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TRABAJOS ORIGINALES

# Effects of photoperiod, plant growth regulators and culture media on *in vitro* growth of seedlings of *Cyrtochilum loxense* (Lindl.) Kraenzl. an endemic and endangered orchid from Ecuador

## Efectos del fotoperíodo, reguladores de crecimiento vegetal y medio de cultivo en el crecimiento *in vitro* de plántulas de *Cyrtochilum loxense* (Lindl.) Kraenzl. una orquídea endémica del Ecuador

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### Abstract

*Cyrtochilum loxense* (Lindl.) Kraenzl. is an endemic and seriously endangered orchid species endemic in the Loja Province (Southern Ecuador). The main goals of this research were to analyze how culture media, plant growth regulators and photoperiod affect the growth of *C. loxense.* Eight month old plants (approximate 1 – 1.5 cm in height) obtained by *in vitro* germination, were cultivated on MS media or Knudson C; MS with three levels of naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP) (2/0.5; 1/0.5 y 0.5/ 0.5 $mg^{-1}L$); and three photoperiodic regimes (24/0, 16/8, 8/16 h) on MS with and without plant growth regulators. No significant differences of shoot induction were observed on media with or without plant growth regulators, and all tested photoperiods. The highest growth (1.2 cm) was observed in plantlets cultivated on growth regulator-free media with a 16/8 photoperiod. Also the shoot and root formation was better in this species in absence of plant growth regulators. Probably this response is due to the endogenous hormone levels in the tissues or due to the kind and concentrations of PGRs used were too low to induce positive morphogenetic responses.

**Palabras clave:** culture media; photoperiod; plant hormones; endemic plant; Orchidaceae.

### Resumen

*Cyrtochilum loxense* (Lindl.) Kraenzl. es una orquídea endémica y críticamente amenazada, distribuida en la provincia de Loja (Sur del Ecuador). Los objetivos principales de esta investigación fueron analizar la influencia del medio de cultivo, reguladores de crecimiento vegetal y fotoperíodo en el crecimiento de *C. loxense.* Plantas de ocho meses (altura aproximada de 1 – 1.5 cm.) obtenidas por germinación *in vitro*, fueron cultivadas en medio MS y Knudson C; medio MS con tres niveles de ácido naftalen acético (ANA) y 6-bencil amino purina (BAP) (2/0.5; 1/0.5 y 0.5/ 0.5 $mg^{-1}L$); y tres fotoperíodos (24/0, 16/8, 8/16 h) en MS con o sin reguladores de crecimiento vegetal (RCV). No se observaron diferencias significativas en la inducción de brotes en medio con o sin RCV, entre todos los fotoperíodos testados. El tamaño más grande (1.2 cm) se registró en plántulas cultivadas en medio libre de reguladores de crecimiento vegetal, incubadas en un fotoperíodo de 16/8. Además, la mayor brotación y enraizamiento se observó para esta especie en medio libre de reguladores de crecimiento vegetal. Probablemente esta respuesta es debida a los niveles endógenos de hormonas vegetales en el tejido o el nivel de RCV usado fue muy bajo para inducir alguna respuesta morfogénica.

**Palabras clave:** Medio de cultivo; fotoperíodo; hormonas vegetales; plantas endémicas; Orchidaceae.

**Información sobre los autores:**

Los autores declaran:

A.C. y Y.G. diseñaron los experimentos, analizaron resultados y escribieron el artículo. Y. G. desarrolló los experimentos.

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## Introduction

Orchidaceae is the largest of all vascular plant families present in Ecuador with nearly 4000 species 40% of which are endemic (Jørgensen & León-Yánez 1999). Approximately 116 species distributed from Mexico to Bolivia belong to the genus *Cyrtochilum* and sixty-five of these are present in Ecuador (Dodson & Escobar 2005). *Cyrtochilum loxense* (Lindl.) Kraenzl. is an endemic species distributed in the high Andean forest of Southern Ecuador, near to Podocarpus National Park (Loja Province) (Endara et al. 2000). At present, due to high rate of deforestation, forest fires and habitat loss, this species is critically endangered (Dodson & Escobar 2005).

Cueva and González (2009) have previously determined the best conditions for germination of *C. loxense*. This study describes the effects of other parameters such as photoperiod, plant growth regulators as well as culture media to obtain optimal *in vitro* growth of this endangered orchid.

## Material and methods

Eight-month old plants (approximately 1.5 cm in height), obtained through *in vitro* germination (Cueva & González 2009) were used for the evaluation of culture media, photoperiod and plant growth regulators treatments.

**Culture media.-** Murashige and Skoog (1962) (MS) and Kundson C (1946) media were tested to evaluate the nutrient influence on growth, shooting and rooting.

**Plant Growth Regulators treatments.-** Effects of plant growth regulators on growth, shooting and rooting were observed in plants cultivated by 60 days on MS with three combinations of α-naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP) (2 – 0.5, 1 – 0.5, and 0.5 – 0.5 $mgL^{-1}$, respectively). All culture media were supplemented with 20 $g^{-1}$ sucrose; the pH was adjusted to 5.8 with 1N KOH or HCl before adding 7 $g^{-1}$ agar (Bacto™ Agar 214010) and autoclaving at 121 ºC for 20 min.

**Photoperiod.-** To determine the influence of photoperiod on the growth, shoot and root formation of *C. loxense,* day lengths of 8, 16 and 24 h, provided by 40W cool white fluorescent lamps (57 µmol $m^{-2}$ $s^{-1}$) were tested. Plants were cultivated on MS medium augmented with 2 $mgL^{-1}$ glycine; 100 $mgL^{-1}$ myo-inositol; 0.05 $mgL^{-1}$ nicotinic acid and 0.05 100 $mgL^{-1}$ pyridoxine; during 60 days, and they were incubated in growth chambers at 22 ± 1 ºC.

Ten replicas (with ten plants per flask) per PGR and culture media treatment and five replicas for photoperiod were cultured. The growth (growth from the initial plant size, in cm), percentage of individuals forming new lateral shoots, shoot number, percentage of individuals forming roots and root number were register in seedling of *C. loxense* in 30 days intervals. All data were submitted of two-way variance analysis. Culture media data were submitted to Student`s t test, with an alpha value of 0.05. Bonferroni's test was used to detect differences ($p \leq 0.05$) among plant growth regulators and photoperiod treatments.

## Results and discussion

**Culture media.-** No significant differences in growth of *Cyrtochilum loxense* were observed using the culture medium MS and Knudson C (Table 1). These results are in contrast with those recorded for other orchid species, like *Catasetum fimbriatum* and *Cyrtopodium paranaensis,* in which MS medium stimulated a better growth with respect to Knudson C medium after six months of culture (Rego Oliveira & Tadeo de Faria 2005). Since sucrose is a crucial component of culture medium, especially so for orchids (Pierik 1990), the same sucrose concentration in MS and Knudson C (2%), might explain the growth results.

The highest percentage of shoot formation after 60 days of culture (23%) was obtained using Knudson C medium as compared with 7% of shoot formation on MS (Figs. 1a, b). Shoot number was similar in MS and Knudson C. The highest percentage of root formation (82%) and the highest number of roots per explants (1.07) were obtained on Knudson C medium. In this medium, roots were longer and thicker than those formed on MS (Figs 2a, b). Contrarily, in *C. fimbriatum* y *C. paranaensis* these two culture media did not induce significant differences on root number and structure. It seems that the development in *C. loxense* could be stimulated by the presence of some nutrients available in Knudson C medium (George 1993b), a hypothesis that is in agreement with results reported for *Epidendrum* and *Laeliocattleya* (Churchill et al. 1972).

**Plant growth regulators treatments.-** None of the plant growth regulators evaluated caused significant differences in

**Table 1.** Effect of culture media Murashige and Skoog (MS). and Knudson C (KC) on growth, shooting and rooting of *Cyrtochilum loxense.*

| Time | Morphogenic Responses | Culture Media | | P-value |
|---|---|---|---|---|
| | | MS | KC | |
| 30 days | Growth (cm.) | 0.38 ± 0.02 | 0.41 ± 0.04 | 0.39 |
| | Shoot formation (%) | 0 | 7 ± 3 | |
| | Shoot number | 0 | 0.19 ± 0.14 | |
| | Root formation (%) | 27 ± 4 | 39 ± 5 | 0.64 |
| | Root number | 0.27 ± 0.04 | 0.41 ± 0.06 | 0.44 |
| 60 days | Growth (cm.) | 0.85 ± 0.04 | 0.77 ± 0.05 | 0.26 |
| | Shoot formation (%) | 7 ± 2.1 | 23 ± 4.4 | **0.007** |
| | Shoot number | 0.27 ± 0.1 | 0.85 ± 0.4 | 0.23 |
| | Root formation (%) | 63 ± 4.9 | 82 ± 4.8 | **0.007** |
| | Root number | 0.79 ± 0.06 | 1.07 ± 0.06 | **0.008** |

Data are present as mean ± standard error. Data were compared with two-sided Student`s *t* test with an alpha value = 0.05 and equal variance assumed

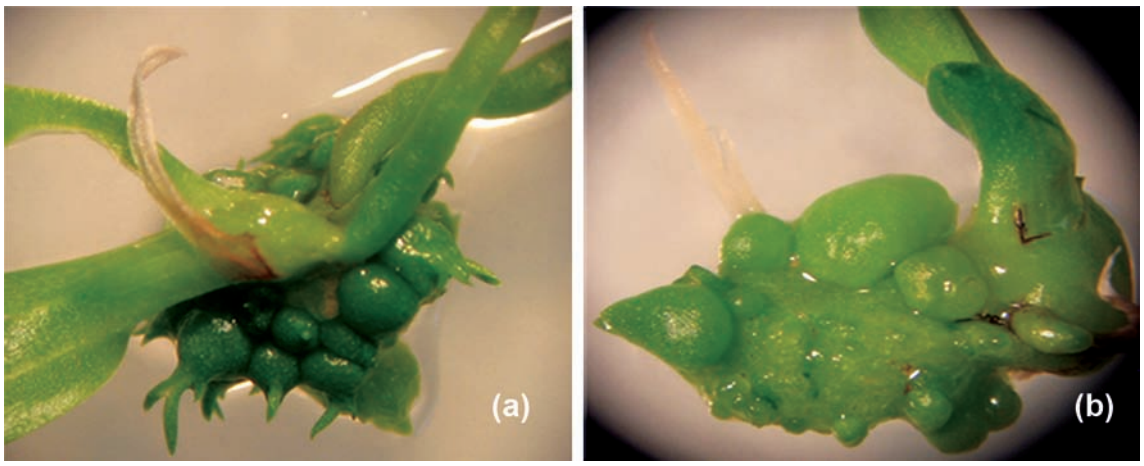


**Figure 1.** Protocorm like body formation of *Cyrtochilum loxense* at 60 days. (a) Kundson C medium. (b) MS medium.

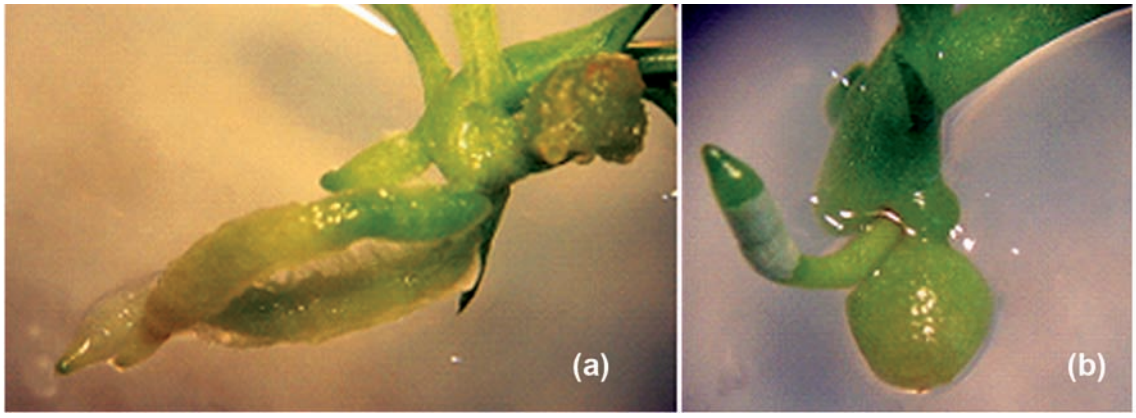


**Figure 2.** Rooting of *Cyrtochilum loxense* plants at 60 days: (a) Knudson C; (b) MS (1962) media.

growth and shoot formation. After 60 days, plant growth ranged between 0.75 – 0.86 cm (Table 2). The highest percentage of shoots (14%) and number of shoots per explants (0.57) were obtained on medium with 0.5 $mg^{-1}L$ BAP and 1 $mg^{-1}L$ of NAA (Table 2). The only statistical difference was observed on percentage of root formation (63%) and number of roots per explant (0.79) both on media without hormones. Krapiec et al. (2003) found the highest shoot induction in *Cattleya walkeriana*, on medium with the same plant growth regulators. Thus these results in the case of *C. loxense* could be attributed to the endogenous hormone concentration, since there are species for which external addition of plant growth regulators is not required (George 1993a).

**Photoperiod.-** To evaluate the effect of the photoperiod on growth, we inoculated explants in MS medium alone and added with PGRs. The growth responses observed on MS without hormones were higher than those on media with plant growth regulators (Table 3). At day 45 the highest growth response was achieved under photoperiod 16/8 h. It is well known how light affects plant development, especially in *in vitro* culture conditions when plants are exposed to continuous light, a condition that can produce significant growth (Clouse 2001; Kim et al. 2002, Nemhauser & Chory 2002), for *C. loxense* at 60 days all plant growth regulators combinations present the highest growth under photoperiod 24/0 h (Table 3, Fig. 3). These results are similar to the results observed for *Psygmorchis pusilla,* for which the highest growth rate was observed with a photoperiod of 22/2 h (Vaz et al. 2004).

Summarizing we can conclude that Knudson C is a good medium for growing *C. loxense in vitro*, moreover it stimulates

**Table 2.** Effect of plant growth regulators on in vitro growth, shooting and rooting of Cyrtochilum loxense after 60 days.

| Plant Growth Regulators $mgL^{-1}$ | | Growth (cm) | Shooting | | Rooting ($p < 0.0001$) | |
|---|---|---|---|---|---|---|
| BAP | NAA | | Shoot formation (%) ($p < 0.074$) | Shoot number/ explant ($p < 0.412$) | Root formation (%) | Root number/ explant |
| 0.5 | 2 | 0.796a | 4 | 0.37a | 16b | 0.17b |
| 0.5 | 1 | 0.751a | 14 | 0.57a | 18b | 0.2b |
| 0.5 | 0,5 | 0.860a | 9 | 0.21a | 27b | 0.29b |
| 0 | 0 | 0.847a | 7 | 0.27a | 63a | 0.79a |

Growth, shoot number, rooting percentage and root number were analyzed by Duncan test; the data within the same column following by the same letter are not significantly different. Shoot formation data were analyzed using the Kruskall Wallis test.

**Table 3.** Effect of plant growth regulators and photoperiod on *in vitro* growth in *Cyrtochilum loxense.*

| Plant Growth Regulators $mg^{-1}L$ | | Photoperiod Light/Dark | Growth (cm) | |
|---|---|---|---|---|
| BAP | ANA | | 45 days | 60 days |
| 0.5 | 2 | 24/0 | 0.6 cd | 0.7 |
| 0.5 | 2 | 16/8 | 0.5 d | 0.6 |
| 0.5 | 2 | 8/16 | 0.6 cd | 0.6 |
| 0.5 | 1 | 24/0 | 0.6 bcd | 0.8 |
| 0.5 | 1 | 16/8 | 0.6 cd | 0.6 |
| 0.5 | 1 | 8/16 | 0.6 cd | 0.7 |
| 0.5 | 0.5 | 24/0 | 0.7 abcd | 0.8 |
| 0.5 | 0.5 | 16/8 | 0.6cd | 0.7 |
| 0.5 | 0.5 | 8/16 | 0.5 cd | 0.6 |
| 0 | 0 | 24/0 | 0.9 ab | 1.2 |
| 0 | 0 | 16/8 | **1 a** | 1.2 |
| 0 | 0 | 8/16 | 0.8 abcd | 1 |

Means in the same column followed by the same letter are not significant different (Duncan p< 0.0001).

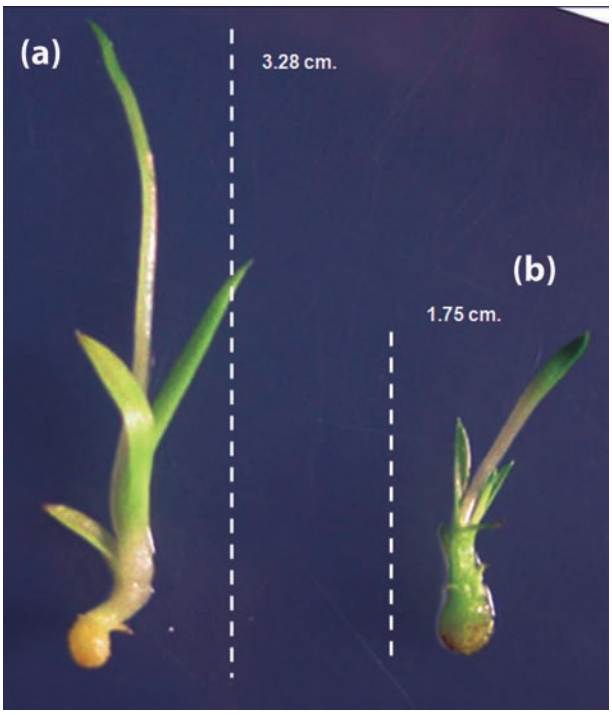


**Figure 3.** High of *Cyrtochilum loxense* growing in MS with PGR at 60 days. (a) 24/0 light/darkness; (b) 8/16 light/darkness.

good shooting and rooting after 60 days. However given that these three experiments were performed simultaneously, we evaluated only MS medium to verify the influence of PGRs and photoperiod. The use of 0.5 $mgL^{-1}$ of BAP and 1 $mgL^{-1}$ of NAA on MS medium improve shooting, on the other hand the best medium for rooting was MS medium without hormones. The best photoperiod for growing was 16/8 h, when MS without PGRs was used.

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