# Pigment Production and Growth of *Alternanthera* Plants Cultured *in vitro* in the Presence of Tyrosine

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## ABSTRACT

*The aim of the present study was to investigate the influence of tyrosine on the in vitro growth and the production of the betacyanin pigment in* Alternanthera philoxeroides *and* A. tenella. *Nodal segments were inoculated in MS medium containing different concentrations of tyrosine (0, 25, 50 and 75 µM), and the number of sprouts and buds, height, root length, fresh matter of shoots and roots and betacyanin content were evaluated. In* A. philoxeroides, *the highest production of betacyanin (51.30 mg 100 $g^{-1}$ FM) was in the stems with the addition of approximately 45 µM tyrosine, while the increase in the leaves was proportional to the tyrosine concentration, and the best average was obtained with a tyrosine concentration of 75 µM (15.32 mg 100 $g^{-1}$ FM). Higher tyrosine concentrations were deleterious to the growth of* A. tenella *plants, and a concentration of 75 µM was considered toxic. However, a tyrosine concentration of 50 µM benefitted betacyanin production, which reached 36.5 mg 100 $g^{-1}$ FM in the plant shoots. These results showed the positive effect of tyrosine on the production of betacyanin in both species; however, application at high concentrations hampered the growth of* Alternanthera *plants.*

**Key words:** betacyanin, elicitors, *in vitro* propagation, alligator weed, joy weed

## INTRODUCTION

The Caryophyllales order in the family Amaranthaceae comprises 65 genera and approximately 1,000 described species that originate from the tropical, subtropical and temperate zones of Africa, South America and Southeast Asia. The genus *Alternanthera* is a prominent member of this family and consists of 80 species (30 of which have been described in Brazil) (Siqueira 1995). *A. philoxeroides* (Mart). Griseb (alligator weed) and *A. tenella* Colla (joy weed) are two species that deserve special attention due to their medicinal and economic importance. The former, a herbaceous and perennial plant, is considered a vigorous invader in

many regions of the world due to its ability to adapt to different ecosystems (Gunasekera and Bonila 2001). *A. philoxeroides* contains flavonoid glycosides and betalains (Blunden et al. 1999; Rattanathongkom et al. 2009), which are known for their antitumor and antiviral properties (Fang et al. 2007) in addition to anti-inflammatory and immunomodulatory activities (Salvador and Dias 2004). *A. tenella* has been used to treat the infections, fevers, bruises and itches and also has diuretic and anti-inflammatory properties (Vendruscolo and Mentz 2006). Studies with the water extract of joy weed demonstrated immunomodulatory and antitumor activities on the rats (Moraes et al. 1994; Guerra et al. 2003) and *in*

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*vitro* antibiotic activity against Gram-positive and Gram-negative bacteria (Silveira and Olea 2009). The major *A. tenella* components that have been studied are tannins, saponins, flavonoid glycosides and heterosides, such as isorhamnetin, quercetin and kaempferol (Biella et al. 2008), as well as other flavonoids, including vitexin and acacetin (Salvador et al. 2006). Studies on the chemical composition of species of *Alternanthera* showed the presence of betalains, betacyanins, betaxanthins, chromo-alkaloids and flavonoids (Brochado et al. 2003; Cai et al. 2005; Salvador et al. 2006). Betalains are N-heterocyclic natural pigments that are derived from the amino acid tyrosine. Under the action of tyrosine hydroxylase, tyrosine forms an intermediate DOPA (4,5 dihydroxyphenylalanine), which is oxidized into cDOPA. This compound leads to the formation of the class of betalains (Tanaka et al. 2008), which are classified into two groups: betaxanthins (yellow) and betacyanins (red). The betacyanins may also be chemically classified into four types: betanin, amaranthine, gonferine and bougainvillein (Volp et al. 2009).

Several authors have studied the bioactivities of these pigments, including their antiviral and antibiotic effects (Strack et al. 2003). Their antioxidant properties have been shown in a wide range of tests (Kanner et al. 2001; Gentile et al. 2004; Tesoriere et al. 2008). For example, Tesoriere et al. (2003) reported that enrichment of human low-density lipoproteins with betalains increased the oxidation resistance, slowing cell aging. Muntha et al. (2005) also documented that natural pigments, such as betanin, could inhibit cell proliferation of a wide variety of human tumor cells (Muntha et al. 2005). Several authors have suggested *in vitro* culturing of betacyanin-producing plants to optimize the large-scale production of these pigments (Santos-Diaz et al. 2005; Savitha et al. 2006; Georgiev et al. 2008; Pavokovi et al. 2009). Chemical synthesis of these molecules does not seem feasible due to a lack of clarification of several steps involved in this synthetic process (Pavokovi and Krsnik-Rasol 2011). Precursor amino acids, which are considered chemical elicitors, have been used successfully both in the cultured plant cells and in intact plants to improve the pigment production (Berlin et al. 1986; Silva et al. 2005). Given the medicinal importance of betacyanin and species of the genus *Alternanthera*, the aim of the present study was to evaluate the influence of

tyrosine on the morphological characteristics of and betacyanin production in *A. philoxeroides* and *A. tenella* cultured *in vitro*.

## MATERIALS AND METHODS

The taxonomy of *A. philoxeroides* (alligatorweed) plants from the municipality of Rio Grande, state of Rio Grande do Sul (RS), Brazil was confirmed by the Amaranthaceae identification key, and the plants were cataloged in the PEL Herbarium under the number 24.53. *A. tenella* (joy weed) plants from the municipality of Pelotas, RS, Brazil had their taxonomy confirmed and were cataloged in the PEL Herbarium under the number 25.26.

Nodal segments of new stems containing one, or two axillary buds of the plants kept for 15 days in the greenhouse were used for *in vitro* establishment of both the species. The segments were washed in tap water and distilled water under mechanical stirring for 15 min. The material was immersed in 70% ethanol for 20 seconds, followed by immersion in 1% sodium hypochlorite with three drops of Tween (20 min). All the procedures were intercalated with autoclaved water baths.

Basic MS medium (Murashige and Skoog 1962) with no growth regulators, pH adjusted to 5.8 and 7.0 g $L^{-1}$ of agar was used for *in vitro* culturing of *Alternathera* plants. Bottles containing 40 mL of culture medium were autoclaved at 121ºC for 20 min. After autoclaving, tyrosine (0, 25, 50 and 75 µM) filtered and solubilized in dimethyl sulfoxide (DMSO) was added to the medium. Experiments with both the species were performed separately as follows.

### Experiment 1
Explants of *A. philoxeroides* were inoculated in the culture medium in a laminar flow hood under aseptic conditions. Bottles with the explants were placed in a growth room kept under a photoperiod of 16 h, photon flux density of 48 µmoL $m^{-2}$ $s^{-1}$ and temperature of 23 ± 2°C. After 40 days of experimental implantation, the average number of auxiliary buds and stems, height (cm), stem fresh matter (mg), root length (cm), root fresh matter (mg) and betacyanin quantification (amaranthine, mg 100 $g^{-1}$ FM) were evaluated. To analyze the betacyanin content, leaves and stems were macerated separately in 0.5 mL of distilled water and then centrifuged at 13.632 g at 4ºC for 25 min. The supernatant was used to quantify the

betacyanins by monitoring the absorbance at 536 nm and 650 nm with an Ultrospec 2100 Pro spectrophotometer (Amersham Biosciences®). Betacyanin concentration was determined using the molar extraction coefficient of amaranthine ($5.66 \times 10^4$) according to the method described by Cai et al. (1998).

The experimental design was completely randomized with four concentrations of tyrosine and five replicates. Each experimental unit consisted of one bottle containing five explants. The results were subjected to analysis of variance (ANOVA) and polynomial regression using WinStat statistical software (Machado and Conceição 2002).

**Experiment 2**

The *in vitro* culturing and growth evaluations of *A. tenella* followed the same method described above for *A. philoxeroides*. However, for betacyanin quantification, stems and leaves were added together and considered as shoots to obtain the amount of fresh matter required for analysis. The experimental design used was completely randomized with four treatments (concentrations of tyrosine) and four replicates. The experimental unit consisted of one bottle containing four explants. The results were subjected to ANOVA, and the means were compared by a Tukey's post-test with a 5% error probability using WinStat statistical software (Machado and Conceição 2002).

**RESULTS AND DISCUSSION**

*In vitro* **culturing of *A. philoxeroides***

The concentration of tyrosine negatively influenced the plant height, with the highest average (6.91 cm) found in the control plants (Fig. 1 and 2A). Although nitrogen was an essential nutrient for plant growth and development, excess organic nitrogen, due to the presence of tyrosine, might have acidified the culture medium, which hampered the growth of *A. philoxeroides* shoots.

The length and fresh matter of roots decreased as the tyrosine concentration increased and no rooting was detected at a concentration of 75 μM. In a study with lentil (*Lens culinaris* Medik), Sarker et al. (2003) found that 20 μM tyrosine combined with other growth regulators showed successful regeneration and *in vitro* rooting. However, in the studies with *A. brasiliana,* Silva et

al. (2005) showed that the plants cultured in MS medium with 10 μM tyrosine under white light showed no difference in the root length compared to the control, with averages of 7.40 cm and 7.05 cm, respectively.



A          B          C          D

**Figure 1 -** Plants of *Alternanthera philoxeroides* grown for 40 days in MS medium with different tyrosine concentrations. Control (A); 25 μM tyrosine (B); 50 μM tyrosine (C); 75 μM tyrosine (D). Scale 1 (cm).

In the present study, the treatment with 25 μM tyrosine led to a significant difference in the root formation of *A. philoxeroides*. As the concentration of tyrosine increased in the culture medium, the length and fresh matter of the roots were significantly reduced (Fig. 2B and 2C). In a study with soy plants (*Glycine max* L.) treated with hydroxylated tyrosine, Soares (2006) found that the average total length and number of roots were 20.9 and 76.7% smaller than the controls for treatments with 25 μM and 100 μM, respectively, indicating that this amino acid might have an adverse effect on rooting. According to Oliveira et al. (2009), direct amino acid absorption by the roots offers advantages to plants because they do not need to metabolize the mineral nitrogen (nitrate and ammonium), thus directing more energy for rooting. However, excess amino acids can cause substrate acidification, leading to toxicity.

Betacyanin production in the stems peaked at approximately 45 μM (51.30 mg 100 $g^{-1}$ FM), while the increase in the leaves was proportional to the tyrosine concentration, with the highest average level obtained with a tyrosine concentration of 75 μM (15.32 mg 100 $g^{-1}$ FM)

(Fig. 2D). Taha et al. (2008) found that the addition of amino acid precursors, such as tryptophan and glutamine, in *Catharanthus roseus* cultured in MS medium, increased the production of vinblastine and vincristine in the treated cells by up to 75%. In callus tissue cultures of velvet bean (*Mucuna pruriens),* Desai et al. (2010) found that the application of 140 μM of various amino acid precursors, including tyrosine, increased the production of L-DOPA by up to 2%. These authors noted that the enzyme triggering the formation of betacyanin, tyrosine hydroxylase, required copper and that copper was a micronutrient in the formulation of MS medium.

Thus, the addition of the substrate tyrosine might have facilitated the action of the enzyme and triggered reactions that increased the content of betacyanin. Similar to what happened in *A. brasiliana* cultured in MS medium containing tyrosine, Silva et al. (2005) found that the accumulation of betacyanin was higher than in the control.
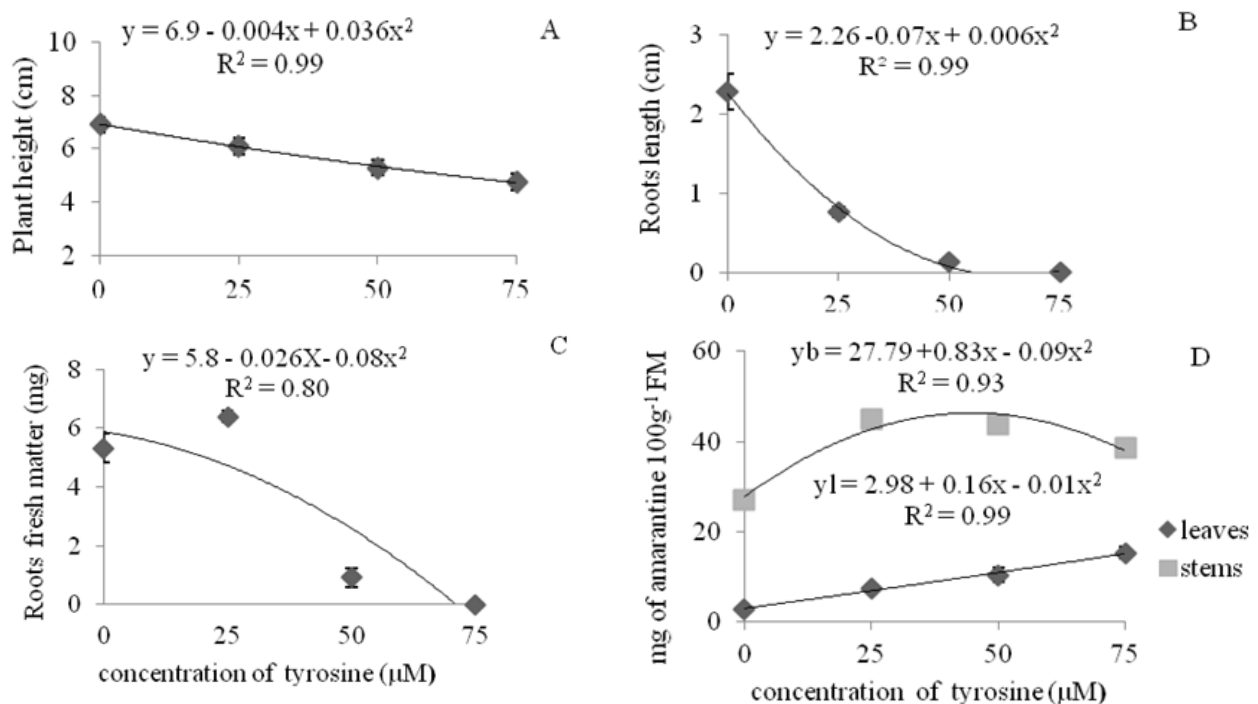


**Figure 2 -** Plant height (A), roots length (B), root fresh matter (C) and betacyanin production (D) of *Alternanthera philoxeroides* grown *in vitro* for 40 days in MS culture medium with different of tyrosine concentrations. Vertical bars represent the standard error for the average of five repetitions.

Tyrosine was used in the present study because it was the precursor amino acid in betacyanin synthesis. According to Georgiev et al. (2008), tyrosine addition should stimulate the synthesis pathway of its corresponding secondary metabolite. This was observed in the leaves and stems of *A. philoxeroides*, except with the highest concentration used in the stem. Certain factors must be considered, such as the cellular capacity for the accumulation of the compound and whether the metabolite of interest is an end product of the biosynthetic pathway (Verpoorte and Maraschi 2001). The target metabolite is not a final product

of the biosynthetic pathway; the rate of its catabolism may become ineffective with the addition of the precursor. Moreover, the cellular capacity for accumulation of this secondary metabolite is currently not well understood. However, limited accumulation of these compounds, which may influence the yield and accumulation of this pigment in *A. philoxeroides*, is expected.

### *In vitro* **culturing of** *A.* **tenella**
The presence of the tyrosine in the culture media had a negative influence on the morphological

characteristics of *A. tenella*. The highest concentration (75 µM) was completely deleterious, preventing the development of the explants. In the present study, the high levels of organic nitrogen in the culture media might have hindered nitrogen assimilation in the form of nitrate, which was preferentially used by the plants (Sen and Batra 2011) and was also present in the MS culture medium formulation. Difficulty in assimilation could account for the deficit in the growth of shoots and roots and the death of nodal segments of *A. tenella* in the presence of 75 µM tyrosine as nitrogen was essential for the growth and development of plants (Schröder et al. 2000).

The formation of new buds was also negatively influenced by the increase in the amino acid concentration in the culture medium and the smallest average was obtained with 50 µM tyrosine. Nitrogen is a constituent of amino acids, nucleotides and coenzymes (Kanashiro et al. 2007); therefore, there is a correlation between the availability of nitrogen and the formation of new organs. The presence of aromatic amino acids with hydroxyl groups, such as Ser and Tyr, in the nutrient solution could have affected the magnesium transporter activity (Garcia et al. 2011), and thus, the development off the plant and the formation of new sprouts and buds (Fig. 3A and Fig. 3B).

The increase in the concentration of organic nitrogen in the medium might have increased the osmotic potential and thereof, hindered the uptake of water by the explants of *A. tenella*. This might have affected their growth in height (Fig. 3C), as previously described for the plants of Brazilian ginseng (*Pfaffia glomerata* Spreng.) where growth was higher at the typical concentration of nitrogen in MS medium and decreased as the organic nitrogen concentration increased (Russowski and Nicoloso 2003).

The al deficiency of *A. tenella* with the treatments used in the present study influenced the fresh biomass of these plants. The biomass decreased with increasing tyrosine, and the lowest average (615 mg) was obtained with 50 µM tyrosine (Fig. 3D). These results corroborated those of Silva et al. (2005), who tested other factors besides tyrosine in the *in vitro* culture of *A. brasiliana*. Regardless of the treatment used, these authors found that the presence of tyrosine in the culture medium led to the lowest values of dry matter.

Berlin et al. (1986) also found this negative effect in *Chenopodium rubrum* L. (red goosefoot) cell culture using 15 µM tyrosine. Their results revealed that high amounts of this amino acid might be toxic to cell proliferation and growth of this species. The rhizogenesis of *A. tenella* was significantly different with the presence of tyrosine in the culture medium. The highest values of fresh root matter and main root length were found in the plants grown in tyrosine-free media (Fig. 3E and Fig. 3F).

However, after 40 days of culture, there was a significant increase in the betacyanin content with 50 µM tyrosine, and a value of 36.95 mg of amaranthine of 100 g $FM^{-1}$ was achieved (Fig. 5).

Tyrosine has also been tested as an elicitor agent for the production of various secondary metabolites in several plant species. In *Cereus peruvianu* callus tissue culture, an increase in the production of tyramine and hordenine alkaloids previously identified in this species was found with the addition of 1.1 M tyrosine (Rocha et al. 2005). Tyrosine and other amino acids were tested to promote protoberberine alkaloid production in the *in vitro* culture of *Thalictrum minusa*. However, Urmantseva et al. (2005) found that the addition of tyrosine hindered the formation of alkaloids compared to the control treatment.

The increase in betacyanin production due to the elicitor action of tyrosine has been reported. Berlin et al. (1986) used 15 µM tyrosine in the culture of *Chenopodium rubrum* callus tissue and demonstrated an increase in the production of betalains of 50 to 100% after 28 days of culture. Silva et al. (2005) found that the production of betalains increased compared to the control in the *in vitro* culture of *A. brasiliana* for 45 days in MS medium supplemented with 10 µM tyrosine. Other elicitors have also been used to increase the production of betalains in the plants. Savitha et al. (2006) tested seven different metal ions in concentrations up to ten times higher than the ones present in MS medium and found that calcium could induce up to a 47% higher betalain production. Using polyamines as elicitor agents, Bais et al. (2000) doubled the productivity of betalains in beet (*Beta vulgaris*) crops. Suresh et al. (2004) found similar results using putrescine and spermidine in bioreactors, increasing the content of betalains in beet cultures by 1.3 times.
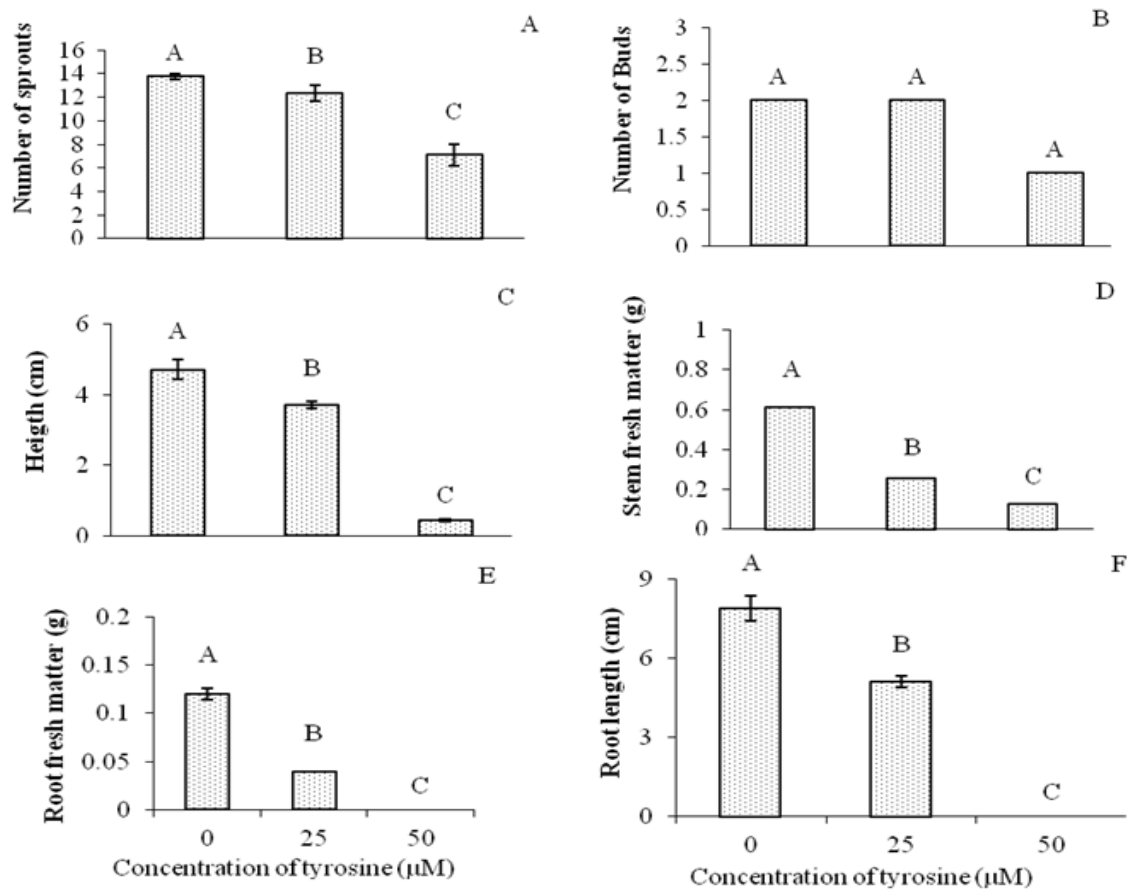
**Figure 3 -** Number of sprouts (A), buds  number (B), height (C), stem fresh matter (D), root fresh matter (E), root length, (F) of  *Alternanthera tenella* grown *in vitro* for 40 days in MS medium with different concentrations of tyrosine. Values  followed  by  different  letters are  significantly  different  (p $\leq$ 0.05;  Tukey  test). Vertical bars represent the standard error for the average of four repetitions.
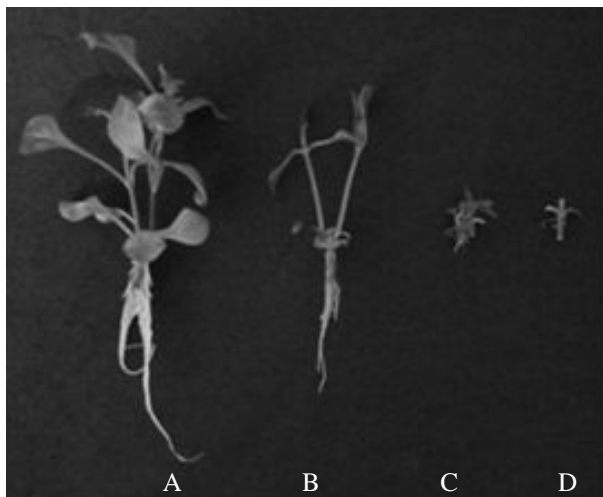


**Figure 4 –** Plants of *Alternanthera tenella* grown for 40 days in MS medium with different tyrosine concentrations. Control (A), 25 µM tyrosine (B), 50 µM tyrosine (C) and 75 µM tyrosine (D). Scale 1 (cm).
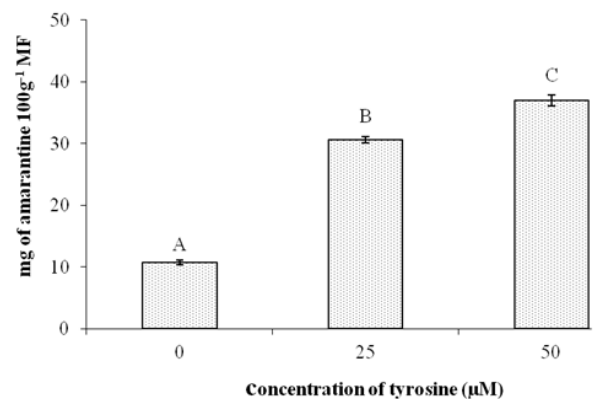


**Figure  5 -** Betacyanin  production  in  plants  of *Alternanthera tenella in vitro* for 40 days  in  MS  medium  with  different concentrations  of  tyrosine.  Values followed  by    different  letters  are significantly  different  (p$\leq$ 0.05; Tukey test). Vertical bars represent the standard error for the average of four repetitions.

In the present study an increased production of betacyanin was found with the addition of tyrosine. Such a response could be related both to the stress caused by the presence of the elicitor in the medium and mainly due to the increased availability of tyrosine, which was the initial substrate for tyrosine hydroxylase (TOH), the enzyme that converted tyrosine to DOPA and led to the formation of betacyanins. By interacting with membrane receptors, the presence of elicitors in the culture medium can trigger responses in the secondary metabolism of the plants. Savitha et al. (2006) noted that stressors would also be able to activate specific genes in the enzymatic machinery involved in the biosynthesis of secondary metabolites such as betacyanins, key pigments in the plants due to antioxidant and free radical-scavenging properties (Gandía-Herrero et al. 2009).

## CONCLUSION

High concentrations of tyrosine had a negative effect for the *in vitro* growth of *Alternanthera* species affecting the formation of shoots and roots, but the addition of this amino acid in the culture medium increased the biosynthesis of betacyanin in the studied two species. The involvement of tyrosine in the  metabolism of natural compounds such as alkaloids and other nitrogen products such as betacyanin has been well established in the literature. But to-date little is known about its mechanism of control and regulation, and the role in secondary metabolism of the two medicinal plant species tested. This study demonstrated that the presence of tyrosine, as an elicitor in the culture medium could cause a considerable increase in the production of amaranthine, a natural derivative important to the nutraceutical industry.

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