division by promoting the growth and number of shoots, since its action is specific in active growth areas as protocorms and root apices (Pierik 1990). Alvarado (2000) was reported to multiply apices of lateral shoots that developed *G. skinneri* protocorms. As in the present investigation, the response was favorable for *G. skinneri* micropropagation, because these growth regulators at low concentrations act as promoters and inducers of cell division. Although the use of $GA_3$ in protocorms from orchids is relatively scarce, the auxins and cytokinins have been widely used in species such as *Barkeria obovata, Catasetum intergerrimun, Cattleya x Esbetts, Epidendrum veroscriptum, Cuitlauzina pendula, Dendrobium sp. Anceps Laelia, Lycaste skinneri, Mormodes tuxtlensis, Oncidium tigrinum Oncidium sp.* and *Stanhopea tigrina.* The basal medium used for *in vitro* cultivation was MS (Murashige & Skoog, 1962) added with vitamins and growth regulators, in order to induce the formation of shoots and increase the number of roots. Each species responds differently, so they have developed 3 to 26 shoots, the above depending on the interaction of type and level of growth regulator, using IAA (1 mg / l) and NAA (0 mg / l) BA (0.5 mg / l) and 2,4-D (0.5 mg / l), BA (0, 0.5, 2, 3 and 5 mg / l) and NAA (0, 0.1, 0.5 mg / l) (Hernández *et al*. 2001, Salazar 2003, Baltazar 2004, Tinoco 2006, Askar *et al*. 2007, Kalimuthu *et al*. 2007, Maza 2008).

## CONCLUSION

It was found that $GA_3$ was the most important factor for shoot and root induction and elongation. However, the optimal concentrations were small and larger concentrations had a negative effect on plant development. The results of this study present new evidence regarding the effect of $GA_3$ to micropropagate *G. skinneri* through protocorms and this protocol could be useful for other orchids.

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