Scientific Article

In vitro development of endangered *Laelia marginata* Lindl. in growth media containing different nitrate/ammonium ratios[1]

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ABSTRACT

In vitro cultivation is a very important method for the conservation of germplasm and the multiplication of endangered plants; however, studies on the adequacy of the nutrients used for a good development of each species are needed. The objective of this study was to evaluate the *in vitro* development of *Laelia marginata* Lindl. in culture media containing different nitrate/ammonium ratios. Seedlings were obtained from seeds germinated *in vitro*. The treatments consisted of different nitrate/ammonium ratios, with five proportions of NO_3^- and NH_4^+: T1-0/100; T2-25/75; T3-50/50; T4-75/25 and T5-100/0. At 200 days after seedling transplanting, height of the aerial part, root length, number of leaves, number of shoots, leaf length, leaf width, aerial and root dry mass and contents of chlorophyll *a*, *b* and carotenoids were evaluated. A completely randomized design was used, with five treatments and ten replicates. The combination of 50/50 nitrate/ammonium resulted in the highest values of aerial part and root length, dry mass of the aerial part and root, as well as leaf length and width. The proportion of 50/50 nitrate/ammonium resulted in the best initial development of *L. marginata* seedlings.

Keywords: nitrogen sources, micropropagation, Orchidaceae

RESUMO

Desenvolvimento in vitro da orquídea ameaça de extinção *Laelia marginata* Lindl. em meios de cultivo contendo diferentes relações nitrato/amônio.

O cultivo *in vitro* é um método de exímia importância na conservação de germoplasma e na multiplicação de plantas ameaçadas de extinção, porém necessita de estudos sobre a adequação dos nutrientes utilizados para um bom desenvolvimento de cada espécie. O objetivo foi avaliar o desenvolvimento *in vitro* de *Laelia marginata* Lindl. em meios de cultura contendo diferentes relações nitrato/amônio. As plântulas foram obtidas a partir de sementes germinadas *in vitro*. Os tratamentos consistiram em diferentes relações nitrato/amônio, sendo cinco proporções NO_3^- e NH_4^+: T1-0/100; T2-25/75; T3-50/50; T4-75/25 e T5-100/0. Aos 200 dias após o transplante das plântulas, foram avaliados altura da parte aérea, comprimento de raiz, número de folhas, número de brotos, comprimento da folha, largura da folha, massa seca da parte aérea e de raiz, teor de clorofila *a*, *b* e carotenoides. Utilizou-se delineamento inteiramente casualizado, sendo cinco tratamentos e dez repetições. A combinação de 50/50 nitrato/amônio resultou nos maiores valores de comprimento de parte aérea e raiz, massa seca de parte aérea e raiz, bem como comprimento e largura da folha. A proporção de 50/50 de nitrato/amônio resultou no melhor desenvolvimento inicial das plântulas de *L. marginata.*

Palavras-chave: fontes de nitrogênio, micropropagação, Orchidaceae.

1. INTRODUCTION

The Orchidaceae family presents species with different growth habits, presenting terrestrial, epiphytic, rupicolous, scandent or even saprophytic species, which allows a cosmopolitan distribution, despite being more abundant and diversified in tropical forests (PINHEIRO et al., 2004).

The different species of orchids present some adaptations that allow them to occupy specific environments, with specializations resulting in restricted distribution, raising the degree of endemism, consequently (PRITCHARD, 1989).

Among the different representatives of the Orchidaceae family, *Schomburgkia* genus contains approximately 18 species, most of them epiphytes, found from Tropical America to West India (JONES, 1973). Amid the different species belonging to this genus, *Laelia marginata* Lindl. is commonly found in gallery forest and dry forest of the

DOI: http://dx.doi.org/10.14295/oh.v23i4.1019

[1] Received in 03/03/2017 and accepted in 17/07/2017

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Cerrado (MENDONÇA et al., 1998), and according to CNCflora (2017) this species is at risk of extinction mainly due to the illegal forest depleting.

The production of seedlings with genetic quality and conservation by means of *in vitro* propagation (STANCATO et al., 2001; MARTINI et al., 2001) have been the primary method used within the different ways of species preservation.

According to Sorace et al. (2008) *in vitro* cultivation is regarded a rapid and practical means of vegetative propagation in floriculture, since it allows the obtaining of a large number of plants with varietal authenticity at any time of the year. However, the formulation of the culture medium used directly reflects on the success or failure of this technique.

The formulation of the culture medium is extremely important for seed germination and plant growth, usually consisting of macronutrients, micronutrients, vitamins, amino acids, sucrose and gelling agent (SORACE et al., 2008).

Nitrogen stand out among the several macronutrients used in the formulation of the culture medium since it is one of the primary essential and active nutrients, formative of several essential biomolecules, such as amino acids, nucleic acids, proteins, enzymes and others, being absorbed in the form of nitrate (NO_3^-) and ammonium (NH_4^+) (VILLA et al., 2009). However, the beneficial effect of the different forms of nitrogen (NO_3^- and NH_4^+) is not well understood, since different formulations and the concentration of nitrogen in the culture medium exert a great influence on growth rate, morphology and cellular totipotency (KIRBY et al., 1987).

Ammonium nitrate, the nitrate and ammoniacal source, is the main form of nitrogen supply in *in vitro* cultivations, resulting in good growth rates in many species, representing the best form of nitrogen for most crops. However, there are species that do not display good growth in the presence of nitrate, such as rice calli (CALDAS et al., 1998).

Studies related to the substitution of ammonium nitrate by other sources of nitrogen in the in vitro cultivation of orchids are scarce in the literature. S studies use urea as a source. According to Araújo et al. (2009), studies that evaluate the possibility of substitution of ammonium nitrate are of great importance, since besides having a high cost, this source of nitrogen has its commercialization controlled by the army.

The objective of this work was to evaluate the in vitro growth of *Laelia marginata* Lindl. in culture media with different nitrate/ammonium ratios.

2. MATERIAL AND METHODS

Seedlings of *Laelia marginata* Lindl. were obtained from seeds germinated *in vitro* in MS growth medium (MURASHIGE and SKOOG, 1962), with half of the concentration of macronutrients. The seeds were obtained from mature capsules obtained by self-pollination of plants grown in a greenhouse located at 23º19’42’’ S, 51º12’11”W latitudes and 594 m above sea level.

Sixty days after sowing, seedlings at protocorm stage were under cultivated in the same MS medium with half of the macronutrient concentration and the nitrogen supply was changed with five different nitrate/ammonium ratios. The treatments consisted of different nitrate/ammonium ratios, with five proportions of NO_3^- and NH_4^+: T1-0/100; T2-25/75; T3-50/50; T4-75/25 and T5-100/0 (Table 1).

Table 1. Salts used for the preparation of culture media with different Nitrate/Ammonium ratios and final concentrations of the elements in each medium.

Salts used (mM)	**Nitrate/Ammonium ratios**				
	0/100	25/75	50/50	75/25	100/0
KH_2PO_4	0.485	0.485	0.485	0.485	0.625
KNO_3	0.000	7.500	9.515	9.515	9.375
KCl	9.515	2.015	0.000	0.000	0.000
$Ca(NO_3)_2$	0.000	0.000	1.500	1.500	1.500
$CaCl_2$	1.500	1.500	0.000	0.000	0.000
$MgSO_4$	0.750	0.750	0.750	0.750	0.750
NH_4Cl	29.860	22.360	14.860	7.360	0.000
$NH_4H_2PO_4$	0.140	0.140	0.140	0.140	0.000
$NaNO_3$	0.000	0.000	2.485	9.985	17.625
NaCl	0.870	0.870	0.870	0.870	0.000
Final concentrations if medium (mM)	**Balance of culture media in ratios Nitrate/Ammonium**				
	0/100	25/75	50/50	75/25	100/0
Nitrate (NO_3^-)	0.0	7.5	15.0	22.5	30.0
Ammonium (NH_4^+)	30.0	22.5	15.0	7.5	0.0
N	30.000	30.000	30.000	30.000	30.000
P	0.625	0.625	0.625	0.625	0.625
K	10.000	10.000	10.000	10.000	10.000
Ca	1.500	1.500	1.500	1.500	1.500
Mg	0.750	0.750	0.750	0.750	0.750
S	0.750	0.750	0.750	0.750	0.750
Cl	43.250	28.245	15.730	8.230	0.000
Na	0.870	0.870	3.355	10.855	17.625

The media base consisted of 30 g L^{-1} of sucrose, 1 g L^{-1} of activated carbon and 7.5 g L^{-1} of agar, pH adjusted to 5.8 (± 0.2) before addition of agar. Glass flasks of 250 ml were filled with 50 ml of the culture medium and were properly autoclaved at 120°C and pressure of 1.05 kg cm^{-2} for 20 minutes. After transplanting the seedlings into the culture medium, the flasks were kept in a growth room at 25 °C (± 2 °C) under a 16-hour of light photoperiod.

At 200 days after seedling transplanting, the following phytometric characteristics were evaluated: a) height of the aerial part: it was measured from the plant neck to the upper end of the largest leaf; b) root length: it was measured from the neck of the plant to the end of the largest root; c) number of leaves: leaf counting per plant; d) number of shoots; e) leaf length: it was measured from the largest leaf, considering from the leaf insertion to its extremity; f) leaf width: it was measured on the largest leaf with the aid of a digital caliper; g) dry mass of the aerial part; h) root dry mass; (the dry mass was measured after drying in a forced ventilation oven at 60 ºC); i) content of chlorophyll *a*, *b* and carotenoids following the methodology described by Meschede et al. (2011) where 0.2 g samples of fresh leaf tissue were placed in capped tubes containing 10 mL of 100% (v/v) acetone. The extracts were filtered and the readings were carried out in spectrophotometer at 663, 645 and 434 nm wavelengths for chlorophyll *a*, *b* and carotenoids, respectively. Determinations of chlorophyll levels (mg gfw^{-1}) were based on the equations described by Whitham et al. (1971): Chlorophyll *a* = (11.24 x A663 - 2.04 x A645), Chlorophyll *b* = (20.13 x A645 - 4.19 x A663) and Carotenoids = (1000 x A434 - 63.14 Chlorophyll *b*) / 214, where A is the absorbance at the indicated wavelength.

The experimental design was a completely randomized one, with five treatments consisted of ten replicates. Each replicate consisted of five seedlings. The analysis of variance was conducted by applying the F test, when significant, it was submitted to the Tukey averages comparison test at 5% of significance. Pearson's correlation was done between the variables and the analysis of main components, using software R (R, 2012).

3. RESULTS AND DISCUSSION

After data analyses, it was observed that the length of the aerial part was 20.75% lower when only ammonium was used as a nitrogen source, when compared with a combination of 50/50 and 75/25 nitrate/ammonium, which presented the highest values (Table 2, Figure 1)

Root length showed a reduction by 43.5% when only ammonium was used as N source, with the combination with 50/50 nitrate/ammonium resulting in the highest values (Table 2).

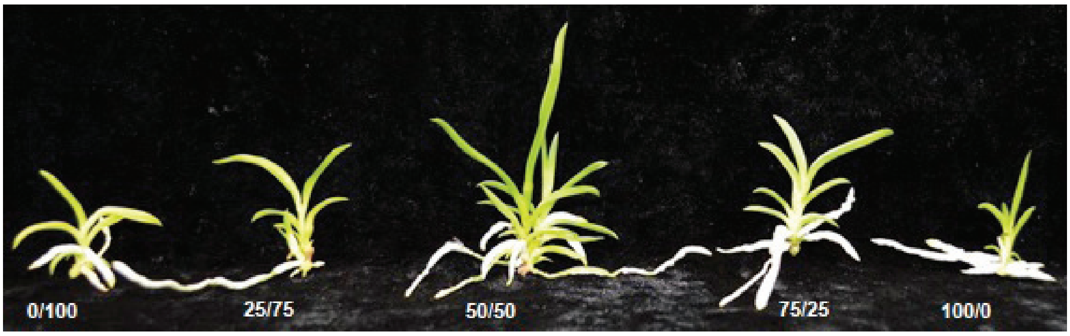

Figure 1. Effect of the combination of 0/100, 25/75, 50/50, 75/25 and 100/0 nitrate/ammonium.

Table 2. Shoot length (CPA), root length (CR), number of leaves (NF), number of buds (NB), leaf length (CF), leaf width (LF), aerial dry mass (MSPA) and root dry mass (MSR), seedlings of *Laelia marginata*, grown in vitro, with different combinations of nitrate/ammonia.

NO_3^-/NH_4^+:	CPA (mm)	CR (mm)	NF	NB	CF (mm)	LF (mm)	MSPA (g)	MSR (g)
0/100	31.10 c	25.30 b	8.70 b	0.30^{ns}	14.50 c	2.76 d	0.015 cd	0.018 bc
25/75	49.20 b	26.10 b	10.90 a	0.60	30.50 b	4.20 b	0.024 b	0.020 b
50/50	67.10 a	44.00 a	7.70 bc	0.20	41.20 a	5.35 a	0.035 a	0.033 a
75/25	63.50 a	29.20 b	6.40 cd	0.00	43.30 a	4.97 a	0.019 bc	0.021 b
100/0	41.70 b	17.80 c	5.40 d	0.00	25.10 b	3.48 c	0.012 d	0.015 c
CV (%)	17.16	17.64	17.46	21.34	22.52	12.74	27.03	19.47

^{ns} not significant, means followed by the same letter in the column did not differ statistically by the Tukey test at 5% probability.

Lewis et al. (1989) observed a greater reduction in the root system of corn and wheat plants grown exclusively with ammonium, resulting in a reduction in the root/aerial part ratio of these plants, and this process is attributed to the fact that carbohydrates translocated from the leaves to the roots are used as carbon skeletons and energy for the ammonium assimilation process in order to avoid their accumulation at toxic levels, and not for the processes associated to root growth, which justifies the reduction in the root length observed in the treatment with ammonium only.

The number of leaves presented the highest values in the 25/75 nitrate/ammonium treatment, but when only nitrate was used, a reduction of 51.45% was observed in the number of leaves. However, for number of shoots, no significant difference was found between treatments (Table 2).

An opposite result was observed by Cruz et al. (2006), who obtained a reduction of 10% in the number of leaves and of 15% in the leaf area of cassava plants supplied only with nitrate, while ammonium provided reductions of 21% and 31%, respectively.

The width and length of the leaf presented the highest values in the 50/50 and 75/25 nitrate/ammonium treatments. On the other hand, the use of nitrate alone resulted in reduction in the order of 48.41% and 66.51% in the width and length of the leaf (Table 2). This result is likely to be related with to the toxic effect of the presence of excess of ammonium in the tissues of the plant. A balance between nitrate and ammonium provides a better use of the nitrogen by the plant.

The dry mass of root and the aerial part showed the highest values with the 50/50 nitrate/ammonium combination. On the other hand, the use of nitrate only as a source of N caused a decrease of 54.54% and 65.71 % (Table 2).

According to Bernardi et al. (2004), balanced doses of nitric and ammoniacal nitrogen promotes the accumulation of fresh and dry matter and increases in the height of seedlings, with emphasis on the development of the root system. Powell et al. (1988) observed in terrestrial orchids of the genus *Cymbidium* that similar doses of nitrate and ammonium had a positive effect on the variables previously mentioned, especially in relation to the development of the root system. These results corroborate with those observed in this study, and the balance between nitric and ammoniacal sources promoted the accumulation of dry mass.

Araujo et al. (2009) state that the combination of ammonium and nitrate stimulate the *in vitro* growth of several species of plants, and the ratio between these two sources of nitrogen seems to be the determining factor in the growth stimulus of plants.

The lower development of plants in the presence of ammonium nitrate alone may be related to the fact that ammonium is assimilated faster than nitrate, because due to its toxicity, as soon as ammonium is absorbed, it is reduced in amino acids. On the other hand, nitrate needs to be reduced to nitrite, and this to ammonium, so, after, it is assimilated, requiring energy for the occurrence of this process (TAIZ and ZEIGER, 2013). Therefore, when the ammonium concentration is increased by the addition of nitrate, the plant spends less energy to assimilate the N, which is directed to the formation of new tissues, consequently increasing the length.

However, according to Barker and Mills (1980), although nitrate assimilation requires a high energy demand, plant growth is higher when it is supplied with nitrate rather than with ammonium. It is believed that the need for detoxification of the plant due to excess of ammonium absorbed may abolish its advantage in terms of energy cost (GUO et al., 2002).

When nitrate availability is reduced, its assimilation occurs mainly in the root, however when nitrate availability increases, most of the absorbed nitrate is translocated to the aerial part, where it is assimilated (TAIZ and ZEIGER, 2013). It is estimated that nitrate reduction and assimilation may consume up to 25% of the processes associated with photosynthesis and electron transport, which occurs at the mitochondria level (BLOOM et al., 1989).

Because ammonium is more toxic to plant cells than nitrate, which can be accumulated in cells or translocated through tissues without damaging the cell, high levels of ammonia may dissipate the proton gradient needed to transport the electrons in photosynthesis and transmembrane respiration, as well as for the capture of metabolites in the vacuoles (TAIZ and ZEIGER, 2013).

Therefore, the knowledge of the energy cost for nitrate assimilation and the toxic effects of ammonium is needed since it is believed that a balance between the two N sources is more advantageous for plant growth than the use of only one of the sources.

In relation to the contents of chlorophyll '*a*', an increase of 27.82% occurred as the nitrate concentration increased (Table 3). For chlorophyll '*b*', a reduction of 26.94% was observed when the 25/75 nitrate/ammonium was used.

Table 3. Levels of chlorophyll *a*, chlorophyll *b* and carotenoids of seedlings of *Laelia marginata* grown in vitro, with different combinations of nitrate/ammonium chloride.

NO_3^-/NH_4^+:	Clorophyll *a* (mg gmf^{-1})	Clorophyll *b* (mg gmf^{-1})	Carotenoids (mg gmf^{-1})
0/100	6.97 cd	4.38 a	5.19 c
25/75	6.23 d	3.20 b	5.20 c
50/50	7.16 bc	4.37 a	6.48 b
75/25	8.59 a	4.15 a	7.50 a
100/0	7.82 ab	4.21 a	6.94 ab
CV (%)	5.04	8.04	4.99

Means followed by the same letter in the column do not differ statistically by the Tukey test at 5% probability.

The levels of carotenoids presented the highest values in the 75/25 nitrate/ammonium treatment, not statistically differing from the 100/0 treatment. On the other hand, the lowest values were observed in the 0/100 and 25/75 nitrate/ammonium treatments (Table 3).

It is believed that the increase in the chlorophyll content with the highest nitrate/ammonium ratio is related to an indirect effect of sulfur (S), which promotes nitrogen absorption, either because it is actively involved in the synthesis of the chlorophylls or because S is part of the ferrodoxin molecule, which is involved in the transfer of electrons in the photosynthesis, that is, the higher availability of S increases the electron transfer, consequently indirectly decreasing the photooxidation of chlorophylls (TAIZ and ZEIGER, 2013).

Based on the Pearson's correlation analysis (Figure 2), it was observed that leaf length showed a positive correlation with length and width of the leaf, with the root and aerial part dry matter, chlorophyll *a* and carotenoids, but it showed a low negative correlation with the number of leaves, number of shoots and chlorophyll *b*. The number of leaves and shoots showed high correlation with each other, but it showed a negative correlation with chlorophyll '*a*" and '*b*'.

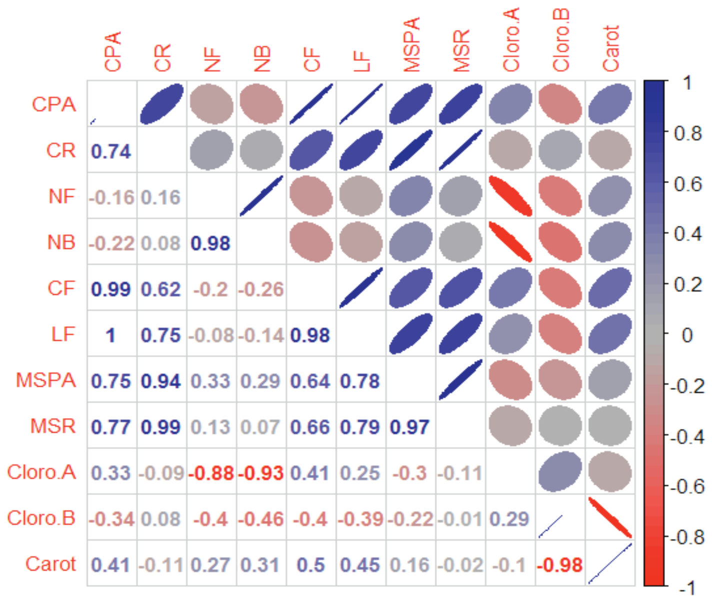

Figure 2. Pearson's correlation coefficients between the variables, length of the shoot (CPA), root length (CR), number of leaves (NF), number of buds (NB), leaf length (CF), leaf width (LF), dry mass of the shoot (MSPA), root dry mass (MSR), chlorophyll a (Cloro. A), chlorophyll b (Cloro. B) and carotenoids (Carot.) seedling *Laelia marginata* grown in vitro, with different combinations of nitrate/ammonium.

Regarding the analysis of the main components (Figure 3), it was verified that 50/50 nitrate/ammonium ratio showed a high correlation with the variables dry mass of the aerial part and root, length of the aerial part and root and length of leaf width.

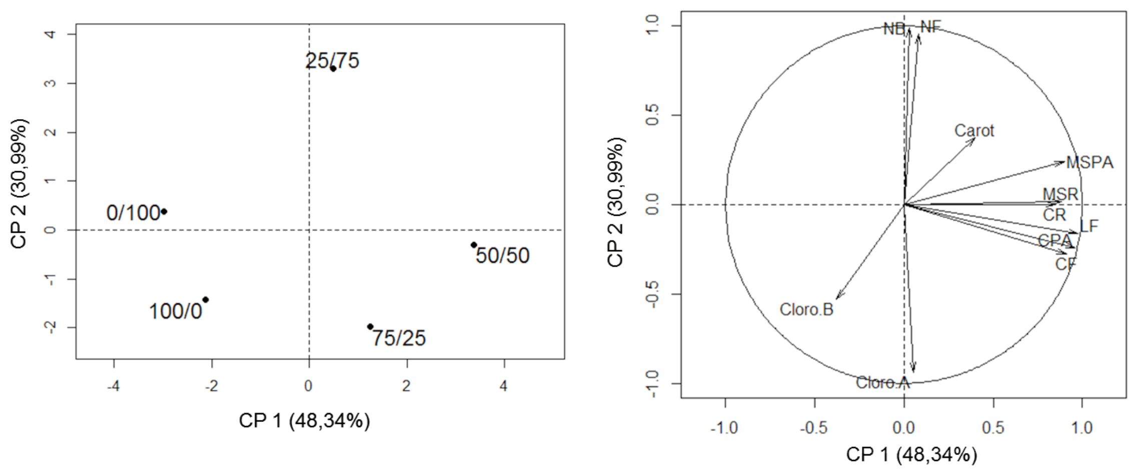

Figure 3. Principal component analysis (CPA) between the variables, length of the shoot (CPA), root length (CR), number of leaves (NF), number of buds (NB), leaf length (CF), leaf width (LF), dry mass of the shoot (MSPA), root dry mass (MSR), chlorophyll a (Cloro. A), chlorophyll b (Cloro. B) and carotenoids (Carot.) seedling *Laelia marginata* grown in vitro, with different combinations of nitrate/ammonium.

On the other hand, no correlation was observed with number of leaves and shoots and chlorophyll ‘a’. This shows that the balance between the nitrate and ammonium sources allows a better development of the seedlings, without causing stress either because of the excess or the lack of nitrate and/or ammonium.

4. CONCLUSION

The 50/50 nitrate/ammonium proportion resulted in the best initial development of *L. marginata* seedlings.

ACKNOWLEDGMENTS

The authors are grateful to the CAPES (Coordination for the Improvement of Higher Education Personnel) and CNPq (Brazilian National Council for Scientific and Technological Development) for financial support.

AUTHORS CONTRIBUTIONS

D.J.B.: cultivation and evaluation of orchids seedlings; paper development. **G.A.C.A.:** cultivation and evaluation of orchids seedlings; paper development. **R.T.H.:** cultivation and evaluation of orchids seedlings; paper development. **G.H.F.:** statistical analyzes. **F.F.F.:** cultivation and evaluation of orchids seedlings; paper development. **V.S.:** cultivation and evaluation of orchids seedlings; paper development. **R.T.F.:** responsible teacher

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