EFFICIENCY OF UREA USAGE AND GROWTH REGULATORS ON *IN VITRO* PROPAGATION OF *ETLINGERA ELATIOR*

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Abstract: Torch ginger is an ornamental plant from Indonesia that belongs to the family Zingiberaceae, which has been having a high acceptance in the Brazilian market. The objective of this work was to develop an efficient protocol for the *in vitro* multiplication of *Etlingera elatior* var. Red Torch. The sproutings from *in vitro* germinated seeds were inoculated in culture medium Murashige & Skoogwith 3% of sucrose, 6% of agar and different concentration of 6-benzylaminopurine and α-naphthaleneacetic acid. As an alternative source of nitrogen, ammonium nitrate was substituted by urea, in different concentrations, without changing the final balance of nitrogen of the medium. After being inoculated, the sproutings were kept in a growth room, under controlled conditions.The characteristics number of sproutings, size of sproutings, number of roots and root length were evaluated in three different subcultures. It was observed the concentration of 13.32 µM of 6-benzylaminopurine favored multiple sproutings. Once α-naphthaleneacetic acid was added from 2.70 µM, a significant increase of all analyzed variables occurred. The substitution of ammonium nitrate by urea up to 50% was favorable for the production of lateral bud sproutings and roots in the propagules, characterizing satisfactory conditions for *in vitro* propagation in a large scale.
Key words: Torch ginger.cultivation *in vitro.* sources nitrogen.

EFICIÊNCIA DO USO DE URÉIA E REGULADORES DE CRESCIMENTO NA PROPAGAÇÃO *IN VITRO* DE *ETLINGERA ELATIOR*

Resumo: O Bastão-do-imperador é uma planta ornamental originária da Indonésia pertencente à família Zingiberaceae, com crescente aceitação no mercado brasileiro. Este trabalho foi realizado com o objetivo de desenvolver um protocolo eficiente para a multiplicação *in vitro* da espécie. As brotações provenientes de sementes germinadas *in vitro* foram inoculadas em meio Murashige & Skoog acrescido de sacarose a 3%, ágar a 6% e diferentes concentrações de 6-benzylaminopurine e α-naphthaleneacetic acid. Como fonte alternativa de nitrogênio foi substituída o nitrato de amônio por uréia, em diferentes proporções, sem alterar o balanço final de nitrogênio do meio. Depois de inoculadas, as brotações foram mantidas em sala de crescimento, sob condições controladas. As características número de brotações, tamanho das brotações, número de raízes e o comprimento das raízes, foram avaliadas em três diferentes subcultivos. Foi observado que a concentração de 13.32µM de 6-benzylaminopurine favoreceu múltiplas brotações. Quando adicionado α-naphthalene acetic acid a partir de 2.70µM, ocorreu aumento significativo de todas as variáveis analisadas. A substituição de nitrato de amônio por uréia até 50% foi

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favorável para produção de multibrotações laterais e raízes nos propágulos, caracterizando condições satisfatórias para a propagação *in vitro* em larga escala.
Palavras-chave: Bastão-do-imperador; cultivo *in vitro;* fontes de nitrogênio.

INTRODUCTION

Torch ginger (*Etlingera elatior* Jack R. M. Smith), also known as Philippine wax flower, is a herbaceous plant from Indonesia that belongs to the familyZingiberaceae. It has inflorescences with pyramidal shape, with green streams and pink, red and porcelain bracts. The reproduction is by seed or rhizome (Lins & Coelho, 2004).

Due to the practice of traditional vegetative propagation, the dissemination of phytopathogenic diseases occurs which has the possibility of compromising the marketing of cuttings and flowers (Lins & Coelho, 2004). The commercial ornamental plants cannot have any little injury or deformation of the leaves and flowers because of the high quality standard demanded by consumers according to Maciel *et al*., (2004).

This quality standard can be reached through the use of tissue culture techniques (Rodrigues *et al.,* 2009), to produce ornamental plants be par excellence, the group of plants in which such procedures, such as *in vitro* propagation, has been having significant expression for the scientific world with direct impact on the economy (Bosa *et al.,* 2003; Silva Júnior *et al.,* 2012).It is also known that changes in the final balance of salt concentration in nutritive media, mainly the ones that contain nitrogen (N), can cause modifications in the growth rate of the plants, in morphology and cell totipotency (Araújo *et al.,* 2009; Villa *et al.,* 2009; Rodrigues *et al.,* 2012).

The absence or low availability of nitrogen (N) in the culture medium can inhibit the growth and development of the plants.The ammonium as the only source of N can cause reduction in the number of cuttings and result in hyperhydricity. Moreover, high concentrations of ammonium can cause toxicity in vegetal species.In relation to the use of nitrate or urea, they can inducemultiple sproutings, which can improve the quality and quantity of cuttings production (Ivanova & Staden, 2009; Rolli et al, 2011; Silva Júnior *et al.* 2013).

In the technique of tissue culture, the mineral nutrition of plants features in strategies for significant increases in production in large scale. One of the most important constituents of plant nutrition is N, as the assimilation of this element is essential for vital processes that

control the growth and development of plants and has marked effects on the biomass and productivity of crops.

Generally in *in vitro* cultivation, use is made of growth regulators to stimulate elongation and rooting of shoots, but other factors can also be used as new sources of N in the basal medium, including the organic source. The culture media usually possess ammonium nitrate and potassium nitrate as a source of inorganic N, but the organic N can alsobe used by the plant. The urea may be used in the medium as of the additional N, and has been tested by some authors (Ramos *et al.,* 2009). The type of nutrition is extremely important for the growth and development phases of plants derived from *in vitro* to support the process of acclimatization (Ramos *et al.* 2009; Rodrigues *et al.,* 2012).

The nutritional deficiency of torch ginger could affect the parameters of growth and production of dry matter. This ornamental species is more demanding in B, K, N, PandSin the initial growth phase instead of Ca and Mg. Thus, N is the third most important element that modulates the increase of carbon in the plant (Frazão *et al.,* 2010).

The intention of producing healthy cuttings in large scale and with low production cost has stimulated the realization of several works using tissue culture techniques. Although the ornamental plants are subjects to a lot of research, there is no work realized with this species trying to study alternative sources of nitrogen in its *in vitro* cultivation.

Therefore, the objective of this work was to study the induction process of *in vitro* multiple sproutings of *E. elatior* var. Red Torch, when ammonium nitrate (NH_4NO_3) was substituted by urea (CH_4N_2O) in different concentrations.

MATERIALS AND METHODS

The work was carried out in the Laboratory of Plant Tissue Culture at the Biology Department, in the Sector of Plant Physiology at the Federal University of Lavras (UFLA), Lavras, Minas Gerais, for three months in 2009. Ripe fruits from commercial planting were used at Monteiro Farm in the county of Messias in Alagoas. The fruits were immersed in sodium hypochlorite solution of 0.2% for 30 minutes, and after that, washed with running water for 1 minute.

In a laminar flow chamber, the seeds were desinfected in the following sequence: 1) wash under distilled water with 3 drops of neutral detergent for 10 minutes; 2) immersion for 5 minutes in the fungicide thiophanate methyl [Cercobim® 700 PM] of 2 $g.L^{-1}$; 3) immersion

for 1 minute in ethanol 70% (v/v); and 4) immersion in sodium hypochlorite solution of 0.6% (v/v) for 15 minutes with 2 drops of detergent Tween 80®. At the end of each stage, the seeds were rinsed three times for two minutes with sterile water.

The seeds were placed to germinate in paper filter substrate previously moistened with sterile water, and packed in sterile Petri dishes (60 x 15 mm), and transferred to test tubes containing 20 mL of the MS culture medium (Murashige & Skoog, 1962),enriched with 8.88 µM of 6-benzylaminopurine (BAP). As source of explant during the subcultives, shoots produced by rhizome cultivated in vitro were used.

The cultures were kept in a growth chamber under photon irradiance of 30 µmol m^{-2} s^{-1}, from light bulbs Philips TDL, in photoperiod of 16 hours and temperature of 25±2°C. Every 30 days, for a total of 90 days, the number and length of shoots and roots produced by the explants were evaluated. The treatments used in this experimente were: 1- (MS without growth regulators); 2- (MS + 4.44 µM of BAP); 3- (MS + 8.88 µM of BAP); and4- (MS + 13.32 µM of BAP).

The best treatment of the last experiment i.e. the one in which MS medium was supplemented with 13.32 µM of BAP was selected for this stage, just varying the concentration of α-naphthaleneacetic acid (ANA).The treatments consisted of: 1- without ANA; 2- 0.54 µM; 3- 2.70 µM; and 4- 5.40 µM. The characteristics evaluated, conditions of cultivation as well as the design used and statistical method, were the same previously mentioned.

The treatments consisted of: control, 25, 50, 75 and 100% of substitution of ammonium nitrate by urea, fixing the concentration 13.32 µM of BAP added to MS medium in all the treatments. The final balance of N in the nutritive medium was not altered, being evaluated the treatments as described in (Table 1). The characteristics evaluated and cultivation conditions were the same as previously mentioned. However, the subculture season lasted 15 days during 45 days.As statistical method of analysis, the data were submitted to the variance analysis and the averages were compared by the test of Scott Knott, at 5% of probability.

Table 1. Different nitrogen sources and concentrations used in the *in vitro* propagation of the explants of *Etlingera elatior*

Sourceof N (%)	Ammonium nitrate(NH_4NO_3)	Urea (CH_4N_2O)
100% NH_4NO_3 (control)	577.7 mgL^{-1}	0 mg L^{-1}
75% NH_4NO_3 + 25% CH_4N_2O	433.2 mg L^{-1}	309.51 mg L^{-1}
50% NH_4NO_3 + 50% CH_4N_2O	288.85 mg L^{-1}	619.02 mg L^{-1}
25% NH_4NO_3 + 75% CH_4N_2O	144.4 mg L^{-1}	928.53 mg L^{-1}
100% CH_4N_2O	0 mg L^{-1}	1228.04 mg L^{-1}

The experiments were carried out in a completely randomized design in bifactorial scheme 4 (BAP or ANA concentrations) x 3 (seasons of subcultures), with five repetitions, being each repetition represented by five bottles containing one explant, evaluated by the linear regression model using the *software* Sisvar (Ferreira, 2011).

RESULTS AND DISCUSSION

A significant result between the BAP concentration and the season of evaluation for the number of buds was observed with the increase of BAP concentration, there was increase of the number of sproutings in the three subcultures. The biggest production of sproutings 9.0 was obtained in MS culture medium containing 13.32 µM of BAP, in the third subculture, at 90 days of incubation (Figure 1A).

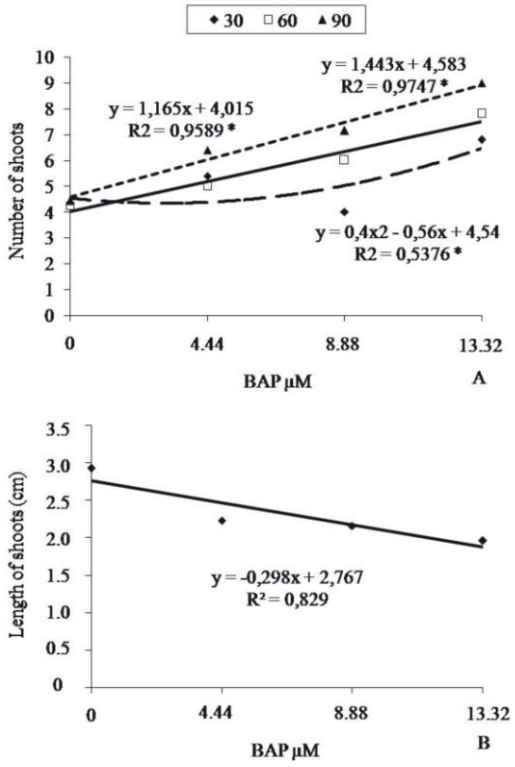

Figure1. Number of buds of torch ginger (A) issued by explants due to different concentrations of 6-benzylaminopurine (BAP), at 30, 60 and 90 days of incubation and average length of the sprouting (B) because of different BAP concentrations at 90 days of incubation

BAP is a cytokinin of quick absorption and activity in the plant metabolism, with signalling and inducing function of multiple sproutings (Rolli *et al.*, 2011). It is mainly noted in the initial growth phase of plants cultivated *in vitro*, the necessity of using and combining growth regulators that can overcome the shortcomings of endogenous contents of hormones in the buds, once they are isolated from the stock plant (Colombo *et al.*, 2010).

However, using MS culture medium in the absence of growth regulators or containing 4.44 µM of BAP *in vitro* cultivation of ginger (*Zingiber officinalle* Roscoe), there was respective induction of (4.1 and 4.2) new buds per explant. Showing that in this case the use of BAP was not significant to induce multiple sproutings (Debiasi *et al.*, 2004).

Moreover, reduction of the number of sproutings of the hybrid *Citrus paradisix* and *Poncirus trifoliate* popularly known as Citrumelo "Swingle", was observed, once BAP concentration was increased in the culture medium (Cantagallo *et al.*, 2005). Probably these species stored high concentration of cytokinins. When growth regulators were applied in the culture medium, it can induce negative feedback in the signalling pathways of sprouting

induction, besides the phytotoxic effects caused by BAP in high concentrations (Rolli *et al.*, 2011).

For the average length of the buds at 90 days of incubation, their reduction was observed once BAP concentration increased. In the absence of the regulator, the average length of the buds was 2.93 cm. When 13.32 µM of BAP was applied, the average length of the buds was 2.0 cm (Figure 1B).

Similar results were described by Debiasi *et al.* (2004), when cultivating ginger in MS culture medium without growth regulators. In this condition, the authors observed larger length of the buds with average 3.2 cm. Thus, high doses of cytokinins can delay the elongation of sprouting (Rolli *et a.,* 2011).

Besides that, sproutings cultivated in MS medium without regulators produced the highest average value 5.45 of roots per explant and average length 3.84 cm at 90 days of cultivation. The lowest results were observed at 30 days of cultivation, with average number of 3.71 roots per explant and medium length 2.52 cm (Figure 2).

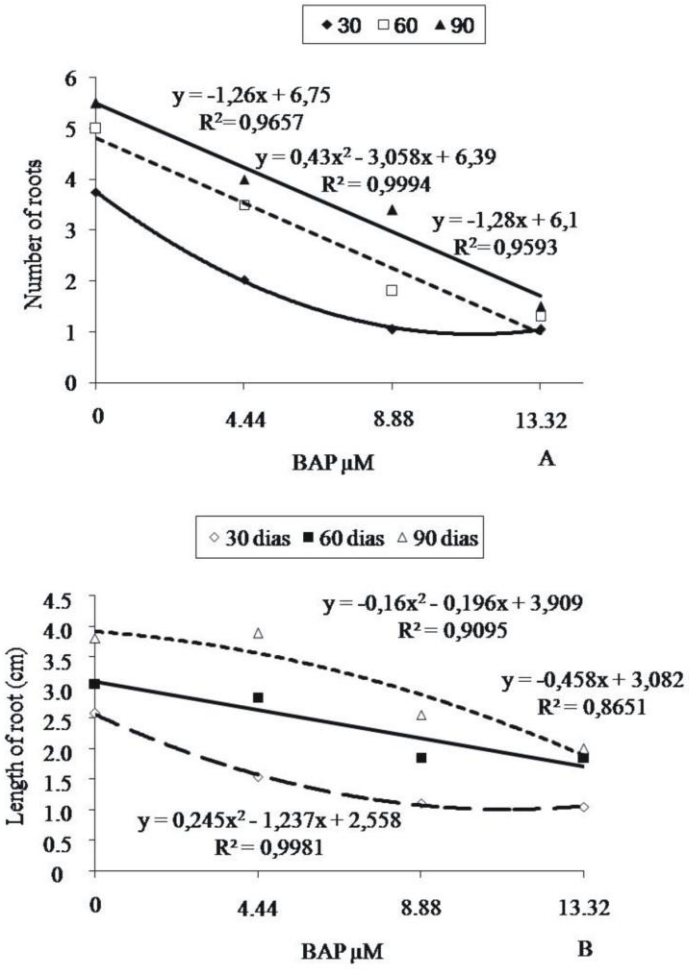

Figure 2. Average number of roots (A) and roots length (B) of torch ginger at 30, 60 and 90 days of cultivation, when different concentrations of 6-benzylaminopurine were applied

It was also observed a direct reduction in the characteristics evaluated of the radicular system in the three subcultivations with the increase of BAP concentration. The lowest values were observed when 13.32 µM of BAP was added to the culture medium. The average number of roots per explant in the first subcultivation was 1.21, in the second one 1.45 and in the third one 1.83. As the length average in the first subcultivation was 1.23 cm, in the second one 2.24 cm and in the third one 2.37 cm. In relation to the number of roots, similar results were obtained with Thai ginger *Alpinia officinarum* Hance, corresponding to 7.1 roots per explants in the absence of regulators. When adding 8.88 µM of BAP, there was a decrease in this number, corresponding to the average value of 3.1 roots per explants (Borthakur *et al.,* 1999). Such results confirm thatcytokinin BAP has inducing effect of sproutings and inhibiting effect to buds and root elongation (Cantagallo *et al.,* 2005).

By adding 13.32 µM of BAP, together with 5.40 µM of ANA, increase in buds production per explant (9.82) was observed. This way, hormone balance between cytokinin BAP and auxin ANA favored higher number of multiple sproutings in torch ginger (Figure 3A). Similar results were found during the initial cultivation phase of the same species when using 21.98 µM of BAP and 4.97 µM of α-naphthaleneacetic acid (AIA). In this case, three to four sproutings during the multiplication phase were obtained. The sproutings rooted and formed clumps in MS medium without growth regulators, originating from eight to ten plants per clump monthly (Colombo *et al.,* 2010).

However, this significant dependence between the levels of cytokinin and auxin in the cultivation of *Cattleya mesquirae* was not observed. Were obtained 3.71 buds per explant were obtained when adding 8.88 µM of BAP, while with the addition of ANA, the sprouting rate was reduced (Ramos & Carneiro, 2007).

In relation to the sproutings length, a significant interaction among the sources of variation (ANA x subcultivation) was observed. The tendency of the third subcultivation was increase in the buds length due to the increase of regulator concentration at 90 days. The highest value observed was 10.03 cm when 5.4 µM of ANA was applied (Figure 3B).

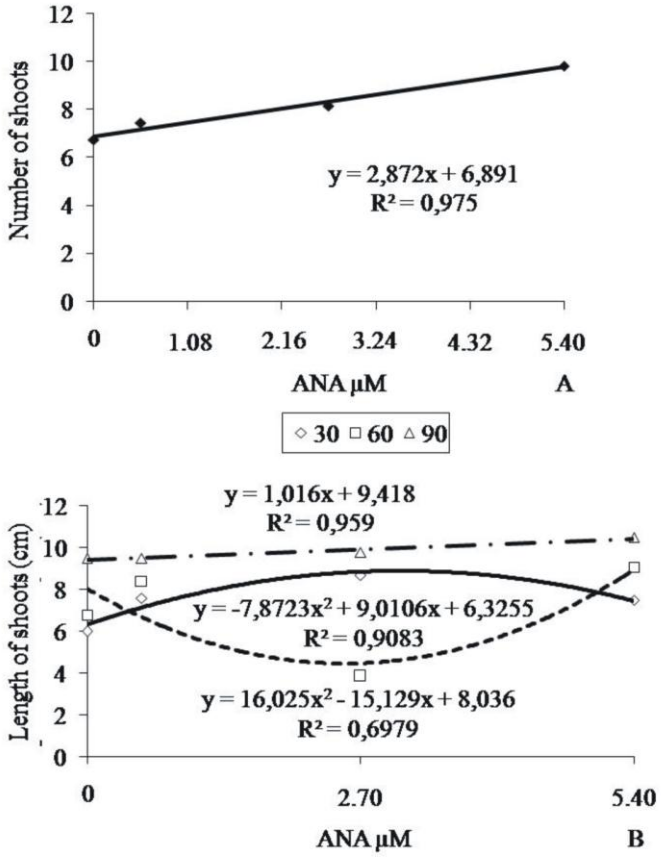

Figure 3.Number of torch ginger buds (A) formed by explants subcultivated in different concentrations of α-naphthaleneacetic acid (ANA) at 90 days of cultivation and average length (B) issued by explants at 30, 60 and 90 days of *in vitro* cultivation due to different concentrations ofα-naphthaleneacetic acid

On the other hand, the second subcultivation showed reduction in the buds length up to the concentration of 2.7 µM of ANA at 60 days. Over this concentration, the explants resumed their growth, reaching the average value of (8.57 cm) when 5.4 µM of ANA was applied. While the third subcultivation without regulator at 30 days had an average (6.2 cm), reaching up to (8.35 cm) by adding 2.7 µM of ANA and (7.1 cm) when5.4 µM of ANA was applied.

The results showed it is favorable to cultivate explants of torch ginger during the first 30 days with BAP associated with low concentration of ANA. From the second subcultive, increase ANA concentration needed to be increased up to 5.4 µM. For the variables: number and root length with the use of ANA, an increasing rectilinear behavior in the regression was observed, displaying that the radicular system of torch ginger is responsive to the increase in

ANA concentration. The highest average value for characteristic number of roots (10.1) was observed when applied 5.40 µM of ANA (Figure 4A).

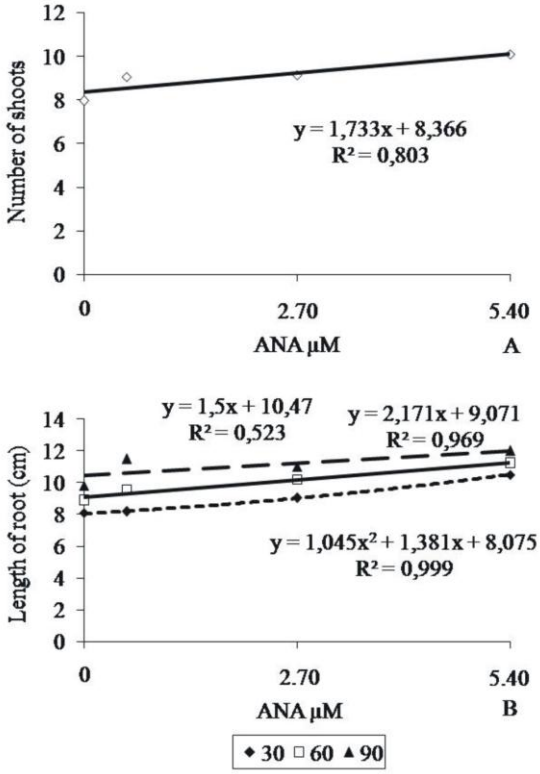

Figure 4. Effect of different concentrations ofα-naphthaleneacetic acid (ANA) in the average number of roots (A) of torch ginger at 90 days of incubation, and average length of the roots (cm) (B) due to different ANA concentrations at 30, 60, 90 days of incubation

In relation to the average root length of 11.23 cm, the highest value was registered at 90 days of incubation according to (Figure 4B). This tendency of the radicular system is also observed in the cultivation of the species *Pyrus communis* L. From the results obtained, the concentrations of 3.2 and 6.4 µM of ANA and absence of activated carbon in the culture medium, a better rooting was possible as it is described by (Erig *et al.,* 2004).

For characteristic number of sproutings, the best results were observed in the treatments with 75 and 100% of ammonium nitrate, which did not differ significantly between them, forming 6.52 and 6.73 sproutings per explant respectively.The use of 50% of ammonium nitrate + 50% of urea provided formation of 5.65 buds per explant, highlighting the reduction of 16% in relation to the use of 100% of ammonium nitrate.

In terms of cost-benefit, the results are promising, indicating viability of *in vitro* propagation technique of torch ginger, with up to 50% of urea in the culture medium as

alternative source of nitrogen. This shows that the species under study has high capacity of resistance and resilience when applying high concentrations of urea (Table. 2).

High contents of urea can cause stability of the chlorophyll content “a” and “b”, which in turn can be partially related to what is called photosynthetic maturity point.This point is characterized when the photosynthetic rate of the plant is kept even with the increase of nitrogen content within the cells (Costa *et al.*, 2001).

Thus, the use of urea can be used as inducer for increase of carbon, favoring the growth and development when it is also associated with the regulators. This was observed during *in vitro* cultivation of *Hyssopus officinalis* L., when adding N-phenyl-N'-benzothiazol-6-il-urea (UNP) in the culture medium. UNP is a new derivate from urea associated with cytokinin, which in this case made a significantly better performance possible in terms of percentage of bud propagation and root formation (Rolli *et al.*, 2011).

Table 2. Averages of number of sproutings, bud length, number of roots and roots lengthwhen substituting ammonium nitrate with urea in MS medium for torch ginger at 45 days of cultivation

Treatment	Number of shoots	Length of shoots (cm)	Number of roots	Length of roots (cm)
100% ammonium nitrate	6.73 a*	7.43 a	7.95 a	8.93 a
75% ammonium nitrate + 25% urea	6.52 a	7.21 a	6.6 b	7.28 b
50% ammonium nitrate + 50% urea	5.65 b	5.82 b	6.0 b	6.85 c
25% ammonium nitrate + 75% urea	5.2 b	5.68 b	4.0 c	4.28 d
100% urea	5.0 b	5.23 b	3.9 c	4.02 d
CV%	20.05	15.36	13.25	18.33

*Mean values followed by the same letter do not differ by Scott Knott test (p value<0.05).

The same behaviorial response to the treatments in relation to the number of buds was observed for characteristic sproutings length.The best results were observed in the treatments with75 and 100% of ammonium nitrate, which did not differ significantly between them with 7.21 cm and 7.43 cm respectively.

However, such tendency in relation to the use of urea as source of nitrogen is not common among the vegetal species. As an example of this adaptive inability, when substituting ammonium nitrate by urea in *Sinningia speciosa* (Lodd), concentrations higher than 20% caused death of the cuttings, highlighting the phytotoxic effect of the compound (Fráguas *et al.*, 2003).

Depending on how much N is available, the plants show signs, e.g. the greater synthesis of chlorophyll molecules, which has its precursor in the initial glutamate. Glutamate is an ammonium compound which, besides being a precursor to other amino acids, is also the precursor δ-aminolevulinic acid (ALA), which in turn is considered tetrapyrrolic universal precursor. Thus, the N deficiency leads to decreased synthesis of glutamate, and ALA porphobilinogen syntheses activity and consequently, a decrease in the biosynthesis of chlorophyll, which leads to the development of chlorosis in plants (Chu *et al.*, 2007).

The use of 50% of ammonium nitrate + 50% of urea resulted in average reduction of 21.6% of sproutings length 5.82 cm, in relation to the use of 100% of ammonium nitrate in the medium. Existing a synergistic effect exists between ammonium and nitrate molecules, stimulating the multiple sprouting (Ivanova & Staden, 2009).In relation to the number and length of roots, the best results were observed in the treatments 100%of ammonium nitrate, differing significantly from the other treatments. The average value of 7.95 roots per explant and average length of 8.93 cm was observed.

There was no significant difference among the treatments with 25 and 50% of urea for variable number of roots. The average values observed were 6.6 and 6.0 respectively. In relation to the radicular system in these treatments, there was significant difference. By using 25% of urea in the culture medium, the average value observed was of 7.28 cm. When using 50% of urea, the average value was of 6.85 cm.

It can inferred that plants of *E. elatior* have urea transporters in the membrane of their cells in root, as mentioned by Witte (2011), capable of hydrolyzing urea and very efficiently can the use urea nitrogen source without experiencing toxicity.

In some groups of plants, the administration of urea as a sole source of nitrogen may cause disturbances in morphophysiology and metabolism. A better understanding of plant urea metabolism involving uptake, storage, internal transport, hydrolysis and assimilation of urea nitrogen will be required to assess and possibly improve direct usage of urea by plants (without prior soil conversion) in agricultural settings employing urea fertilization. (Wang *et al.*, 2008; Witte, 2011).

The lowest results observed for all the characteristics were in the treatments with 75 and 100% of urea.In these treatments, the leaves showed chlorotic and early senescence aspect, displaying the intolerance of the species to high levels of this compound in the culture medium.

CONCLUSIONS

The use of 13.32 µM of BAP in MS medium facilitated *in vitro* production of *E. elatior* var. Red Torch in a large scale with average rate of 9 buds per explant. By adding 5.40 µM ANA, this sprouting rate was even higher. Moreover, there was significant increase in the sproutings length with the application of ANA.

In relation to different sources of nitrogen, the treatment with 100% of ammonium nitrate showed the best results for the characteristics evaluated. However, the best cost-benefit of *in vitro*cultivation of torch ginger was highlighted with the use of urea up to 50% in substitution of ammonium nitrate for commercial production in large scale.

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