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In vitro growth of *Brassocattleya* orchid hybrid in different concentrations of KNO_3, NH_4NO_3 and benzylaminopurine

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ABSTRACT

One of the most important applications of plant tissue culture is mass propagation of ornamental plants. This experiment evaluated the effect of different concentrations of NH_4NO_3 and KNO_3 and BAP on the *in vitro* growth of orchid hybrid *Brassocattleya* 'Pastoral'. Seedlings of this orchid hybrid were used as explants and cultivated in medium with mineral salts and vitamins from the MS medium (Murashige & Skoog, 1962), with the macronutrients P, Ca and Mg reduced by half, and with an addition of 25 g L^{-1} of sucrose, 0.1 g L^{-1} of myo-inositol and 1.5 g L^{-1} of activated charcoal. Agar-agar was added (6.5 g L^{-1}) and the pH was adjusted to 5.8. As treatments, four concentrations of the NH_4NO_3 and KNO_3 (2x; 1x; ½ and ¼ MS medium) and three concentrations of BAP (0.0; 0.5 and 1.0 mg L^{-1}) were assayed. The multiplication, growth in height, fresh and dry weight and sugar level in dry weight of sprouts were evaluated. There occurred a higher growth in height with 0.25x NH_4NO_3 and KNO_3 salts concentrations of MS medium and higher rate of multiplication with combination of NH_4NO_3 and KNO_3 reduced by half of the MS medium concentration and 1.0 mg L^{-1} BAP.

Keywords: tissue culture, macronutrients, cytokinins, multiplication, growth.

RESUMO

Cultivo *in vitro* de *Brassocattleya* (Orchidaceae) em diferentes concentrações de KNO_3, NH_4NO_3 e benzilaminopurina

Entre as maiores aplicações da cultura de tecidos de plantas está a propagação massal de mudas de plantas ornamentais. O objetivo deste trabalho foi avaliar o cultivo *in vitro* de um híbrido de orquídea *Brassocattleya* em diferentes concentrações de NH_4NO_3, KNO_3 e BAP. Foram utilizadas sementes do híbrido de orquídea *Brassocattleya* 'Pastoral' e as plantas foram cultivadas em meio MS com redução pela metade das fontes de P, Mg e Ca e adição de 25 g L^{-1} de sacarose, 100 mg L^{-1} de mio-inositol, 1,5 g L^{-1} de carvão ativo e 6,5 g L^{-1} de ágar-ágar, sendo o pH ajustado para 5,8. Como tratamentos foram usados quatro concentrações dos sais NH_4NO_3 e KNO_3 (2x; 1x; ½ e ¼ do meio MS) e três concentrações de BAP (0,0; 0,5 e 1,0 mg L^{-1}). Avaliou-se a multiplicação, o crescimento em altura, massa fresca e seca, além dos teores de açucares redutores na massa seca das mudas. Observou-se grande influência das doses de NH_4NO_3 e KNO_3 sobre o crescimento em altura das mudas, massa fresca e seca e teores de açucares redutores em *Bc.* ('Pastoral' x Auto). A dose de ¼ da utilizada no meio MS promoveu aumento significativo do crescimento das plantas. Para multiplicação, houve melhor resultado com a dose de ½ dos sais NH_4NO_3 e KNO_3 utilizados no meio MS e 1,0 mg L^{-1} de BAP.

Palavras-chave: cultura de tecidos, nitrogênio, citocinina, multiplicação, crescimento.

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Brazilian floriculture has increased participation in the Brazilian GNP recently with flower production in different regions of the country both for the domestic market and for export. Exportation revenue from Brazilian floriculture products was US $31.5 million in 2009, but the quantity of imports in the sector (US$ 20 million) is still high (Kiyuna *et al*., 2010).

Among the orchids, the commercial group called Cattleyas that includes different species and interspecific and intergeneric hybrids including the *Brassocattleya* genus is very important in orchid commercial production and genetic breeding in this group and is extensively propagated in commercial laboratories (Raposo, 1993; La Croix, 2008).

A plantlet production by tissue culture has various applications in agriculture and horticulture and accelerates orchid propagation and makes it possible to obtain high quality plantlets derived from seeds or shoot tip culture (Torres *et al*., 1998).

Macronutrients are included in the culture medium in the form of inorganic salts and nitrogen can be supplied in the nitric, ammonium and organic forms in the culture medium. Both the N quantity and the ion ratio should be adjusted for each species, optimizing the *in vitro* growth and morphogenesis processes (Torres *et al*., 1998; Pasqual, 2001). Disarz & Corder (2009) obtained higher plantlet multiplication rates for Black Acacia (*Acacia mearnsii*) in MS culture medium with 3/4 the original nutrient concentration and the best multiplication rate of *Stevia reubaudiana* was obtained in a treatment with ¼ the N concentration used in the MS culture medium (Bespalhok *et al*., 1993). The concentrations of other macronutrients in the culture medium also seem to be too much for most cultures and reductions are needed to obtain the best results (Torres *et al*., 1998).

BAP (6-benzylaminopurine) is the most effective cytokinin for multiplication, followed by kinetin

and 2-ip (isopenthenyladenine) (Hu & Wang, 1983; Schuch & Erig, 2005). Sato *et al*. (2001) obtained about 200% increase in the fresh matter and 62.5% in the number of sprouts in *in vitro* gerbera culture using BAP compared to the culture medium that did not contain cytokinins.

The objective of the present study was to assess the *in vitro* development of plantlets obtained from *Brassocattleya* 'Pastoral' seeds under the concentrations of NH_4NO_3, KNO_3 and BAP in the culture medium.

MATERIAL AND METHODS

The study was carried out in the Biotechnology Sector of the 'Fundação Shunji Nishimura de Tecnologia', Pompéia, São Paulo State, Brazil.

The Murashige & Skoog (1962) culture medium was used with macronutrients reduced by half and addition of 25 g L^{-1} sucrose, 100 mg L^{-1} mio-inositol, 1.5 g L^{-1} activated charcoal and 6.5 g L^{-1} agar-agar. The pH was adjusted to 5.8±0.1 before adding the agar.

Culture flasks with 600 mL, 13.6 cm tall, 8.5 cm diameter and 6.8 cm opening diameter were used for the experiment. The flasks were sealed with polypropylene caps. To each flask 65 mL culture medium was added and autoclaved at 121°C and 1 kgf cm^{-2} for 20 minutes.

The explants were obtained from four month-old plantlets, approximately 1.0 cm tall, derived from seeds germinated *in vitro*. The seeds were obtained from the self-pollinating of the *Brassocattleya* 'Pastoral' ('Pastoral' x Auto) hybrid. Seed-derived material was used because there was a sufficient quantity of *in vitro* material available to carry out the experiment. In spite of the genetic segregation, the experiment with seeds in orchid permitted widening the range of genotypes that respond to the treatments applied, serving as reference for application in shoot tip cultures of many hybrids of the *Brassocattleya* genus.

The plants were kept in a growth chamber with a 16-hour light period, day/night temperature 25/20°C, 60% relative humidity and artificial lighting from Gro-lux-type Sylvania light bulbs, with 35 µmol/m/s irradiance.

The experiment was carried out in complete randomized blocks with 12 treatments and four replications. Each replication consisted of one culture flask containing ten plants.

Four doses of NH_4NO_3 and KNO_3 were evaluated: 2x, 1x, ½ and ¼ of the MS culture medium concentration, and three BAP concentrations: 0, 0.5 and 1.0 mg L^{-1}. The salt and BAP concentrations were tested alone and together and possible interactions were verified between the two components, because both are related to the development of the shoots.

The *Brassocattleya* ('Pastoral' x Auto) plant development was assessed by the multiplication rate, plantlet height, fresh and dry matter and an analysis of the reducing sugar contents contained in the dry matter using the methodology by Nelson & Somogy (Nelson 1944; Somogy 1952).

The multiplication rate and plantlet height were assessed during two consecutive replications in the culture medium containing the treatments and the other items were assessed at the end of the experiment. The replications were carried out every 120 days.

The means obtained of the multiplication rate, growth in height and reducing sugar content were submitted to regression analysis to compare the results. The Duncan test (5%) was used to compare the fresh and dry matter average of the treatments.

RESULTS AND DISCUSSION

There was significant influence from the different NH_4NO_3 and KNO_3 doses on the *in vitro* development of the *Brassocattleya* ('Pastoral' x Auto) orchid (Figure 1). The NH_4NO_3, KNO_3 and BAP concentrations in the culture medium directly influenced the multiplication rate and growth in height of the plants cultured. There were salts and BAP interaction, mainly for *Bc*. ('Pastoral' x Auto) plantlet multiplication.

The best results for the multiplication rate in the treatment were obtained with the addition of 1.0 mg L^{-1} BAP. For these the best responses, multiplication rates of around 5.0 and 5.4:1 occurred at the NH_4NO_3 and KNO_3 concentrations equivalent to half and once of those of the culture medium. In the treatments with BAP added to the culture medium, the worst performance was at the concentrations of twice and ¼ the NH_4NO_3 and KNO_3 concentration of the culture medium, and these were approximately 2:1. However, without plant regulator addition, the best performance was at the concentrations of ½ and twice the NH_4NO_3 and KNO_3 concentration of the MS culture medium, with multiplication rates obtained from two consecutive replications and at 120 days culture of 3.4 and 3.5:1, respectively.

There was significant interaction among the NH_4NO_3 and KNO_3 doses and BAP concentrations on the multiplication rate obtained. The doses of these salts could be reduced to half when 1.0 mg L^{-1} BAP was used, enabling a good multiplication rate with reduced costs for the NH_4NO_3 and KNO_3 salts used in the culture medium formulation (Figure 1). In the treatments without the Plant Growth Regulator, the dose corresponding to half the MS culture medium also presented the best result but approximately 1.5 shoot per explant less than when 1.0 mg L^{-1} BAP was used. The use of 0.5 mg L^{-}1 BAP did not increase the multiplication rate compared to the culture medium without BAP. In a study with *Oncidium* genus orchids, the best multiplication rate was obtained using MS culture medium with the addition of 2 mg L^{-1} BAP (Kalimuthu *et al*., 2007). Saiprasad & Polisetty (2003) obtained a greater number of plantlet using MS culture medium with 1.0 mg L^{-1} BAP for the *Dendrobium* and *Oncidium* orchid genera and the *Cattleya leopoldii* species. Hu & Wang (1983) observed differences among the cytokinins and BAP induced high multiplication rates while kinetin and 2-ip stimulated only plant growth.

The NH_4NO_3 and KNO_3 salt concentration also influenced the multiplication rate and there was

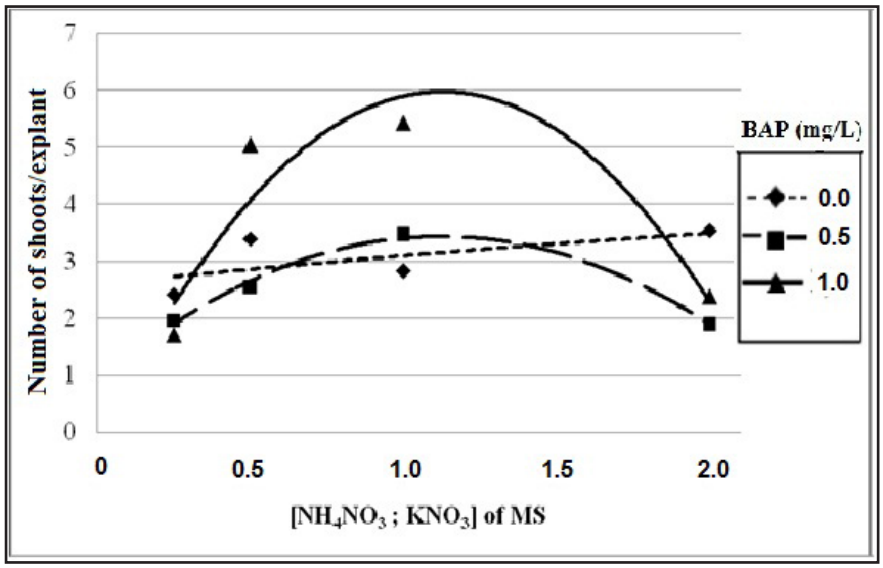

Figure 1. Multiplication rate of *Brassocattleya* ('Pastoral' x Self) orchid in MS culture medium with varying NH_4NO_3, KNO_3, and BAP concentrations (taxa de multiplicação de orquídea *Brassocattleya* ('Pastoral' x Auto) em meio de cultura MS com diferentes concentrações de NH_4NO_3, KNO_3 e BAP). Pompéia, USP, 2008.
BAP 0,0 mg L^{-1} y = -0,053x^2 + 0,562x + 2,591, R^2 = 0,427ns; BAP 0,5 mg L^{-1} y= -2,015x^2 + 4,529x + 0,89, R^2 = 0,987**; BAP 1,0 mg L^{-1}, y = -4,831x^2 + 10,89x -0,161, R^2 = 0,86**

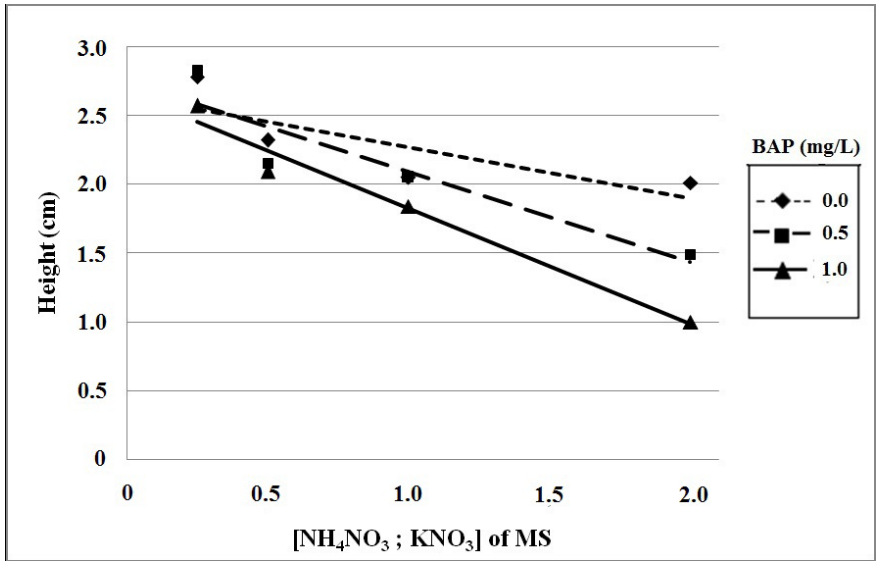

Figure 2. Shoot height of *Brassocattleya* ('Pastoral' x Self) orchid in MS medium with varying NH_4NO_3, KNO_3, and BAP concentrations (altura de brotos de orquídea *Brassocattleya* ('Pastoral' x Auto) cultivadas em meio MS com diferentes concentrações de NH_4NO_3, KNO_3 e BAP). Pompéia, USP, 2008.
BAP 0,0 mg L^{-1}, y = -0,368x + 2,635, R^2 = 0,647*; BAP 0,5 mg L^{-1}, y = -0,653x + 2,743, R^2 = 0,84**; BAP 1,0 mg L^{-1}, y = -0,836x + 2,659, R^2 = 0,97**

significant interaction among the salt doses and the BAP concentration at 1.0 mg L^{-1}. There was only high multiplication rate with this regulator when the salt doses were between ½ and once that of the MS culture medium. Nevertheless the plantlet cultured at the lower (¼) and the higher (twice) doses did not respond to BAP for multiplication and the results were below the multiplication of the culture medium without this plant growth regulator. Sato *et al*. (2001) also observed interaction between the total N concentrations in the culture medium and the presence of BAP in *in vitro* gerbera culture, and satisfactory multiplication rates were obtained using 2.0 mg L^{-1} BAP only when the total N concentration was close to ½ the concentration of the MS culture medium.

The effect of the cytokinins (CK) on breaking correlative inhibition and inducing multiple shoot sprouting is known, but the process of interaction between the N and K nutrients and the cytokinins in *in vitro* morphogenesis has not yet been elucidated. Thus according to the results obtained in the present and in other correlated studies, the explanation is not limited to the nutritional aspect (Kerbauy, 2008). Recent studies have shown that in *Arabidopsis thaliana*, nitrate and ammonium presence significantly stimulated the expression of the *AtIPT* genes, responsible for the key enzyme of the CK biosynthesis, isopenthenyl transferase that promotes CK accumulation in the roots and later translocation to the shoots inducing shoot development of the plants as a whole (Takey *et al*., 2004).

Greater growth was observed in *Bc.* ('Pastoral' x Auto) plantlet height when the smaller dose of the NH_4NO_3 and KNO_3 salts was used (¼ MS culture medium concentration) and there was 150 to 170% average growth compared to the initial explants (Figure 2). Increase in the salt doses was inversely proportional to the growth in the plantlet height. The presence or absence of BAP in the culture medium did not significantly influence the growth in height of the plantlets. This result confirmed the hypothesis that BAP is responsible for inducing multiple sprouting and it is not very efficient on the *in vitro* plantlet growth (Hu & Wang, 1983). However, reductions have been reported in other studies in plantlet height of different species with the use of BAP in the culture medium (Leitzke *et al*., 2010).

Bc. ('Pastoral' x Auto) orchids presented best plantlet growth with doses lower than those used in the MS culture medium. Other authors have also reported better growth of other species

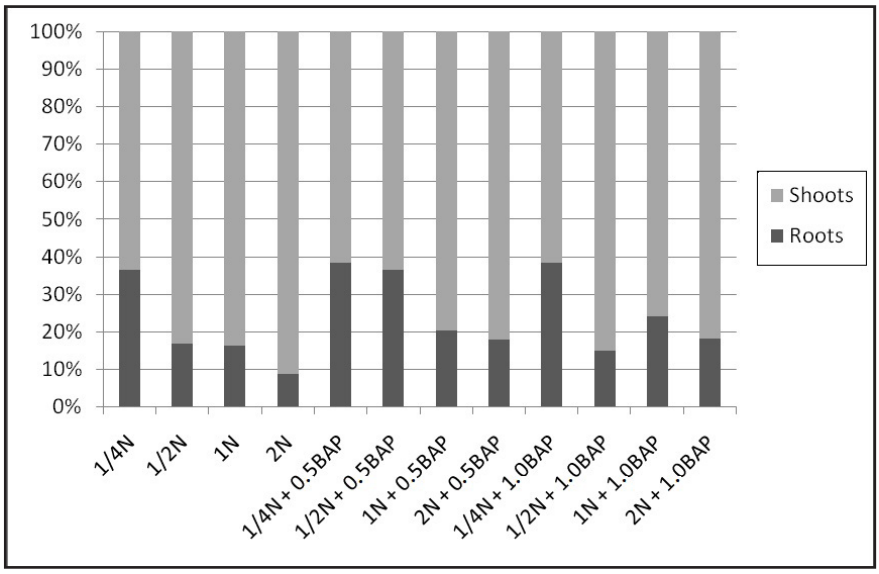

Figure 3. Shoot dry matter and root ratio of *Brassocattleya* ('Pastoral' x Self) orchid in MS medium with several NH_4NO_3, KNO_3, and BAP concentrations (proporção entre matéria seca da parte aérea e raízes em orquídea *Brassocattleya* ('Pastoral' x Auto) em meio MS com diferentes concentrações de NH_4NO_3, KNO_3 e BAP). Pompéia, USP, 2008.

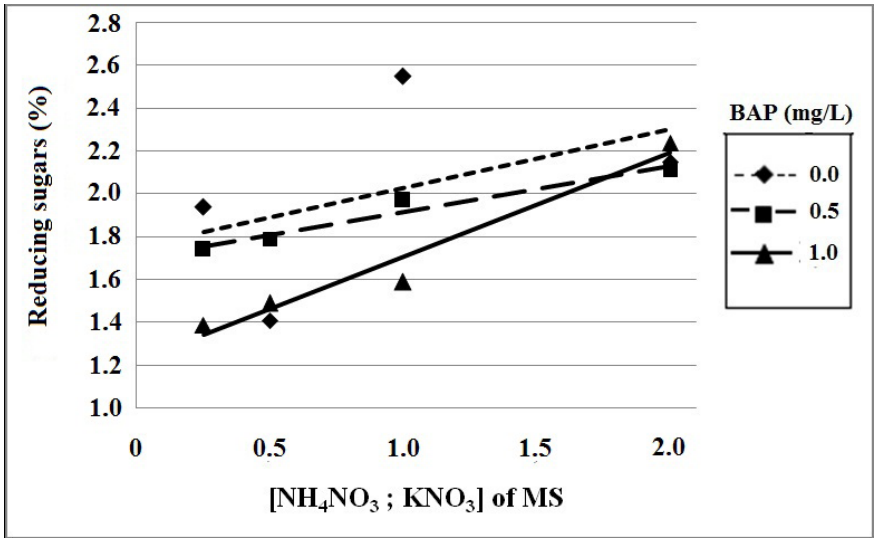

Figure 4. Reducing sugars (%) in dry matter of *Brassocattleya* ('Pastoral' x Self) orchid in MS culture medium with several NH_4NO_3, KNO_3 and BAP concentrations (açúcares redutores (%) na matéria seca em orquídea *Brassocattleya* ('Pastoral' x Auto) em meio MS com diferentes concentrações de NH_4NO_3, KNO_3 e BAP). Pompéia, USP, 2008.

with reduced concentrations of nutrients used in the culture medium (Biasi *et al.*, 1998; Schuch & Peters, 2002). Reduction in the NH_4NO_3 and KNO_3 salts also resulted in significant increase in root production, that may have favored a greater nutrient absorption in general, because significant increases were also observed in the fresh and dry matter with the lower concentrations of these salts (Table 1).

For the other items assessed, fresh matter and dry matter and dry matter percentage, a gradual increase was observed with decrease in the NH_4NO_3 and KNO_3 concentrations in the culture medium, and for the shoots the best results occurred at the concentration of ½ to once that recommended in the MS culture medium. In a study by Russowski & Nicoloso (2003) with *in vitro* Brazilian ginseng (*Pfaffia glomerata*) culture, dry matter increased when the nitrogen was reduced to 80% of the MS culture medium concentration. For the roots, an even greater reduction of around ¼ that used in the MS culture medium, was positive for the increase in the fresh and dry matters. The cytokinins also influenced the *Bc*. ('Pastoral' x Auto) fresh and dry matters and the best results were obtained with the 0.1 mg L^{-1} BAP concentration where the fresh matter increased from 1.30 g (s/ BAP) to 1.79 g (1.0 mg L^{-1} BAP) and the dry matter from 0.13 g (s/ BAP) to 0.17 g (1.0 mg L^{-1} BAP) (Table 1).

The shoot/root ratio tended to be greater with increases in the N and K concentrations in the culture medium, probably because it inhibited adventitious root production and lengthening in the culture medium. Using BAP used in the culture medium, regardless of the concentration used, 0.5 or 1.0 mg L^{-1}, resulted in more uniform root and shoot development, specially at concentrations higher than ¼ of the NH_4NO_3 and KNO_3 salts that generally raised the ratio between these two parts of the plantlet, probably showing a better redistribution of the assimilates and nutrients (Figure 3 and Table 1).

The relatively high values of the coefficients of variation for the data in Table 1 were due in part to the fact that the explants used for the experiment were derived from seeds.

The reducing sugar contents decreased with reduction in the NH_4NO_3 and KNO_3 concentrations (Figure 4). However, this seems to have been more an indirect effect of the nitrogen, because N decrease caused an increase in the plant dry matter. As most of the dry matter consists of reserve carbohydrates and proteins and other carbonic chains, they accumulated and the reducing sugar contents decreased. In addition there was a greater use of the reducing sugars in respiration to obtain energy, spent in the formation of complex molecules. This inverse ratio between dry matter and reducing sugar contents was also demonstrated in an experiment with potatoes (Salamoni *et al.*, 2000).

The results of the present study verified the nitrogen and potassium

Table 1. fresh weight (MF), dry weight (MS), % of dry weight (%MS) and shoots/roots ratio (Pa/Rz) in *Brassocattleya* ('Pastoral' x Self) orchid in MS culture medium with several NH_4NO_3, KNO_3 and BAP concentrations (matéria fresca (MF), matéria seca (MS), porcentagem de matéria seca (%MS) e relação parte aérea/raízes (PA/Rz) em orquídea *Brassocattleya* ('Pastoral' x Auto) em meio de cultura MS com diferentes concentrações de NH4NO3, KNO3 e BAP). Pompéia, USP, 2008.

Treatments	MF (g)	MS (g)	MS (%)	PA/Rz
¼ N	1.3ab	0.131ab	10.195a	1.84c
½ N	1.34ab	0.131ab	9.818ab	7.48ab
1N	0.935cd	0.087bc	9.325ab	7.2ab
2N	0.805d	0.072d	9.013ab	10.86a
¼ N + 0.5BAP	1.201ab	0.107bc	9.695ab	2.15c
½ N + 0.5BAP	1.155bc	0.1bc	9.103ab	1.81c
1N + 0.5BAP	0.934cd	0.087bc	9.515ab	4.27bc
2N + 0.5BAP	0.723d	0.06d	8.358cd	5.23bc
¼ N + 1.0BAP	1.792a	0.167a	9.358ab	1.64c
½ N + 1.0BAP	1.605ab	0.133ab	9.443ab	4.08bc
1N + 1.0BAP	1.454ab	0.123ab	8.658bc	3.17bc
2N + 1.0BAP	1.051bc	0.078cd	7.728d	4.93bc
CV (%)	31.58	28.76	8.206	62.33

nutritional requirements of the hybrid orchids of the *Brassocattleya* genus that has high ornamental potential and is related to a large part of the total of orchids produced in Brazil, but has been very little studied for its nutritional needs either in the field or *in vitro*. It was verified that orchids such as *Bc*. ('Pastoral' x Auto) respond positively in growth when they are submitted to reduced doses of the NH_4NO_3 and KNO_3 salts in the MS culture medium. For multiplication, an increase was observed in the number of plantlet per explants with the application of 1.0 L^{-1} BAP in culture medium and the concentrations of ½ and once of the quantity of the ammonium nitrate salts (NH_4NO_3) and potassium nitrate salts (KNO_3) used in the MS culture medium. For the evaluated items growth in height, fresh and dry matter, an even greater decrease, around ½ of the salts used, gave the best results for the *in vitro* culture of these orchids. Therefore the MS culture medium with ½ the macronutrients and the addition of 1.0 mg L^{-1} BAP was effective for the multiplication stage while for the rooting stage MS culture medium with ¼ reduction of the NH_4NO_3 and KNO_3 salts and the addition of 1.0 mg L^{-1} BAP is recommended for the culture of this orchid.

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