

Trypsin inhibitor in *Enterolobium contortisiliquum* calli grown in the presence of plant growth regulators

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Abstract – The objective of this work was to investigate the effect of plant growth regulators (PGR) on the induction of calli from cotyledons of *Enterolobium contortisiliquum* grown in vitro, as well as to evaluate the inhibitory activity of trypsin in these calli. Cotyledons of *E. contortisiliquum* were cultivated in culture medium supplemented with dichlorophenoxyacetic acid (2,4-D), picloram (PIC), kinetin (KIN), and 6-benzylaminopurine (BAP). Callus mass, inhibitory activity of trypsin, morphogenetic and cytochemical observations, and biological activity were measured after 60 days. The Pareto diagram showed that only 0.5 mg L⁻¹ PIC influenced the increase of fresh mass, whereas 0.5 mg L⁻¹ BAP influenced dry mass. The principal component analysis plot showed the formation of two clear clusters. The inhibitory activity of trypsin is achieved at a higher intensity in the treatment with 0.5 mg L⁻¹ 2,4-D + 2.0 mg L⁻¹ PIC + 0.5 mg L⁻¹ KIN + 2.0 mg L⁻¹ BAP. The combination of PGR at different rates causes an intense callus formation. Low concentrations of PGR result in a higher fresh mass, dry mass, and biological activity. The use of PGR is efficient in obtaining *E. contortisiliquum* calli with a satisfactory inhibitory activity of trypsin.

Index terms: biospeckle laser, plant growth regulators, secondary metabolites.

Inibidores de tripsina em calos de *Enterolobium contortisiliquum* cultivados na presença de reguladores de crescimento de plantas

Resumo – O objetivo deste trabalho foi investigar o efeito de reguladores de crescimento de plantas (RCP) na indução de calos a partir de cotilédones de *Enterolobium contortisiliquum* cultivados in vitro, bem como avaliar a atividade inibitória de tripsina nestes calos. Cotilédones de *E. contortisiliquum* foram cultivados em meio de cultura suplementado com ácido diclorofenoxiacético (2,4-D), picloram (PIC), cinetina (KIN) e 6-benzilaminopurina (BAP). Foram mensuradas, após 60 dias, massa de calo, atividade inibitória de tripsina, observações morfogenéticas e citoquímicas, e atividade biológica. O diagrama de Pareto mostrou que apenas 0,5 mg L⁻¹ de PIC influenciou o aumento da massa fresca, enquanto 0,5 mg L⁻¹ de BAP influenciou a massa seca. O gráfico de análise de componentes principais mostrou a formação de dois clusters claros. A atividade inibitória de tripsina é alcançada em maior intensidade no tratamento com 0,5 mg L⁻¹ de 2,4-D + 2,0 mg L⁻¹ de PIC + 0,5 mg L⁻¹ de KIN + 2,0 mg L⁻¹ de BAP. A combinação de RCP em diferentes doses causa intensa formação de calo. Baixas concentrações de RCP resultam em maior massa fresca, massa seca e atividade biológica. A utilização de RCP é eficiente na obtenção de calos de *E. contortisiliquum* com satisfatória atividade inibitória de tripsina.

Termos para indexação: biospeckle laser, reguladores de crescimento de plantas, metabólitos secundários.

Introduction

Human population has been growing at an alarming rate, requiring food production to be further leveraged to meet food demand (Crist et al., 2017). In conventional agriculture, to successfully achieve high yields, most plants need to be cultivated with the concomitant use of some type of agricultural pesticide (Campos et al., 2019). However, these agrochemicals can be over- and incorrectly used, causing harm to the environment due to soil and water contamination, toxicity to plants, and loss of resistance in genetically improved plants (Bhushan & Pathma, 2021), besides being toxic to humans and animals (Gupta et al., 2019).

Therefore, this conventional agricultural model needs to be revised. In this context, sustainable agriculture emerges, which is a model based on improving the efficient use of agrochemicals with innovative and less harmful alternatives (Ganguly et al., 2020). Among these alternatives, the use of pesticides produced from molecules derived from the secondary metabolism of plants is one of the most important and promising (Costa et al., 2019).

In this line of research, protease inhibitors are among the most studied secondary metabolites, along with essential oils. Those inhibitors are mainly composed of proteins or peptides with properties that inhibit the catalytic action of enzymes by interacting at varying degrees of affinity with reactive sites (Singh et al., 2020). The most studied protease inhibitors belong to the Fabaceae, Solanaceae, and Poaceae families (Singh & Yadav, 2020); however, the amount and type of inhibitor varies among plant species. The response induced by these inhibitors can result in a resistance against pathogens and/or insects, which is expressed systematically by the plant and protects all of its tissues against the attack of herbivorous insects due to the specificity of the inhibitors to most of the digestive proteases of these pests (Singh et al., 2020). The use of inhibitors to combat insects by marking their digestive enzymes has been highlighted in the scientific community, with studies showing how the growth and development of these pests is affected when they are fed with transgenic plants that express these proteins (Nunes et al., 2021).

In Brazil, notably in the Cerrado region, a wide range of plant species has been the target of researches. The species *Enterolobium contortisiliquum* (Vell.) Morong. (Fabaceae), also known as “black ear”,

is a tree that can exceed 20 m in height and has a large amount of protein in its seed cotyledons. In a previous study, Batista et al. (1996) isolated, for the first time, a Kunitz-type trypsin inhibitor from *E. contortisiliquum* seeds. Kunitz domains present in *E. contortisiliquum* are considered secondary metabolites, despite their protein nature (Oliveira et al., 2013), since they do not play a primary role in plant metabolism. When these substances are extracted from a natural source, the production of metabolites in vitro has the potential to overcome low yields and production seasonality. Moreover, the production of the inhibitor in vitro can guarantee the continuous availability of the product, besides allowing the use of numerous strategies to stimulate the specific production of the necessary metabolite.

In in vitro cultures that aim to obtain metabolites, callus culture is one of the most preferred techniques. A callus is a disorganized mass of cells with some degree of differentiation and it can be formed, for example, by the stimulus of a combination of plant growth regulators (PGRs) (Gorelick & Bernstein, 2014). PGRs play a significant role in inducing morphogenetic responses in explants. In general, auxins and cytokinins are the most used regulators for the majority of plants, acting in the development and accumulation pathways of bioactive molecules (Chaâbani et al., 2015). Akinropo et al. (2020) used these PGRs in experiments with plants of the *Enterolobium* genus, and Shim et al. (2010) found that the callus culture is currently an important bioprospecting strategy for natural products, which has been used as an alternative in the production of several secondary metabolites.

The objective of this work was to investigate the effect of plant growth regulators on the induction of calli from cotyledons of *Enterolobium contortisiliquum* grown in vitro, as well as to evaluate the inhibitory activity of trypsin in these calli.

Materials and Methods

Seeds of *E. contortisiliquum* harvested in 2016, in the municipality of Coronel Murta, in the north of the state of Minas Gerais, Brazil (16°36'31.5"S, 42°11'45.7"W), were established in vitro. The tegument of the seeds was removed with a scalpel (Figure 1 A and B), and seeds were subjected to

asepsis, consisting of immersion in distilled water with two drops of Tween 20 (Sigma-Aldrich Brasil Ltda., São Paulo, SP, Brazil) for 1 min, immersion in 70% (v/v) alcohol for 1 min, and immersion in 1.0% sodium hypochlorite solution for 20 min. Subsequently, the disinfectants were leached by triple washing in sterile distilled water.

After asepsis in a laminar flow chamber, the cotyledons were separated from the embryonic axis to avoid seed germination, since the inhibitor was previously located in the cotyledons (Batista et al., 1996). The cotyledons were then placed individually in 25x150 mm test tubes containing 15 mL of the Murashige & Skoog culture medium (Murashige & Skoog, 1962), supplemented with PGR according to each treatment (Table 1). In addition, 5.5 g L⁻¹ agar and 30 g L⁻¹ sucrose were added to the medium. The pH of the medium was adjusted to 5.7±0.3 before autoclaving at 121±1°C and 1.05 atm pressure for 20 min. After cotyledon inoculation, the test tubes were conditioned in a growth room at 25±2°C, without light.

The experiment was performed in a 2⁴⁻¹ fractional factorial design with a center point (Table 1), using four growth regulators: two auxins – 2,4-dichlorophenoxyacetic acid (2,4-D) and picloram (PIC); and two cytokinins – kinetin (KIN) and 6-benzylaminopurine (BAP). The used concentrations were: minimum of 0.5 mg L⁻¹, maximum of 2.0 mg L⁻¹, and center point of 1.25 mg L⁻¹. This procedure was adopted because, in preliminary tests, no callus formation was observed in the explant culture in the medium without growth regulators (Figure 1 C); the other treatments presented the formation of a compact callogenic mass (Figure 1 D).

The phytotechnical evaluations of callus mass were performed on an analytical balance, by measuring first the fresh mass and then the dry mass after drying in a convection oven until reaching constant mass. Data were subjected to the analysis of variance and, when significant, means were compared by Scott-Knott's test, at 5% probability, using the R software (R Core Team, 2020).

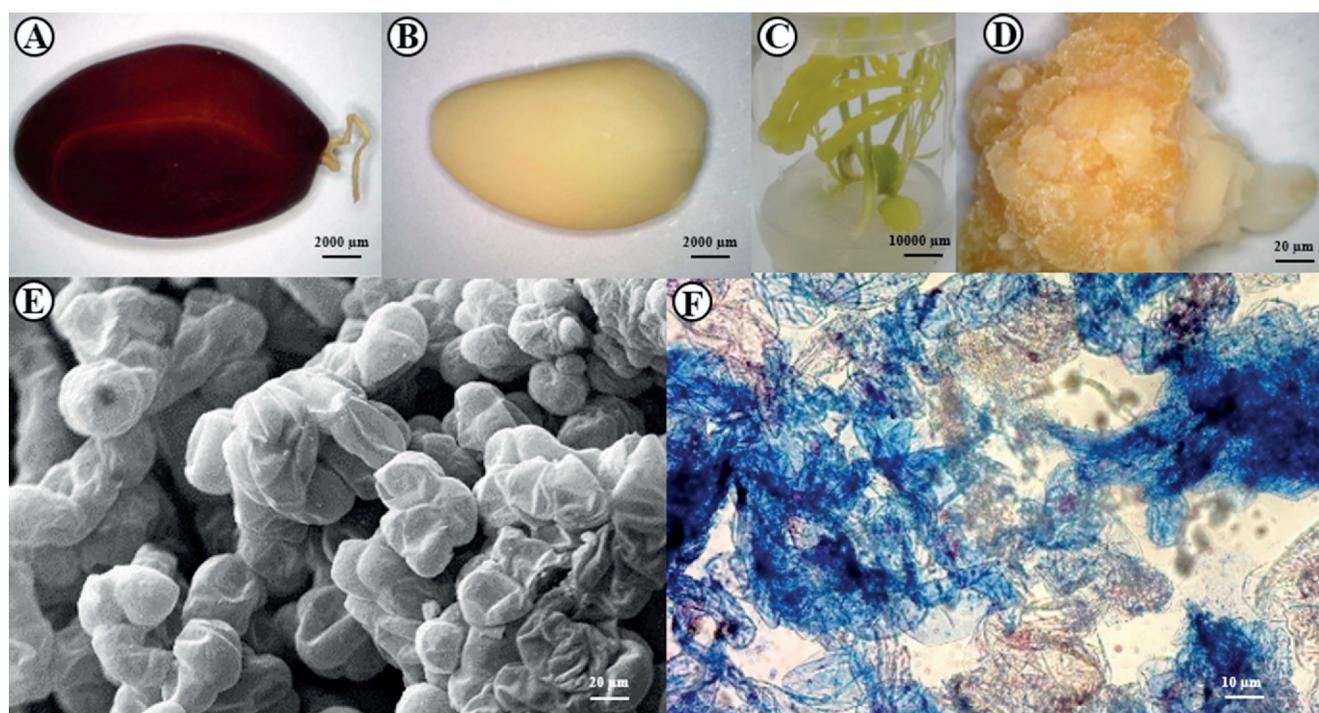


Figure 1. Morphology and histology of cotyledons and callus of *Enterolobium contortisiliquum*, showing: seed with presence of tegument (A); cotyledon (B); seedling germinated in the absence of growth regulators (C); callus obtained from cotyledon cultured with combinations of growth regulators (D); scanning electron microscopy (E); and cytochemical test evidencing the predominance of Evans blue dye, indicating the presence of non-embryogenic cells (F).

To obtain the extract containing trypsin used in all enzymatic analyses, the digestive tubes of sixth-instar *Spodoptera frugiperda* (J.E. Smith) caterpillars reared in a laboratory were removed after immobilization at -20°C for 10 min. This procedure was carried out since, in enzymatic assays, the use of a commercial enzyme, such as bovine trypsin, may provide imprecise data due to differences in insect physiology and protein structure. The crude extract obtained after maceration in a Potter homogenizer – one digestive tract to 4.0 mL distilled water at 4°C – was filtered on a 100 μm nylon mesh and centrifuged at 10,000 g, at 4°C , for 30 min; the trypsin enzyme extract (supernatant) was stored at -20°C until its use (Rossi et al., 2010). All reagents used in enzymatic assays were purchased from Sigma-Aldrich Brasil Ltda. (São Paulo, SP, Brazil). A solution with N- α -benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPNA) at 1.25 mmol L⁻¹ and 0.1 mol L⁻¹ glycine-sodium hydroxide (Gly-NaOH) buffer (pH 9.7) was used in the trypsin inhibition assays. A total of 54.5 mg BAPNA were diluted in 1.25 mL dimethyl sulfoxide (DMSO), and the volume was filled to 100 mL with Gly-NaOH buffer. In the assay, the periods of reaction time were 30, 60, 90, and 120 min. In order to initiate the reaction, 200 μL suitably diluted enzyme extract, 200 μL *E. contortisiliquum* extract containing the inhibitor, and 800 μL substrate solution were added to the reaction mixture. The control test was analyzed in the absence of the inhibitor. A 30% acetic acid

Table 1. Combinations of the auxins 2,4-dichlorophenoxyacetic acid (2,4-D) and picloram (PIC) and the cytokinins kinetin (KIN) and 6-benzylaminopurine (BAP) used for callus induction in cotyledons of *Enterolobium contortisiliquum*.

Treatment ⁽¹⁾	Plant growth regulator (mg L ⁻¹)			
	2,4-D	PIC	KIN	BAP
1	0.5	0.5	0.5	0.5
2	2.0	0.5	0.5	2.0
3	0.5	2.0	0.5	2.0
4	2.0	2.0	0.5	0.5
5	0.5	0.5	2.0	2.0
6	2.0	0.5	2.0	0.5
7	0.5	2.0	2.0	0.5
8	2.0	2.0	2.0	2.0
Center point	1.25	1.25	1.25	1.25

⁽¹⁾Replicates = 12.

solution (200 μL) was used to stop the reaction, and absorbance was determined at a wavelength of 410 nm (Erlanger et al., 1961). The trypsin inhibitor unit (TIU) was calculated according to the following equation: $\text{TIU} = (I_c - I_i)/I_E$, where I_c is the slope of the line from the plot absorbance x time (min) related to the control assay, I_i is related to the inhibition assay, and I_E is the slope of line obtained with the p-nitroanilide standard by Erlanger et al. (1961). The results were expressed in mTIU g⁻¹ of fresh seed or callus.

For the analysis of the morphogenetic nature of the callus, samples were collected and dehydrated in a growing ethanolic series using a critical point (Horridge & Tamm, 1969), then metallized and observed with the LEO Evo 40 scanning electron microscope (Zeiss, Jena, Germany).

For the cytochemical analysis, 100 mg callus fractions were collected from each treatment and macerated on watch glass with the aid of a stick. Three drops of 0.1% Evans blue dye were added for 3 min and its excess was removed; three drops of 2.0% acetic carmine dye were added at the same time (Steiner et al., 2005). Finally, slides were mounted and photographs were taken using the CX31 light microscope (Olympus, Tokyo, Japan), to which the Eurekam 5.0 digital camera (BEL Engineering s.r.l., Monza, Italy) was attached.

For the analysis of biological activity, the biospeckle laser technique was used (Ansari et al., 2018). The test tubes were illuminated by a coherent light, and the interference patterns, formed by the interaction of light with the callus, were captured by a digital microscope arranged in the backscattering configuration. The used coherent light came from a red laser diode with a wavelength of 632 nm, magnified by a set of lenses sufficient to cover the entire sample. The distance between the lens and the sample was 0.75 m. The interference patterns formed by the interaction of light with the callus were collected with the AM-413zt digital portable microscope (Dino-Lite, São Paulo, SP, Brazil). For each light session, a set of 128 grayscale images, with a resolution of 1,280x1,024 pixels and intervals of 0.08 s between frames, were stored. Data analysis and interpretation were performed by image analysis with numerical approximations, which returned information related to the variability of the biological activity of the callus according to the tested growth regulators.

Results and Discussion

Regardless of the combinations of the tested growth regulators, there was a large production of fresh and dry masses of calli (Table 2). For fresh mass, the highest values were found in treatments 1, 2, 4, 5, and 6 and, for dry mass, in 1, 4, and 7. For callus induction, among the classes of growth regulators most commonly used, auxins and cytokinins stand out. However, their use may be limited due to the opposing effects between them and the secondary metabolism (Raj et al., 2015). Therefore, to increase the productivity of bioactive compounds, it is necessary to supplement cultures with balanced auxin/cytokinin concentrations (Murthy et al., 2014).

In the analysis of biological activity, the highest values were observed in treatments 1, 5, 7, 8, and 9 using the biospeckle laser technique, which allows measuring the degree of particle movement inside the cells of the studied material. The evaluated activities are not related to a specific phenomenon, but to a set of phenomena, including cell growth and reproduction and processes related to organelle movement, cytoplasmic flow, or even chemical reactions (Braga et al., 2009).

The Pareto chart indicates which variables were significant (Figure 2). From this diagram, it was possible to identify the parameters or the interactions between parameters that most influenced the fresh and

dry masses, as well as biological activity. An analysis based on linear and quadratic effects was performed in the construction of the diagram, resulting in the influence of an isolated factor for fresh and dry masses and in the interaction among three factors for biological activity. The diagram analysis revealed that only PIC at the lowest concentration of 0.5 mg L⁻¹ influenced the increase of fresh mass, i.e., the addition of 2,4-D, KIN, and BAP in the culture medium did not affect fresh mass production (Figure 2). For dry mass, only BAP, also at the lowest concentration, influenced dry mass production. However, in the analysis of biological activity, there was an interaction between 2,4-D, KIN, and PIC. Biological activity was the highest with 2,4-D at the lowest concentration, also being affected by KIN and PIC at higher concentrations, but not by BAP.

The principal component analysis (PCA) showed that 63.31% of the variance was explained by the first (PC1) and third (PC3) components, totaling 43.47 and 19.84%, respectively (Figure 3). The positioning of each variable in the plot represents its behavior against the others, so that a greater proximity among variables indicates a greater correlation among them. Variables that are on opposite sides have a negative correlation, whereas those positioned on the same side have a positive one. The PCA plot showed that two clusters were formed (Figure 3). The first, including treatments 2, 3, 4, and 6, is positioned at a greater distance from the biological activity vector but closer to the

Table 2. Fresh mass, dry mass, and biological activity of *Enterolobium contortisiliquum* callus cultured in Murashige & Skoog medium with different combinations of growth regulators⁽¹⁾.

Treatment	2,4-D	PIC ----- (mg L ⁻¹) -----	KIN	BAP	Fresh mass (g)	Dry mass (g)	Biological activity ⁽²⁾ (no unit)
1	0.5	0.5	0.5	0.5	7.3315a	0.5720a	18.05a
2	2.0	0.5	0.5	2.0	7.8870a	0.3916b	8.89d
3	0.5	2.0	0.5	2.0	6.0228b	0.3408b	12.24b
4	2.0	2.0	0.5	0.5	7.4000a	0.4362a	10.37c
5	0.5	0.5	2.0	2.0	7.5642a	0.3124b	17.00a
6	2.0	0.5	2.0	0.5	7.4600a	0.3130b	9.94c
7	0.5	2.0	2.0	0.5	6.6540b	0.4895a	18.33a
8	2.0	2.0	2.0	2.0	5.5107b	0.3593b	17.70a
Center point	1.25	1.25	1.25	1.25	6.1017b	0.3080b	17.63a
Coefficient of variation (%)					15.82	34.49	6.36

⁽¹⁾Averages followed by equal letters, in the columns, do not differ by Scott-Knott's test, at 5% probability. ⁽²⁾Biological activity is a variable without unit and its analysis allows measuring the degree of particle movement inside the cells of the studied material. This movement is related to cell growth and reproduction, as well as to processes related to organelle movement, cytoplasmic flow, or even chemical reactions. 2,4-D, dichlorophenoxyacetic acid; PIC, picloram; KIN, kinetin; and BAP, 6-benzylaminopurine.

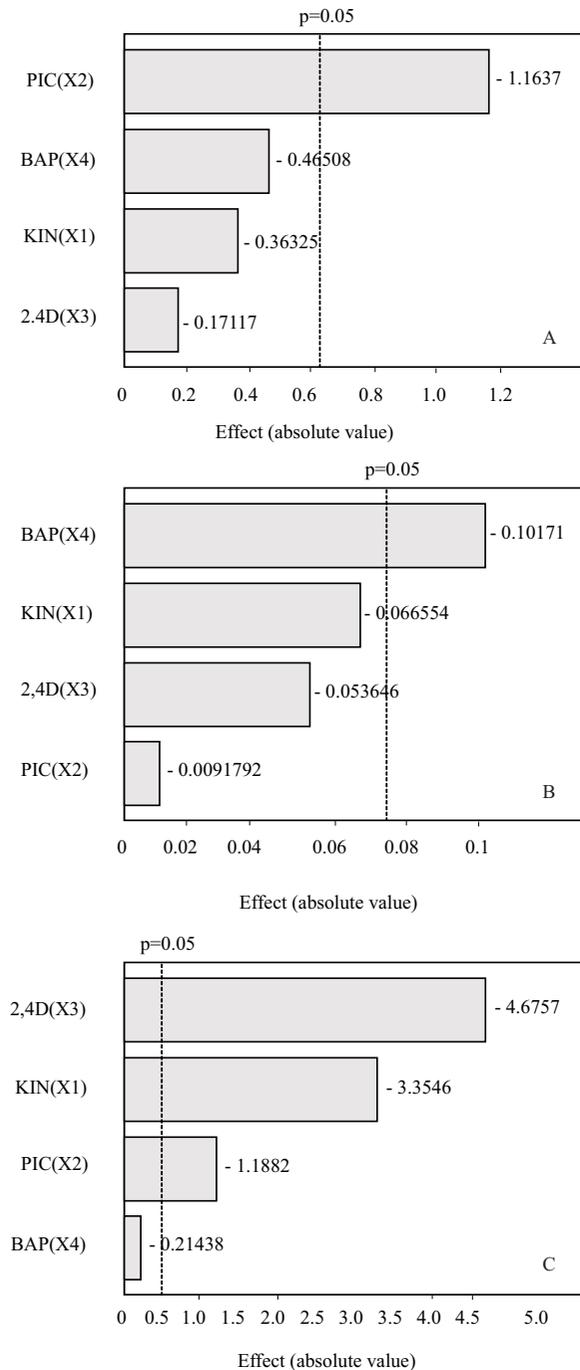


Figure 2. Pareto chart showing the significant variables in the factorial design for: fresh mass (A), dry mass (B), and biological activity of *Enterolobium contortisiliquum* callus cultured in Murashige & Skoog medium with different combinations of growth plant regulators (mg L^{-1}) (C). The tested growth plant regulators are displayed on the Y-axis: PIC, picloram; BAP, 6-benzylaminopurine; KIN, kinetin; and 2,4-D, dichlorophenoxyacetic acid. The bars beyond the dotted line indicate that that growth regulator showed a significant effect.

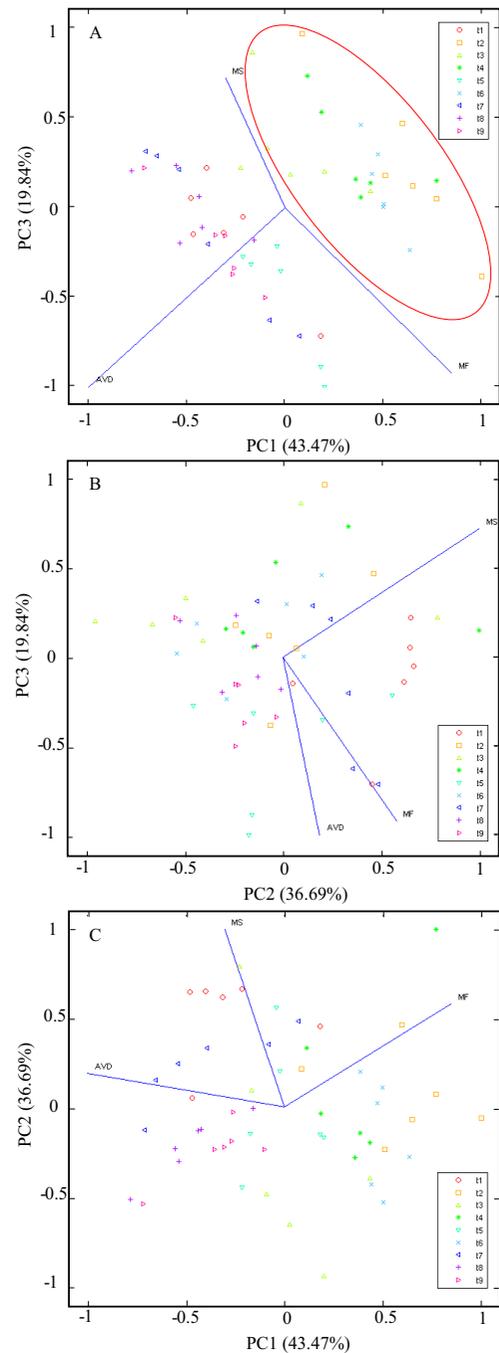


Figure 3. Plot of two principal component vectors describing the relationship between fresh mass (A), dry mass (B), and biological activity of *Enterolobium contortisiliquum* callus cultured in Murashige & Skoog medium with different combinations of growth regulators (mg L^{-1}) (C). The positioning of each variable in the plot represents its behavior against the others, so that a greater proximity among the variables indicates a greater correlation among them. Legend symbols indicate the nine treatments (T1 to T9) in the study (Table 1).

fresh and dry mass vectors in the graph, indicating, respectively, lower values in these treatments for biological activity and a negative correlation to this variable, i.e., higher masses show lower biological activities. The second cluster is formed by treatments 1, 5, 7, 8, and 9, being positioned next to the biological activity vector, indicating higher biological activities and, consequently, lower fresh and dry masses. These results corroborate the test of means (Table 2).

The PC2 and PC3 components (36.69 and 19.84% of explained variance, respectively) of fresh mass, dry mass, and biological activity did not allow observing any clustering of treatments. Components PC1 and PC2 (43.47 and 36.69% of explained variance, respectively)

of these same variables showed a slightly different cluster consisting of treatments 2, 4, and 6, closer to fresh mass. This cluster was positioned opposite to the biological activity variable, indicating a high fresh mass and a lower biological activity. However, since some samples from other treatments are included in this cluster, a clear separation of treatments was not possible.

The TIA response is shown in Figure 4. The TIA obtained directly from the cotyledon extract was used as a control, since the occurrence of TIA in cotyledons of *E. contortisiliquum* has been observed in the literature. However, there are no known reports on the existence of this activity in cotyledon-derived calli. These

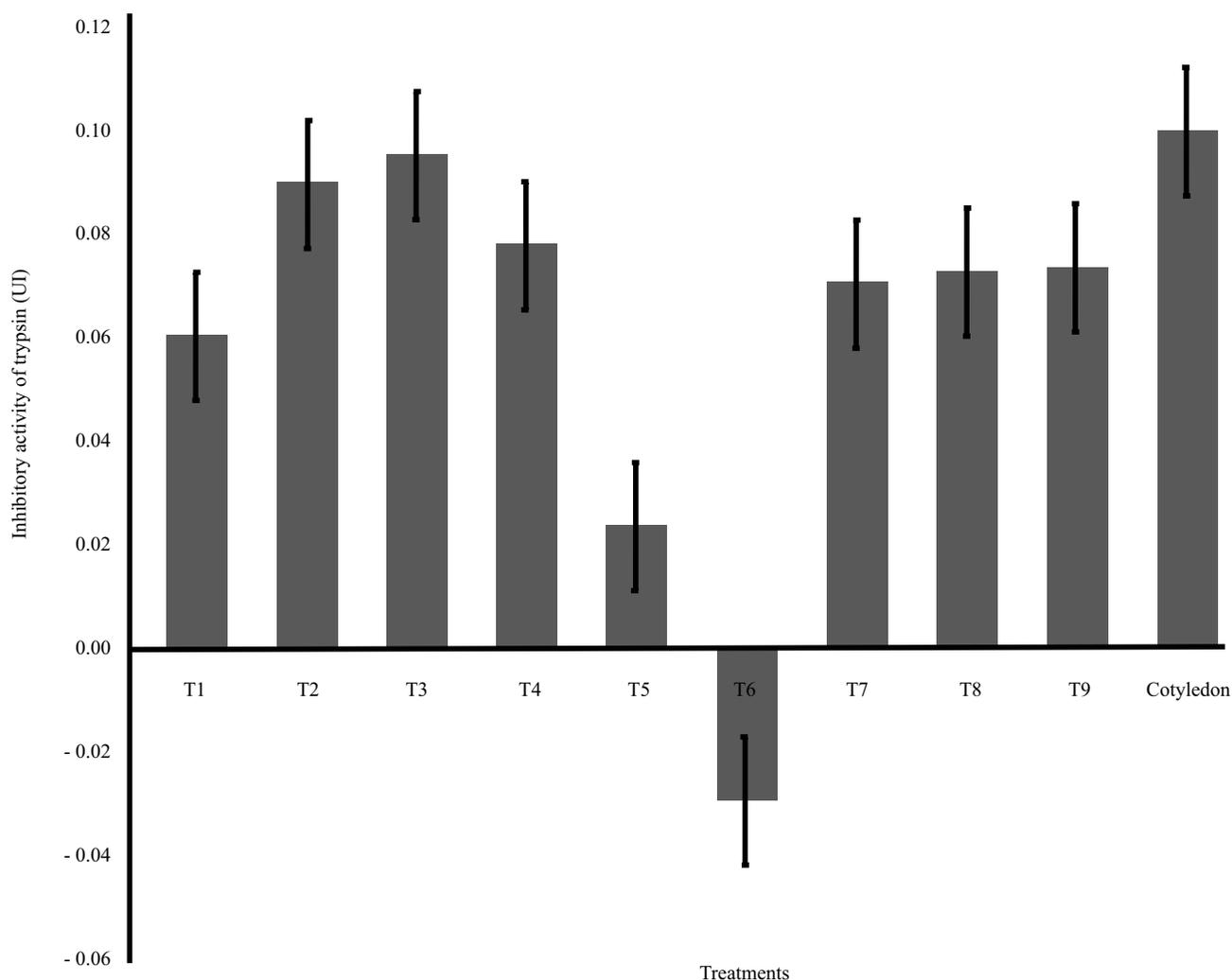


Figure 4. Means of trypsin inhibitory activity found in the callus of *Enterolobium contortisiliquum* under different treatments (T1–T9) and in cotyledon cultured with different growth regulators.

results are relevant since the production of secondary metabolites can be achieved more easily from non-embryogenic calli, as previously shown (Ng et al., 2016). Although cotyledones had a higher TIA, other treatments showed promise as a source of metabolite production. In addition, all treatments, except 5 and 6, presented similar inhibitory activities. However, the highest values of 0.0978 and 0.0934 IU were obtained in the cotyledon and in treatment 3, respectively, being only 4.49% lower than that of the control. Positive values indicate that trypsin was inhibited, and negative ones that it was activated. It is interesting to note that only treatment 5 showed trypsin activation and that the concentrations of growth regulators in this treatment are exactly opposite to those of treatment 3, which resulted in the highest inhibitory activity.

Growth regulators may inhibit or stimulate the production of active compounds in the cultured biomass, which unfortunately is not always correlated with the induction or inhibition of cell multiplication (Stalman et al., 2003), as observed in the present study (Table 2 and Figure 4). Therefore, it is difficult to select experimental models that ensure both a good yield of biomass and the production of a particular compound (Luczkiewicz et al., 2014).

The manipulation of in vitro culture conditions aiming to increase callogenic mass affects the metabolism of metabolites and may influence the production of some specific metabolite of interest (Li et al., 2015). It is important to highlight that callus induction for the production of some compound is related to the morphogenetic nature of the callus. According to Ng et al. (2016), non-embryogenic calli are more likely to produce some metabolites than embryogenic ones. Furthermore, callogenic cells can be distinguished according to their morphogenetic nature by scanning electron microscopy (Santos et al., 2015). In the present study, this analysis allowed stating that *E. contortisiliquum* callus cells are non-embryogenic, since they did not show any structure type resembling a pro-embryo or some embryonic structure (Figure 1 E). Embryogenic cultures can be distinguished using acetic carmine and Evans blue dyes. Cells that react strongly to carmine and weakly to Evans blue are considered embryogenic, whereas those that react weakly to the first and intermediately to the latter are non-embryogenic (Steiner et al., 2005). The cytochemical test showed that the obtained callus

were non-embryogenic, indicating the potential of the *E. contortisiliquum* callus in producing secondary metabolites (Figure 1 F).

The present study is the first comprehensive report describing the effect of auxins and cytokinins both on callus formation and on its biological activity, as well as on its TIA. In general, the results obtained here have confirmed previous reports in the literature that show the difficulties in the development of a culture medium that promotes intense callus formation and a high accumulation of secondary metabolites (Luczkiewicz et al., 2014; Raj et al., 2015). According to Stalman et al. (2003), this limitation may be explained by the fact that the primary and secondary metabolic pathways compete for precursors, which is mainly noted in the accumulation of biomass in the callus. Other authors have shown that the addition of 2,4-D at high concentrations of 5.0 mg L⁻¹, for example, in the culture medium negatively influences the production of secondary metabolites (Raj et al., 2015).

Only treatment 1 (combination of regulators at lower concentrations) showed the highest fresh mass, dry mass, and biological activity (Table 2), indicating that the greater cell multiplication increased biological activity. However, this treatment did not have a satisfactory TIA in relation to the cotyledon (control), allowing to suggest that the inhibitory activity does not increase biological activity. This behavior of biomass increase and decrease in metabolic accumulation was also observed in the study of Raj et al. (2015), who found a negative correlation between cell growth and alkaloid accumulation in the callus of *Sesamoides suffruticosa* (Lange) Kuntze cultured with different combinations of auxins and cytokinins. The same authors concluded that the culture medium could not be optimized simultaneously for a high biomass production and accumulation of metabolites.

Conclusions

1. The in vitro cultivation of cotyledons of *Enterolobium contortisiliquum* in the presence of different rates of the dichlorophenoxyacetic acid (2,4-D), picloram (PIC), kinetin (KIN), and 6-benzylaminopurine (BAP) plant growth regulators (PGR) is an efficient strategy for callus mass production.

2. Lower concentrations of PGR result in a higher fresh mass, dry mass, and biological activity of the callus of *E. contortisiliquum*.

3. The greatest trypsin activity is found in cotyledons and callus cultivated in the presence of 0.5 mg L⁻¹ 2,4-D + 2.0 mg L⁻¹ PIC + 0.5 mg L⁻¹ KIN + 2.0 mg L⁻¹ BAP.

4. The *in vitro* callus cultivation of *E. contortisiliquum* is a viable tool for the production of metabolites of interest.

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