PERACIO RAFAEL BUENO FERREIRA

# EFEITO DE ESTIMULADORES E INIBIDORES DE PRODUÇÃO DE ETILENO NO DESENVOLVIMENTO *IN VITRO* DE *CANAVALIA ENSIFORMIS*

Dissertação apresentada a Universidade Federal de Viçosa como parte das exigências do Programa de Pós-Graduação em Agroquímica, para obtenção do título de *Magister Scientiae*.

VIÇOSA
MINAS GERAIS - BRASIL
2012

**Ficha catalográfica preparada pela Seção de Catalogação e Classificação da Biblioteca Central da UFV**

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2012

Ferreira, Peracio Rafael Bueno, 1988-
Efeito de estimuladores e inibidores de produção de etileno no desenvolvimento *in vitro* de *Canavalia ensiformis* / Peracio Rafael Bueno Ferreira. – Viçosa, MG, 2012.
ix, 34f. : il. (algumas col.) ; 29cm.

Inclui apêndice.
Orientador: Antonio Jacinto Demuner.
Dissertação (mestrado) - Universidade Federal de Viçosa.
Referências bibliográficas: f. 26-31.

1. Feijão-de-porco. 2. *Canavalia ensiformis*. 3. Etileno. 4. Aminas. 5. Crescimento (Plantas). I. Universidade Federal de Viçosa. II. Título.

CDD 22. ed. 635.652

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APROVADA: 24 de julho de 2012.

Prof. Alberto Oliveros Bastidas

Prof. Sérgio Antonio Fernandes

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(Orientador)

# Dedico

Aos meus pais

Marlene Simao Gomes e Maruzam Bueno Ferreira, e aos dois anjos eternos em minha vida, que apesar de terem sido levadas pelos ventos como pétalas, o perfume ainda permeia em meu coração: Ingrid e Lorena Peracio

# AGRADECIMENTOS

Meus agradecimentos ao meu Bom Pastor, que antes conhecia somente em palavras, mas que hoje meus olhos podem ver. A minha dependência a Ele foi total, para que hoje eu pudesse chegar aonde cheguei. E que me ensinou com muito amor, que uma obra que Ele começou, jamais ficará inacabada, pois Ele não é homem para mentir. É Deus de aliança e promessas. À Maria Santíssima e todos os santos em especial São Bento e Santa Terezinha do Menino Jesus, pela intercessão em minha vida e benção derramadas na forma de pétalas.

Ao Acampamento Maanaim e Grupos de Oração Universitários Cenáculo do Senhor e Imaculado Coração de Maria, que me mostraram o poder que o Espírito Santo tem em minha vida.

À Universidade Federal de Viçosa por toda a infraestrutura disponível para execução do trabalho e o Conselho Nacional de Pesquisa e Desenvolvimento (CNPq) pela concessão de bolsa de estudos e CAPES e FAPEMIG pelos recursos financeiros.

Aos Prof. A.J. Demuner, Prof. L. C. A. Barbosa e Profª C. R. A. Maltha e todos os estudantes e funcionários do Laboratório de Análise e Síntese de Agroquímicos (LASA/DEQ) em especial a minha irmã Ruth, Ulisses, Luciana, Simone, Betina, Raphael, Diego, Fabrício, Montanari, Michele, Silvana.

Ao Prof. W.C. Otoni por todo o apoio e amizade e ao Laboratório de Cultura de Tecidos (LCT/Bioagro) e seus estudantes em especial Crislene, Diego, Marcela, Lorena, Cléber, Marcos, Leonardo, Elyabe, Leydi.

Ao Prof. F. L. Finger e ao Laboratório de Fisiologia da Pós-colheita de Produtos Hortícolas, pelo suporte e apoio nas análises etileno. Em especial as alunas Tereza Drumond e Tânia Pires por toda ajuda.

À Profª E. I. S. Floh e o Laboratório de Biologia Celular (Biocel/USP) pela recepção e ajuda essencial na análise de poliaminas, em especial a técnica Amanda Ferreira Macedo.

À minha família em especial meus pais e irmãos Junior e Danusa. Os meus sobrinhos Lothar Matthaeus e Deborah e meus primos amados.

À mulher que faz da minha vida um sentido a mais, Itainá Gonçalves Andrade, por todo carinho, companheirismo, palavras duras nos momentos certos, pela ajuda durante as fotografias. Por simplesmente me fazer sorrir quando quero chorar. Por

nunca ter me abandonado e sempre ter acreditado em mim. Mostrando que eu poderia vencer.

A todos os meus amigos. Aos antigos e aos novos. A garota de Comercinho que esteve ao meu lado durante dois anos (até a eternidade), Ruth. Ao Jorcien, Karla, Giulia, Uyra, Isadora, Lays, Luana, Patrícia, Priscila, Ulisses, Éder e Luciana pelo grande apoio. Pelos momentos de oração agradeço a Aline, Guilherme, Rodrigo e Roni. Ao Wilson Vicente de Souza Pereira por todo amparo na escrita da dissertação, principalmente na estatística. E de forma especial agradeço ao meu irmão, nascido pela fé, Leandro Vieira, pelo companheirismo, amizade verdadeira e fiel, pelo apoio nos momentos de tribulações e risadas nos momentos de descontração. Palavras são poucas para expressar o quanto sou grato a esse garoto, amado por Deus, e violeiro das altas horas. Obrigado, amigo e irmão.

Em especial a minha irmã caçula Dayane Franciele que nos deixou, mas que estava ao meu lado a todo o momento. Garota sinônimo de bravura e coragem. E que hoje nos braços do Pai e no regaço acolhedor da mãe, continua rezando por mim. Brilhando como uma estrela nos céus, com uma luz que jamais se apaga. Amo-te eternamente.

A todos que pela minha memória caduca, não coloquei o nome, mas que estão presentes em lugares especiais no meu coração. Agradeço.

# BIOGRAFIA

PERACIO RAFAEL BUENO FERREIRA, filho de Maruzam Bueno Ferreira e Marlene Simão Gomes, nasceu em 29 de fevereiro de 1988 na cidade de Contagem, estado de Minas Gerais.

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# ÍNDICE

## LISTA DE ABREVIATURAS

1M – uma membrana

2M – duas membranas

ABA – ácido abscísico

ACC – ácido 1-aminociclopropano-1-carboxílico

ADC – arginina descarboxilase

AVG – aminoetoxivinilglicina

CAD – cadaverina

CHA – clicloexilamina

DAH - 1,7-diaminoheptano

DFMA – difluorometilarginina

DFMO – difluorometilornitina

ET – etileno

FW – peso fresco

HPLC – Cromatógrafo Liquido de Alta Eficiência

MGBG – metilglioxal bis-(guanilidrazona)

MP – perclorato de mercúrio

GC – grupo controle

PA – poliaminas

pH – potencial hidrogeniônico

PUT – putrescina

SAM – S-adenosilmetionina

SPD – espermidina

SPM – espermina

STS – tiossulfato de prata

## RESUMO

FERREIRA, Peracio Rafael Bueno, M.Sc., Universidade de Viçosa, julho de 2012. **Efeito de estimuladores e inibidores de produção de etileno no desenvolvimento *in vitro* de *Canavalia ensiformis*.** Orientador: Antonio Jacinto Demuner. Coorientadores: Wagner Campos Otoni e Luiz Cláudio de Almeida Barbosa.

O etileno é conhecido por controlar o crescimento vegetal e sua relação com poliaminas tem sido estudada porque essas duas classes de hormônios compartilham um precursor biossintético comum, a S-adenosilmetionina. A fim de avaliar a influência de etileno e poliaminas sobre as respostas de crescimento *in vitro* de *Canavalia ensiformis* foram utilizados diferentes compostos que atuam sobre a biossíntese de etileno e poliaminas. Os tratamentos com o inibidor de etileno, aminoetoxivinilglicina, perclorato de mercúrio, sistema de membranas (uma e duas membranas), espermina e inibidor de poliaminas acarretaram no crescimento das plântulas, coincidindo com alto conteúdo de poliaminas, principalmente espermina e putrescina, e uma elevada razão entre Putrescina e espermidina + espermina. O tratamento com o precursor de etileno, ácido 1-aminociclopropano-1-carboxílico, apresentou baixo crescimento. Os resultados apresentados destacam as interações entre etileno, poliaminas e crescimento *in vitro* de *Canavalia ensiformis*. A adição de poliaminas exógenas acarretou efeitos estimulantes de crescimento na parte aérea e raízes de *C. ensiformis*. Portanto, pode-se utilizar poliaminas, capturadores e inibidores de etileno e sistema de membranas na melhoria do crescimento *in vitro* de *Canavalia ensiformis*.

## ABSTRACT

FERREIRA, Peracio Rafael Bueno, M.Sc., Universidade Federal de Viçosa, July, 2012. **Effect of stimulators and blockers of ethylene production on the growth *in vitro* of *Canavalia ensiformis.*** Adviser: Antonio Jacinto Demuner. Co-adviser: Wagner Campos Otoni and Luiz Cláudio de Almeida Barbosa.

Ethylene is known to control plant growth and its relationship with polyamines has been studied because the two classes of hormones share a common biosynthetic precursor, S-adenosylmethionine. In order to assess the influence of ethylene and polyamines on *in vitro* growth responses of *Canavalia ensiformis*, different inhibitors and stimulators were used that act on ethylene and polyamines biosynthesis. Treatment with the ethylene inhibitor, aminoethoxyvinylglycine, mercury perchlorate, membrane system (one and two membranes), polyamine (spermine) and polyamines inhibitor triggers increased plant growth, coinciding with high polyamine contents, mainly spermine and putrescine, and a high Putrescine/(Spermidine + Spermine) ratio, whereas treatment with the ethylene precursor 1-aminocyclopropane-1-carboxylic-acid triggers lessened growth characteristics. The results presented here highlight the interactions between ethylene, polyamines and plant growth of *C. ensiformis* vitroplants. Exogenous polyamines presented stimulatory effects on shoots and roots of *C. ensiformis*. Thus, polyamines, ethylene scavengers, ethylene inhibitors and membrane systeGC can be useful for improving *in vitro* growth in *Canavalia ensiformis*.

## INTRODUÇÃO GERAL

*Canavalia ensiformis* L. DC (Leguminosae) é conhecida popularmente no Brasil como fava branca, feijão bravo, feijão de porco, feijão holandês, entre outros (Lorenzi e Matos, 2008). *C.ensiformis* é relativamente resistente a doenças e pragas e, além disso, apresenta alta toxicidade, relacionada à presença de compostos como concanavalina A, canatoxina e canavalina (Oliveira et al., 1999a). Essa leguminosa possui também uso medicinal devido ao seu alto conteúdo de ureases e lectinas (Fujimura et al.,1993). Atividades biológicas como antibacteriana (Prabhu et al., 2010), antioxidante (Sowndhararajan et al., 2011), antitumoral (Yudovin-Farber et al., 2005), alelopática (Santos et al., 2011), inibição de receptor de dopamina D1 (Pattamadilok et al., 2011) estão associadas a *C. ensiformis* e outras espécies do gênero.

Um estudo com cultura de calos provenientes de hipocótilo de *C. ensiformis* induziu a produção do isoflavonoide medicarpina por elicitação com esporos de fungos e $HgCl_2$ (Gustine 1976). Esse composto não é produzido normalmente pela planta e sua regulação foi estudada por Gustine et al. (1978). Outros estudos com cultura de tecidos *in vitro* demonstraram o acúmulo de L-canavanina (Ramirez et al., 1992) e lectina (Silva et al., 2005) em *C. brasiliensis*.

Nesse trabalho, inicialmente com o objetivo de produzir compostos secundários a partir de cultura de tecidos, foi perceptível a inibição do crescimento de *C. ensiformis* durante a micropropagação *in vitro* nos frascos de cultivo completamente vedados. Esta observação suscitou o estudo sobre a influência do etileno e poliaminas no desenvolvimento de *C. ensiformis*.

Etileno (ET) e poliaminas (PAs) são conhecidos por controlar crescimento vegetal (Hu et al., 2006). Alterando-se os níveis de etileno ou poliaminas pode-se consequentemente contribuir para controle do crescimento da planta (Burstenbinder et al., 2010). O ET é um hormônio gasoso que é produzido em quase todos os tecidos vegetais com taxa de produção dependente do estágio de desenvolvimento (Bleecker and Kende, 2000). Já as PAs são compostos alifáticos que desempenham vários papeis no crescimento e desenvolvimento vegetal (Bregoli et al., 2006). Elas estão envolvidas na resposta de defesa, pois os níveis de PAs são notavelmente aumentados durante condições de estresse (Dias et al., 2010), e auxiliam na modulação do sistema fotossintético em plantas superiores (Ioannidis et al., 2012).

Embora o ET e as PAs tenham funções biológicas opostas, a síntese de ambos está relacionada, uma vez que o mesmo precursor, S-adenosilmetionina (SAM), é usado em suas biossínteses (Dias et al., 2010). A rota biossíntética do ET e das PAs é conhecida (Figura 1), tornando-se possível a manipulação dos níveis dessas duas substâncias pela adição de inibidores e promotores de seu metabolismo ao meio de cultura. Sendo assim, seu conhecimento é útil para o estudo das relações entre etileno e poliaminas.

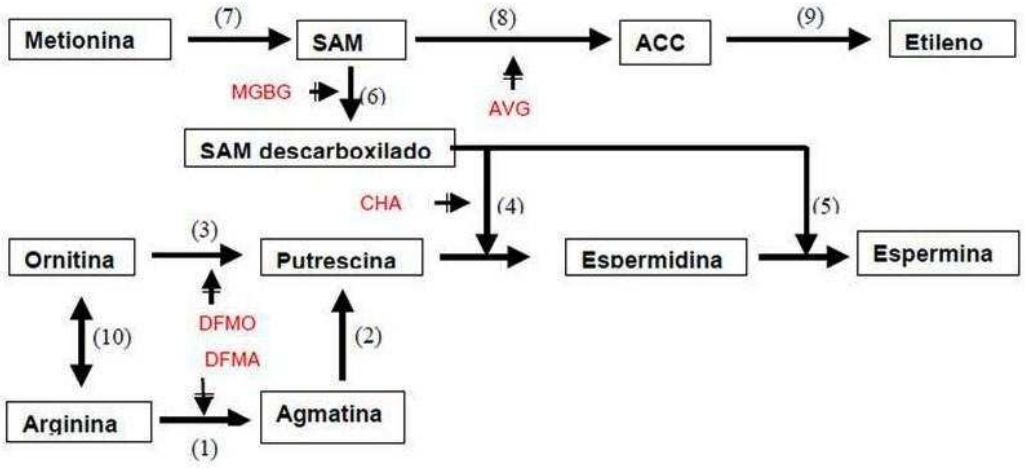


**Figura 1**. Rotas de biossíntese de etileno e poliaminas. As enzimas envolvidas são (1) arginina descarboxilase, (2) agmatina iminohidrolase, (3) ornitina descarboxilase, (4) espermidina sintase, (5) espermina sintase, (6) SAM descarboxilase, (7) SAM sintase, (8) ACC sintase, (9) ACC oxidase, (10) arginase. Em vermelho estão representados os inibidores e seus respectivos pontos de ação: DFMA (difluorometilarginina), DFMO (difluorometilornitina), CHA (cicloexilamina), MGBG (metilglioxal-bis-guanil hidrazona), AVG (aminoetoxivinilglicina). Modificado de Martin-Tanguy, (2001).

Nesse contexto, o presente trabalho é apresentado na forma de artigo, onde se utilizam inibidores e promotores da biossíntese de etileno e poliaminas e são avaliadas suas influências no desenvolvimento de raízes e parte aérea de *Canavalia ensiformis.*

**Effect of stimulators and blockers of ethylene production on the growth *in vitro* of *Canavalia ensiformis***

## INTRODUCTION

Ethylene (ET) is a gaseous hormone that is produced in almost all plant tissues, although the production rate depends on the developmental stage (Taiz and Zeiger 2004). Ethylene is also involved in several other stages of the plant life cycle, including germination, seedling growth, leaf and petal abscission, organ senescence, and responses to biotic and abiotic stresses (Schaller and Kieber, 2002). Different types of stresses like flooding, mechanical injuries, pathogen induced damage, water deficit or drought, mineral toxicity, and salinity are known to increase the endogenous levels of ethylene (Siddikee et al., 2011).

The quantity of ethylene accumulated in the culture recipient depends on the production rate in the tissues and the gas exchange rates in the cultures. Although culture flasks do allow exchange of ethylene, its production frequently exceeds losses, resulting in accumulation in the culture flasks (Pua, 1999). Use of gas-permeable membranes increases natural ventilation in culture vessels, photosynthesis, growth rates and ethylene release, avoiding its accumulation (Iarema et al., 2012). Ethylene production can be inhibited at different stages of its biosynthetic pathways. In order to study the effects of ethylene release on plant growth, inhibitors of the first two steps of the biosynthetic pathway, i.e., formation of S-adenosyl-methionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) have been widely exploited. ACC activity is inhibited by aminoethoxyvinylglycine (AVG) or aminooxyacetic acid. These compounds prevent ACC formation and may play an important role in research on ethylene and its effects (Lin et al., 2009).

Ethylene and polyamines (PAs) are known to control plant growth. Altered ethylene or polyamine level could hence contribute to growth inhibition (Burstenbinder et al., 2010). PAs are aliphatic compounds that play various roles in the growth and development of plants (Bregoli et al., 2006). They are involved in defense responses since PA levels are significantly increased during stress conditions (Evans and Malmberg 1989), and assist in modulation of the photosynthetic apparatus in higher plants (Ioannidis et al., 2012) altering the partitioning of proton motive force (energy).

Although ET and PAs have apparently opposite biological functions, the synthesis of these phytohormones is related to the same precursor, SAM (Dias et al., 2010). PA production may play an important role in regulating *in vitro* regeneration by leading to cell elongation and division through the inhibition of ET production (Hu et al., 2006).

The *Canavalia* genus is comprised of 40 plant species, of which eight have been studied, where the species *C. ensiformis* is most investigated due to its high nutritional value and the first crystal urease was isolated from this plant (Summer, 1926).

This species presents medicinal use because of its high urease and lectin contents (Fujimura et al., 1993). Additionally, it exhibits antitumor (Yudovin-Farber et al., 2005), antiplasmodial (Berger, 2000), antioxidant (Sowndhararajan et al., 2011) and antibacterial (Prabhu et al., 2010) properties, as well as its inhibitory activity of the dopamine D1 receptor (Pattamadilok et al., 2011).

Studies have shown that the toxicity of *C. ensiformis* may be useful in the prevention of insect attack both in the field or in storage (Kay, 1979) and is related to the presence of a multiplicity of compounds, including proteins such as concanavalin A, canatoxin and canavaline (Oliveira et al., 1999b) with insecticide (Fitches et al., 2001) and antifungal activities (Oliveira et al., 1999a). *C. ensiformis* has toxic effects on colonies of *Atta sexdens* (Hebling et al., 2000) and nematicidal activity (Arim et al., 2006) against *Meloidogyne* spp (Rodriguez-Kabana et al., 1992). *C. ensiformis* contains secondary metabolites such as cyanogenic glycosides and tannins, commonly known as deterrents to insects (Swain, 1979), as well as the polysaccharide galactorhamnan which is toxic to beetles (*Calosobruchus maculatus*) (Oliveira et al., 2001).

Tissue culture has shown to be an important tool for *in vitro* production of *C. ensiformis* compounds of medicinal importance including medicarpine (Gustine, 1976) and L-canavanine (Ramirez et al., 1992). However, the propagation of this species *in vitro* and its sensitivity to ethylene and polyamines levels were not reported.

In order to verify this potential effect, the objectives of the present study were to investigate the effect of ethylene on *in vitro* growth in *C.ensiformis* via supplementation with a promoter of ethylene action ACC, with an ethylene biosynthesis inhibitor -AVG, an ethylene scavenger - mercury perchloride (MP), as well as polyamines spermine and polyamine inhibitors (MGBG), and system of membranes.

## MATERIALS AND METHODS

### Germination and growth *in vitro*

Seeds were obtained from mature jackbean fruits (*Canavalia ensiformis*) from "Horta Velha" (Federal University of Viçosa) and were disinfected under aseptic conditions by immersion in 70% (v/v) alcohol for 60 seconds, followed by immersion in a 5% (w/v) commercial sodium hypochlorite solutions (Super Globo®, Brazil) for 15 minutes. The seeds were then rinsed 3 times with deionized autoclaved water and left to soak for 24 h. These procedures were performed in a laminar flow hood. The seeds were transferred to 250 mL glass flasks and completely sealed with polypropylene lids (Fig. 2A), containing 50 mL of Murashige and Skoog basal salts solution (Murashige and Skoog, 1962) containing B5 vitamin complex (Gamborg et al., 1968), 30 g $L^{-1}$ of sucrose, 100 mg $L^{-1}$ of myo-inositol, and 8 g $L^{-1}$ of agar (the pH of the medium was adjusted to 5.7 ± 0.1 and it was autoclaved at 120 °C and 1.1 kPa for 20 minutes). Seeds were germinated in growth room environment with light regime of 16 h and irradiance of 60 μmol $m^{-2}$ $s^{-1}$ provided by 2 fluorescent lamps (HO Sylvania T12-110 W) at 25 ± 2 °C.

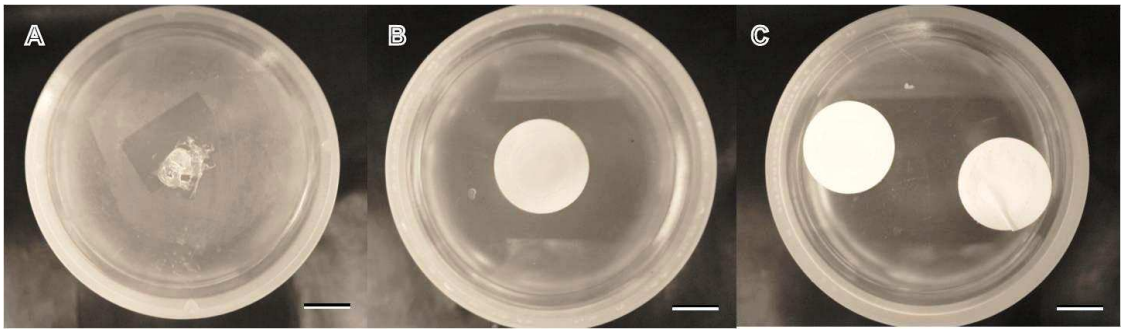


**Figure 2** - Detail of the rigid polypropylene lids without membrane (**A**); and with one (1M) (**B**) and two (2M) (**C**).

### Bioassay

Treatments consisted of: 1) the ethylene inhibitor AVG (3 µM); 2) the ethylene promoter ACC (3 µM), 3) ethylene scavenger MP (250 mM), 4) inhibitor of polyamine biosynthesis MGBG [Methylglyoxalbis-(guanylhydrazone)] (1 mM), 5) spermine (1 mM), and sealing with 6) one (Fig. 2B) and 7) two (Fig. 2C) 0.45 µm fluoropre membrane (MilliSeal® AVS-045 Air Vent, Japan) systeGC, as well as the control in

which no substance was added. These substances, except for MP, were filter sterilized through a Millex-GS filter (Millipore, USA) with a 0.22 µm diameter pore membrane, and then added to the culture medium during the cooling process (approximately 45 °C), in previously autoclaved glass flasks (60 x 60 mm, 250 mL). The MP was introduced to the culture system by attaching a previously autoclaved 1.5 mL microtube (Eppendorf, Deutschland) containing a 100 µL aliquot of MP to the inside of the culture flasks.

All treatments were maintained in a controlled-environmental growth room with 16 h photoperiod and 60 µmol $m^{-2}$ $s^{-1}$ irradiance (HO Sylvania T12-110 W) at 25 ± 2 °C. Five replicates were used per treatment, where each replicate consisted of a glass flask containing 2 seeds. Plant growth was evaluated by length of shoot and root, and fresh and dry weight after 22 days of seed inoculation.

**Ethylene determination**

Ethylene levels were measured daily up to the 22$^{nd}$ day of cultivation, according to the methods of Reis et al. (2003). Ethylene levels were measured by withdrawing air samples from the containers using a 1 $cm^3$ sterilized syringe. Concentrations were then determined in a Hewlett-Packard 5890 series II gas chromatograph, equipped with a flame ionization detector at 150 °C, injector at 110 °C and Porapak Q 80/100 (1.5 m x 4 mm) column at 60 °C. Nitrogen carrier gas and hydrogen flow rates were 30 mL $min^{-1}$ and air 320 mL $min^{-1}$, respectively. Mean peak areas of ethylene are shown in Table 3 in Appendix. To calculate the ethylene concentration was used the following formula:

$$ethylene = \frac{\frac{PASa \; x \; SC}{PASt} \; x \; V}{FM} \; x \; t$$

**Legend**: PASa (Peak Area of Sample [chromatogram from Figure 14 on Appendix]); SC (Standard Concentration); PASt (Peak Area of Standard) V (volume [L]); FM (Fresh Matter [kg]) and t (Time [h])

**Polyamine determination**

Polyamines were quantified in the roots and shoots of *C. ensiformis* according to Silveira et al. (2004). Fresh mass samples of 0.2 g for each treatment at the different periods were ground in 1.4 mL of 5% (v/v) perchloric acid. Free and conjugated polyamines were derivatized by a 5 mg $mL^{-1}$ solution of dansyl chloride in acetone (4º C).

Free polyamines were determined directly from the supernatant. Conjugated polyamines were extracted by hydrolyzing 200 µL of the supernatant with 200 µL of 12 M HCl for 18 h at 110 °C. A 40µL aliquot of sample was added to 100 µL of dansyl chloride. 20 µL of 0.05 mM 1,7-diaminoheptane (internal standard) and 50 µL of saturated sodium carbonate. The samples were incubated in the dark for 50 min at 70 ºC. The excess of dansy chloride was converted to dansylproline by adding 25 µL of proline (100 mg $mL^{-1}$). After 30 min incubation, dansylated polyamines were extracted with 200 µL of toluene. The toluene phase was collected and dried under nitrogen. The dansylated polyamines were solubilized in 200 µL of acetonitrile.

These were analyzed by high-performance liquid chromatography (HPLC) in a 5 µm C18 column (Shimadzu Shin-pack CLC ODS), using a gradient with increasing proportions of absolute acetonitrile to 10% acetonitrile in water (pH 3.5) like Table 1. The flow rate was 1 mL $min^{-1}$ at 40 °C, and the fluorescence detector was calibrated to 340 nm (excitation) and 510 nm (emission). Typical chromatogram of the polyamines standard pool are listed in Figure 15 (Appendix) and the standard curve to calculate concentration and mean peak areas of the chromatograms are set out in Tables 4 and 5 respectively in the appendix.

| Acetonitrile | Acetonitrile (10 %) pH = 3.5 | Time (min) |
|---|---|---|
| **65%** | 35% | 0 a 10 |
| **65 a 100%** | 35 a 0% | 10 a 13 |
| **100%** | 0% | 13 a 21 |

**Table 1**. Mobile phase gradient for polyamines determination according Silveira et al. (2004).

A mixture of putrescine (PUT), spermidine (SPD), spermine (SPM) and cadaverine (CAD) was used as a standard.

**Data analysis**

Data was tested for normality by the Shapiro Wilk test and data not showing normality ($p > 0.05$) was converted to $\log_{10}(x)$. Normal data (or standard) were analyzed by analysis of variance and when there was no difference between treatments by the F-test, the Tukey test was performed at 5% probability (qualitative data) or regression analysis (quantitative data). Data not normalized even after conversion were analyzed using generalized linear models and when observing differences between treatments the Tukey test and regression analysis were used.

Analyses were performed using the R software version 2.12.0 for Windows (R Development Core Team, 2011) and the graphics were made with the software Microsoft Office Excel® 2011.

**RESULTS**

**1) Ethylene production**

The present study showed inhibition of ethylene accumulation in treatments with AVG, MP, 1M, 2M, SPM. The treatments presenting ethylene accumulation in the vessels were the control, ACC and MGBG. Dynamics of ethylene production are shown in Figure 3.

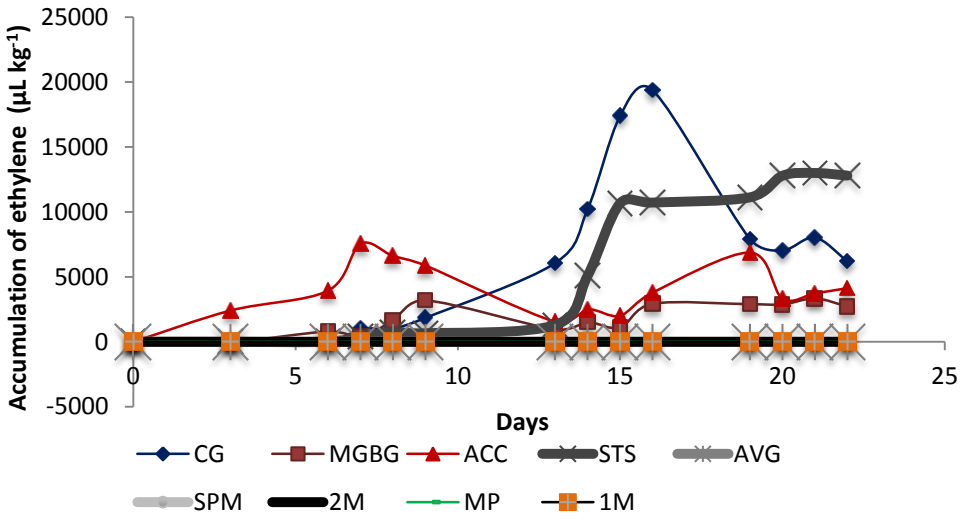


**Figure 3 -** Dynamics of production of ethylene during germination and growth of *C. ensiformis*. Ethylene accumulation levels (µL $kg^{-1}$ fresh weight) measured over the growth period of 22 days. GC (control group); 1M (one membrane); 2M (two membranes); ACC (1-aminocyclopropane-1-carboxylic acid); AVG (aminoethoxyvinylglycine), SPM (spermine); MGBG (methylglyoxal-bis(guanylhydrazone)); MP (mercury perchlorate) and STS (silver thiosulfate)

The highest ethylene level was observed in the control group at 16 days of cultivation (19374 µL $kg^{-1}$) followed by STS to 21 days (13000 µL $kg^{-1}$), ACC at 7 days (7542.2 µL $kg^{-1}$) and MGBG at 21 days (3340.8 µL $kg^{-1}$). Ethylene production started on day 3 for the ACC treatment, on day 6 for MGBG and on day 7 in the control group. There were no significant differences in dry weight ($p > 0.05$). The greatest fresh weight was obtained for treatment 2M (8.82 g) (Fig. 4).

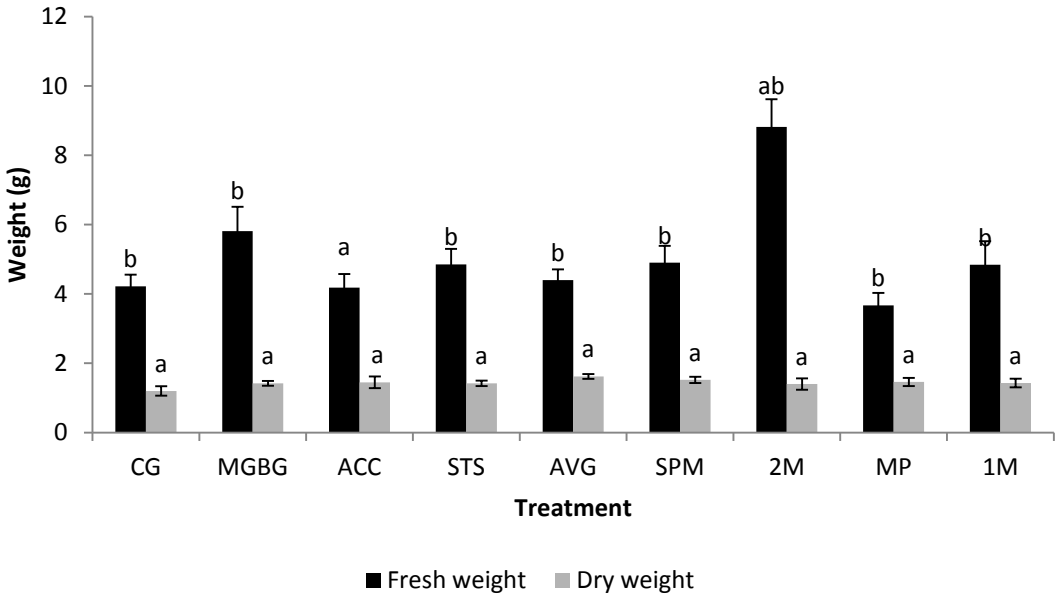


**Figure 4 -** Influence of ethylene in fresh and dry weight of *Canavalia ensiformis* seedlings. GC (control group); 1M (one membrane); 2M (two membranes); ACC (1-aminocyclopropane-1-carboxylic acid); AVG (aminoethoxyvinylglycine), SPM (spermine); MGBG (methylglyoxal-bis(guanylhydrazone)); MP (mercury perchlorate) and STS (silver thiosulfate). Means indicated by the same letter for treatments are not significantly different (Tukey's test, $p<0.05$).

**2) Effect on growth of *C. ensiformis***

The effect of ethylene on growth of *Canavalia ensiformis* is shown in Figure 5. Variations in stem growth were observed between treatments. There was a high rate of growth in the following treatments: 2M, SPM, MGBG and MP. The longest stem was observed in treatment 2M, measuring approximately 14.5 cm. Lower growth rates were observed in the GC (2.58 ± 0.74 cm) and ACC (2.74 ± 0.95) treatments, where these values do not differ significantly ($p> 0.05$). Root growth was homogeneous in all treatments except for 2M, which presented longer roots (5.75 ± 0.78 cm).

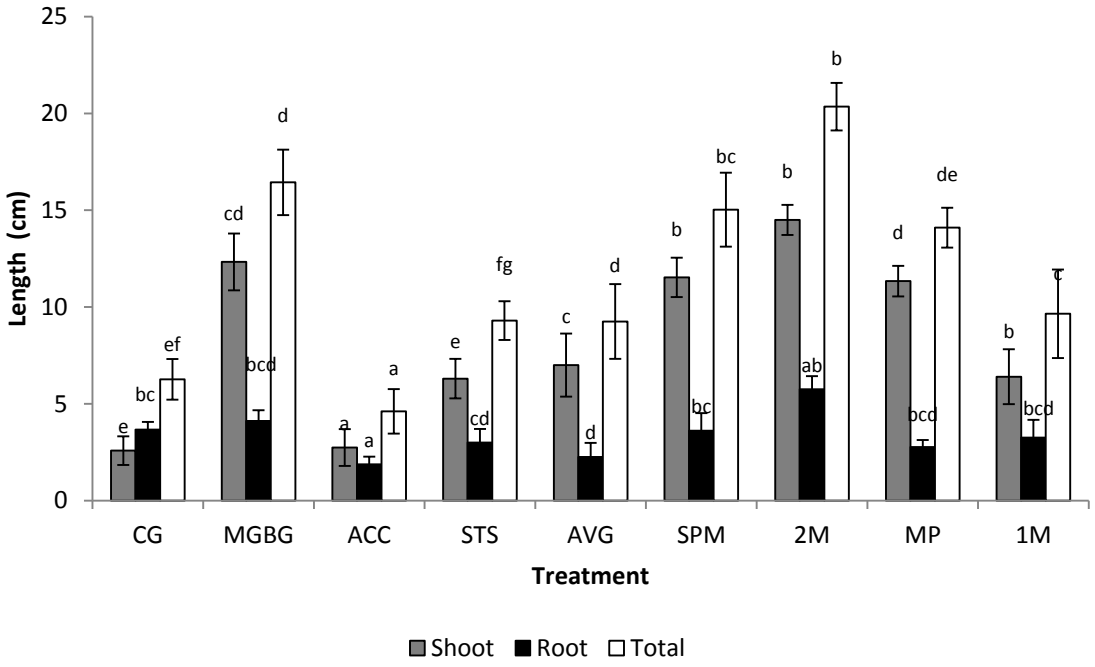


**Figure 5 -** Influence of ethylene on the growth of *Canavalia ensiformis* seedlings. GC (control group); 1M (one membrane); 2M (two membranes); ACC (1-aminocyclopropane-1-carboxylic acid); AVG (aminoethoxyvinylglycine), SPM (spermine); MGBG (methylglyoxal-bis(guanylhydrazone)); MP (mercury perchlorate) and STS (silver thiosulfate). Means indicated by the same letter for treatments are not significantly different (Tukey's test, at $p < 0.05$).

Values of total length are greater in treatments with increased root and shoot lengths. Seeds submitted to the 2M treatment had a higher total growth (20.35 ± 1,23 cm), followed by MGBG (16.44 ± 1.69 cm), SPM (15.03 ± 1.91 cm), MP (14.1 ± 1.03 cm), AVG (9.25 ± 1.93 cm), 1M (9.65 ± 2.29 cm), GC (6.26 ± 1.05 cm) and ACC (4.61 ± 1.15 cm). The treatments presenting accumulation of ethylene also had lower overall growth. Plants with high levels of ethylene, as in the ACC and control treatments, did not develop well after the emergence of the radicle (Fig. 6), reaching lengths of 4.6 ± 1.15 cm, compared to treatments where there was no accumulation of ethylene as in 2M. Mercury perchloride efficiently captured the ethylene produced, and accumulation levels were not detectable. The treatments with MP showed well-developed seedlings measuring 14 ± 1.03 cm in length.


**Figure 6 -** Influence of ethylene on seedling development. **A**- AV; **B**- GC; **C**- 1M; **D**- 2M; **E**- SPM; **F**- STS; **G**- ACC; **H**- MGBG; **I** – MP. 22 days after seed inoculation.

**3) Effect of polyamines**

In this work, increased polyamine concentrations coincide with lower ethylene levels in the headspace of the culture flasks, and consequently better development of *C. ensiformis* roots and shoots, on Table 2.

| | polyamine (μg $g^{-1}$ FW) | | ethylene ($\mu L^{-1} Kg^{-1} hr^{-1}$) | length (cm) | |
|---|---|---|---|---|---|
| | shoot | root | | shoot | root |
| GC | 332.01 | 164.07 | 7032.4 | 3.02 | 10.8 |
| MGBG | 347.11 | 116.84 | 2874.23 | 5.9 | 8.7 |
| ACC | 223.63 | 40.40 | 3332.2 | 6.5 | 13.0 |
| STS | 204.75 | 240.14 | 12788.01 | 8.6 | 4.0 |
| AVG | 132.34 | 142.65 | 0 | 10.2 | 5.0 |
| SPM | 314.29 | 43.29 | 0 | 23.0 | 18.0 |
| 2M | 172.13 | 15.53 | 0 | 19.02 | 12.0 |
| MP | 183.70 | 101.89 | 0 | 16.8 | 9.0 |
| IM | 97.55 | 48.18 | 0 | 7.0 | 13.2 |

**Table 2 -** Relationship between the levels of polyamines and ethylene and the *Canavalia ensiformis* roots and shoots length.

In general, the response of total polyamines is stronger in shoot than in root (Fig. 8). The highest total putrescine (Put) content was in the culture medium containing spermine (Fig. 9), followed by MGBG, MP, 2M, ACC, AVG, STS and 1M for the shoots. Put concentrations were higher in the roots for treatments with MGBG, SPM and 2M. However, among the roots there were no differences with regards to Put concentrations. The same behavior can be noted for free and conjugated polyamines.

Free and total (CAD) were found in greatest concentration in the control group and ACC treatments, while for the shoots and roots were GC and STS treatments (Fig. 10). Both presented high ethylene content (Fig. 3). Differences between CAD levels in the roots and shoots were noted in SPM, 2M, 1M and MP (Fig. 10C). *Canavalia ensiformis* presented cadaverine conjugate only in the shoots treated with STS and MP.

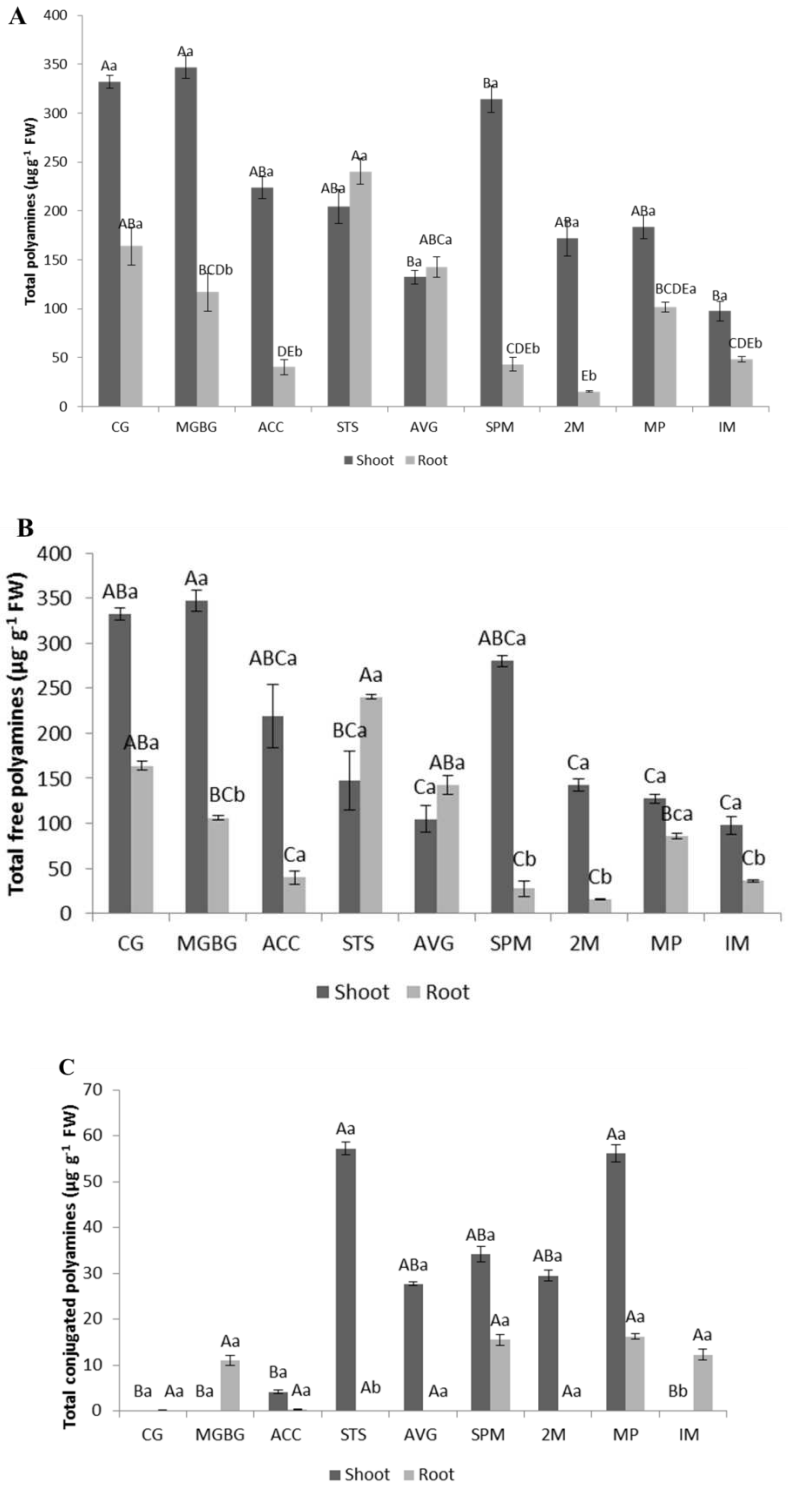


**Figure 8 -** Levels of polyamines in the *Canavalia ensiformis* roots and shoots. **A**. Total Polyamines; **B**. Total Free Polyamines; **C**. Total Conjugated Polyamines. Means indicated by the same capital letters for all treatments and lowercase letter for a specific treatment (roots and shoots) are not significantly different as assessed by the Tukey's test at 5% probability level.

No differences were observed between treatments regarding total spermidine (SPD) content in the shoots (Fig. 11). In the roots, concentrations were lower for SPM and 2M, and did not differ between shoot and roots in the treatments with GC, STS and MP. The concentration of spermidine free presented the same profile but differed from the conjugate, since the conjugate showed no SPD in GC, ACC and 2M (root).

The control group (GC) showed a higher concentration of total and free spermine (SPM) in the shoots, with no difference between the roots (Figs. 12A and 12B). Regarding the conjugate, it was not produced by the treatments with STS, AVG, 2M, while no production was observed by the shoots in treatments with MGBG and 1M (Fig 12C).

*Canavalia ensiformis* presented a high Put / (SPD + SPM) ratio for total and conjugated polyamines in the shoots for treatments with spermine and MP, respectively, and there were no differences in the roots (Fig. 13).

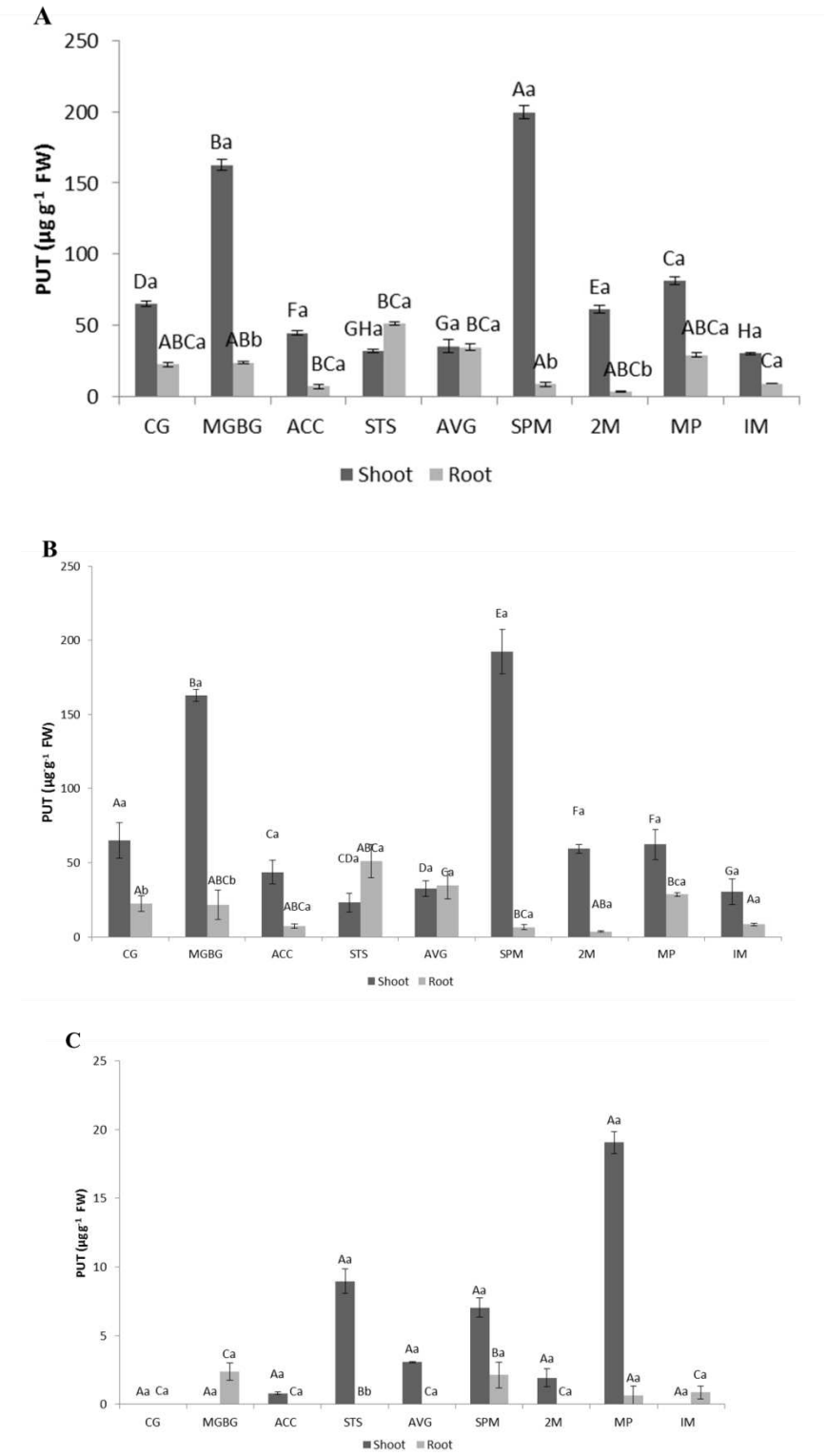


**Figure 9** - Levels of polyamines in the *Canavalia ensiformis* roots and shoots. A. Total Putrescine; B. Free Putrescine; C. Conjugated Putrescine. Means indicated by the same capital letters for all treatments and lowercase letter for a specific treatment (roots and shoots) are not significantly different as assessed by the Tukey's test at 5% probability level.

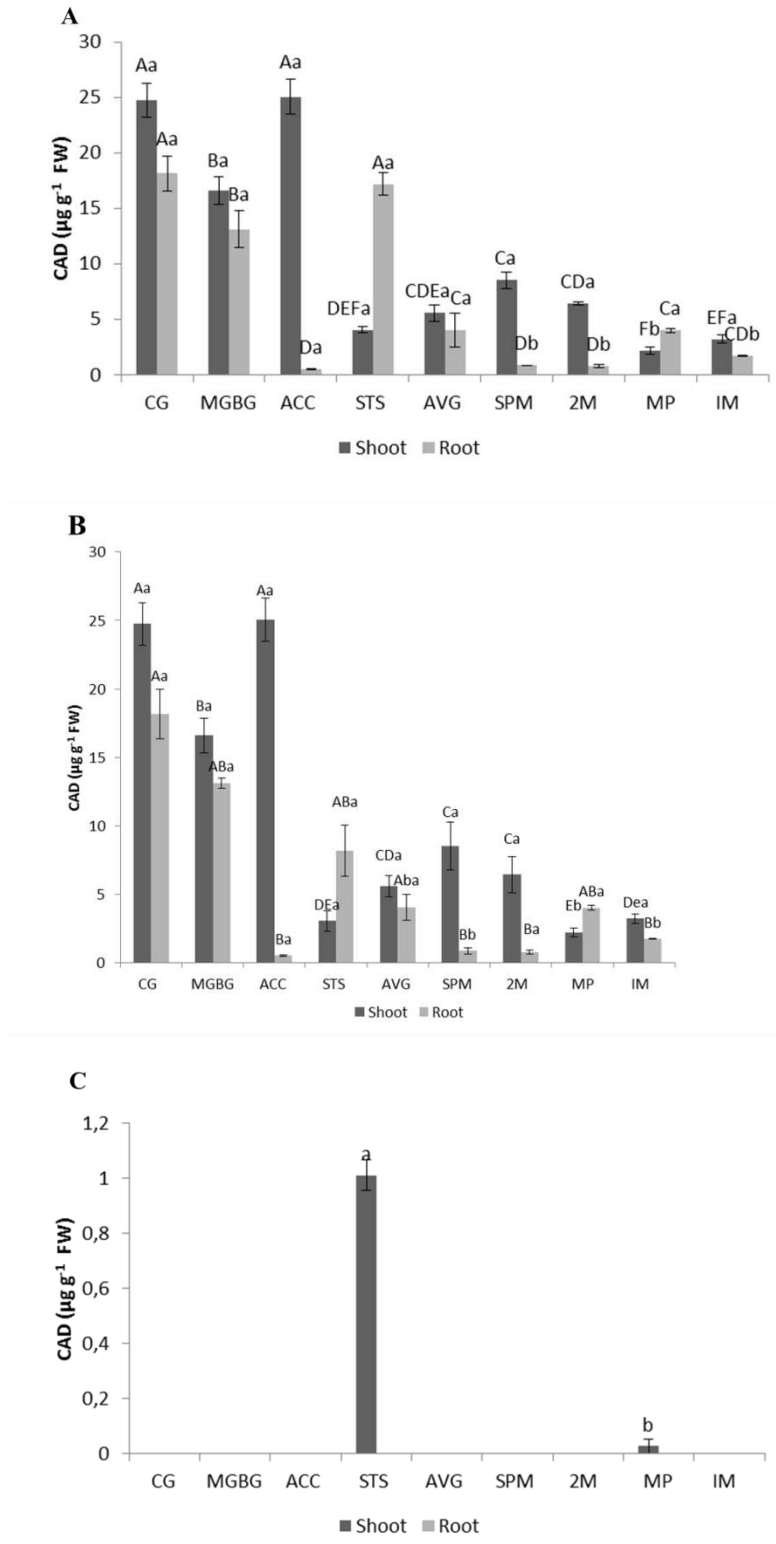


**Figure 10 -** Levels of polyamines in the *Canavalia ensiformis* roots and shoots. A. Total Cadaverine; B. Free Cadaverine; C. Conjugated Cadaverine. Means indicated by the same capital letters for all treatments and lowercase letter for a specific treatment (roots and shoots) are not significantly different as assessed by the Tukey's test at 5% probability level.

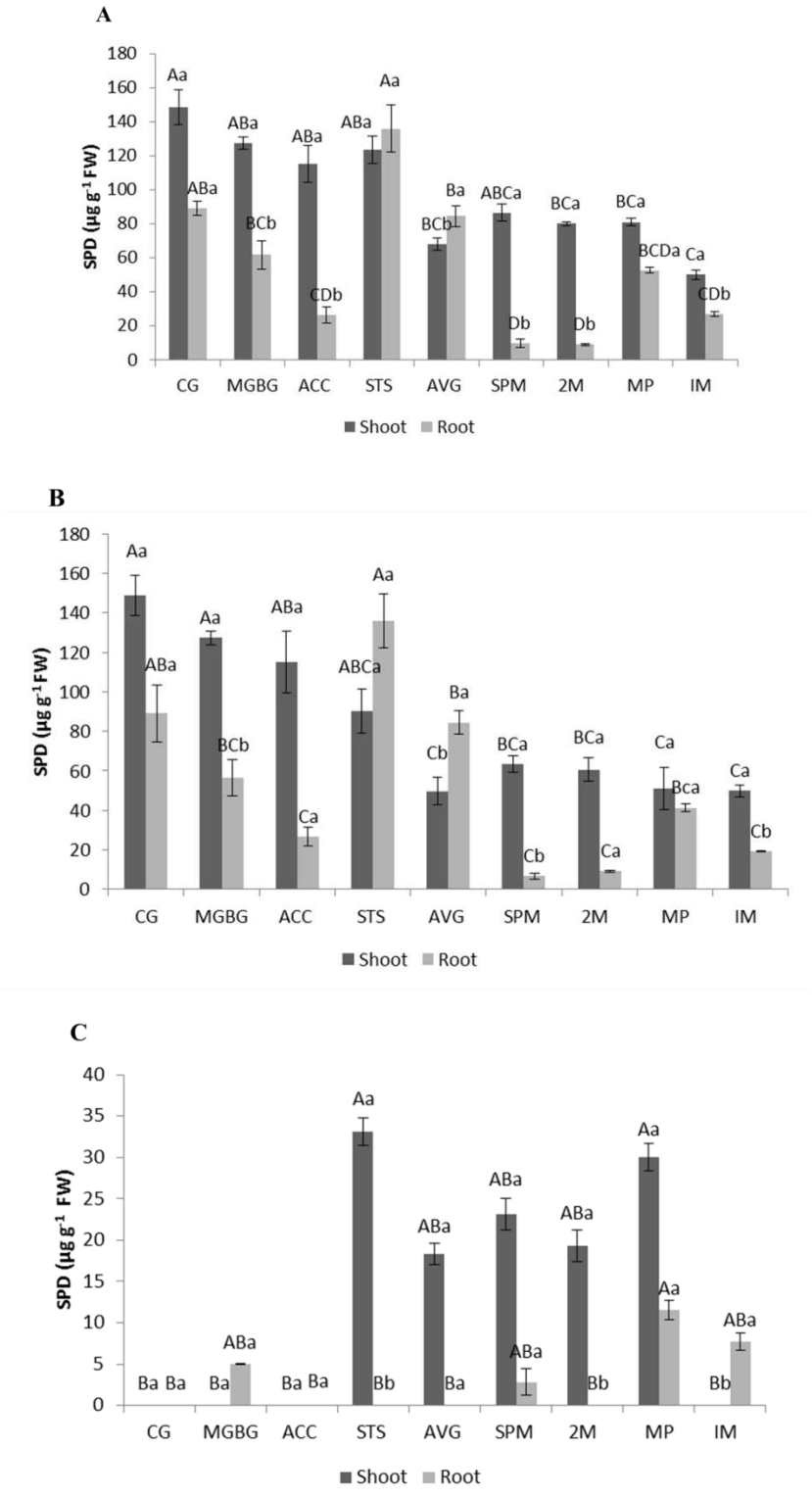


**Figure 11** – Levels of polyamines in the *Canavalia ensiformis* roots and shoots. A. Total Spermidine; B. Free Spermidine; C. Conugated Spermidine. Means indicated by the same capital letters for all treatments and lowercase letter for a specific treatment (roots and shoots) are not significantly different as assessed by the Tukey's test at 5% probability level.

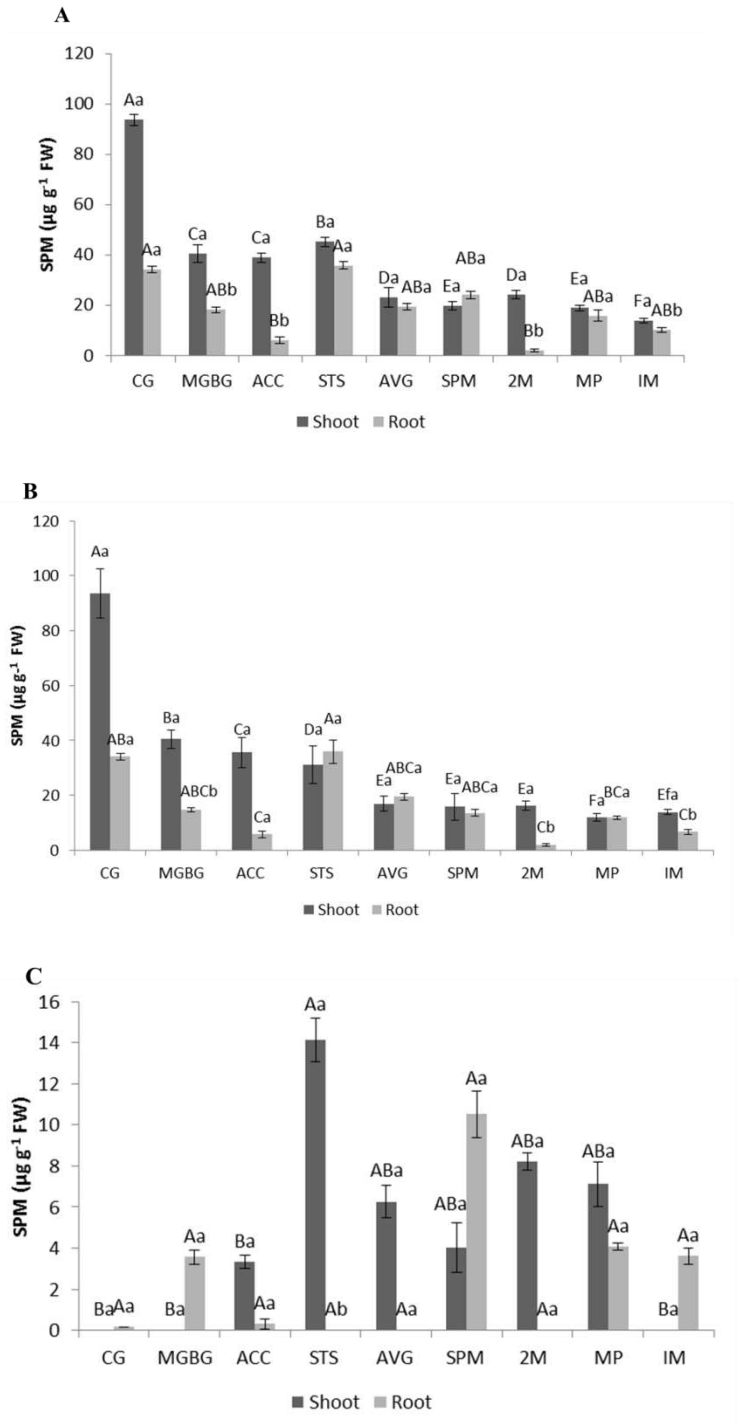


**Figure 12** - Levels of polyamines in the *Canavalia ensiformis* roots and shoots. A. Total Spermine; B. Free Spermine; C. Conjugated Spermine. Means indicated by the same capital letters for all treatments and lowercase letter for a specific treatment (roots and shoots) are not significantly different as assessed by the Tukey's test at 5% probability level.

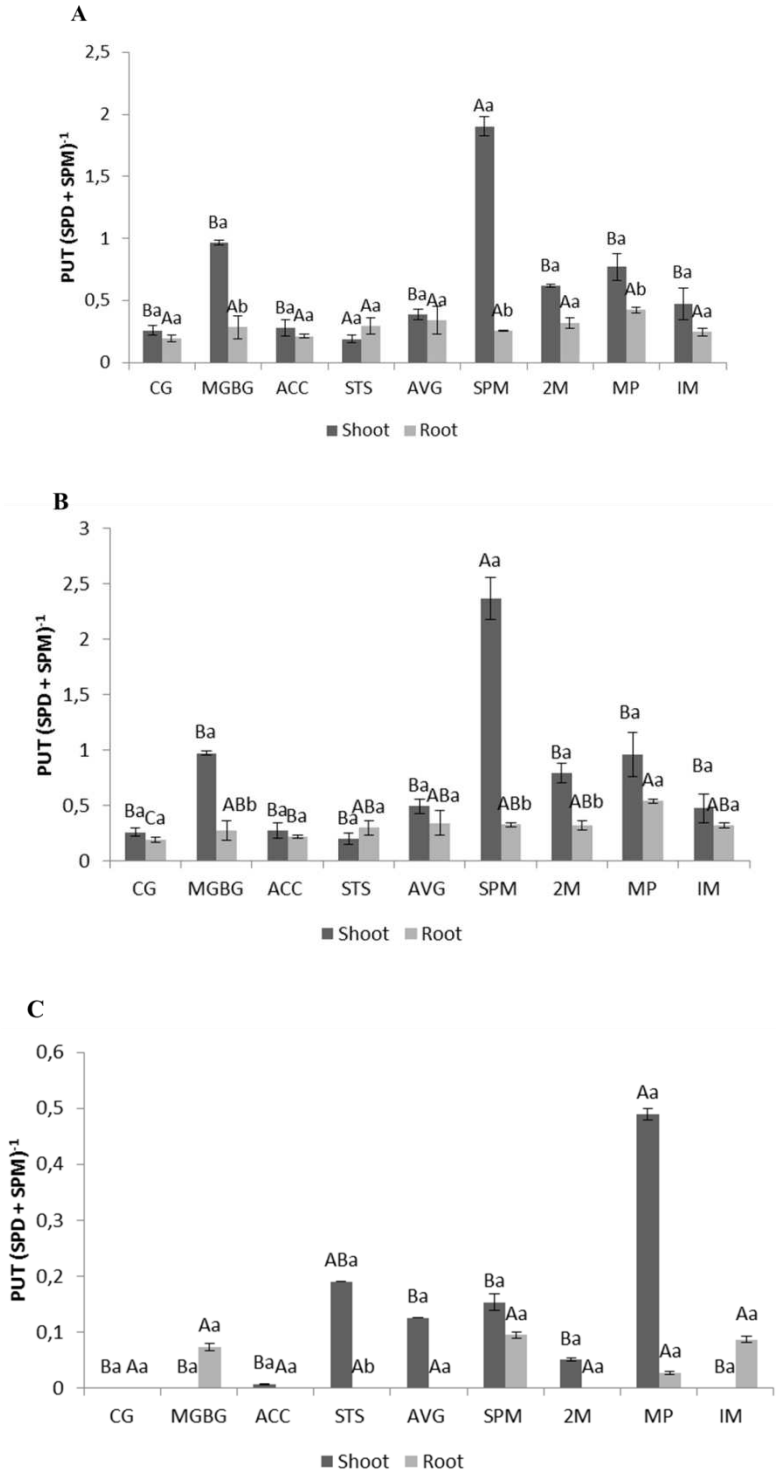


**Figure 13** - Ratio of polyamines in the *Canavalia ensiformis* roots and shoots. A. Ratio of total Put/(SPD + SPM); B. Ratio of free Put/(SPD + SPM); C**.** Ratio of conjugated Put/(SPD + SPM). Means indicated by the same capital letters for all treatments and lowercase letter for a specific treatment (roots and shoots) are not significantly different as assessed by the Tukey's test at 5% probability level.

## DISCUSSION

Seedling growth is a complex process in which endogenous plant growth regulators are known to play important roles (Bewley, 1997). One of these growth regulators is ethylene which is produced by most plant tissues during seed germination and can have profound effects on plant physiology (Locke et al., 2000).

Detectable quantities of ethylene were produced by *C. ensiformis* after the third day, which coincides with the onset of germination. Accumulation of ethylene increased with shoot length *C. ensiformis* and these results are consistent with findings for *Populus tremula* (González et al., 1997). This behavior was noted only in the treatments that produced ethylene.

The treatment with ACC increased ethylene levels in *C. ensiformis* and the same was noted in *Passiflora cincinatta* (Dias et al., 2010). This treatment caused growth inhibition, (Figs 6B and 6G) in some samples and even tissue necrosis. This suggests that ethylene levels in this treatment led to the inhibition of the morphogenic responses, in accordance with observed by Ortega-Martinez et al. (2007). The same behavior was shown by the control group, where full sealing prevented the release from the ethylene flasks, affecting plant development (Fig. 6B). The presented results confirm the conclusion reached by George and Sherrington (1984), that sufficient quantities of ethylene can accumulate in the culture flasks to regulate plant growth.

Inclusion of ACC in the germination medium resulted in reduced shoot and root growth in *Canavalia ensiformis* (Fig 5). In shoots, ethylene is usually associated with inhibited stem growth in both monocotyledonous and dicotyledonous plants (Locke et al., 2000). The inhibition of root growth is a normal characteristic response of plants to high amounts of ethylene (Abeles, 1973). It is thought that the reason for this is that mechanical stress results in enhanced ethylene production in the root, and the consequent thickening of the root leads to enhanced mechanical strength (Smalle and Van der Straeten, 1997).

No ethylene was detected in culture flask treated with MP, indicating that MP effectively scavenged the produced ethylene. Similar results were obtained by Dias et al. (2010). Treatments 1M and 2M, like that with MP, did not accumulate ethylene and permitted increased *C. ensiformis* growth (Figs 6C, 6D and 6I, respectively). Thus, these treatments containing one and two membranes improved the ventilation of *in vitro* culture flasks to allow greater gas exchange and no ethylene accumulation (Xiao et al.,

2011). Gas-permeable membranes have previously been used for improved culture of eggplant (Ribeiro et al., 2009) and passion fruit (Pinto et al., 2008).

AVG efficiently inhibited ethylene biosynthesis, resulting in no accumulation as was noticed by Locke et al. (2000), thus the *C. ensiformis* seedlings in this treatment developed more than the control treatment (Fig. 6B). The efficacy of AVG action on ethylene production is due to the high specificity of ACC synthase activity for ethylene biosynthesis (Huai et al., 2001). Seeds subjected to treatment with AVG, developed well after germination, indicating that ethylene has an effect on *C. ensiformis* growth inhibition. In sensitive grasses, overproduction of cyanide, which is formed at physiologically damaging concentrations as a co-product of ethylene during the oxidation of ACC, is implicated in phytotoxic growth inhibition (Grossmann and Kwiatkowski, 2000).

Other studies suggest that stimulation of ethylene, via induction of ACC synthase, triggers an increase of endogenous abscisic acid (ABA), which causally leads to growth inhibition (Hansen and Grossmann, 2000). As shown previously and according to the obtained results (Fig 4), ABA affects biomass production might be due by reducing stomatal aperture and consequently $CO_2$ assimilation (Grossmann and Scheltrup, 1995). However, the effects of ethylene inhibitors and biosynthesis inhibitors prompted the investigation of another class of endogenous compounds of these processes: polyamines.

Since the biosynthetic pathways of ethylene and polyamines are linked through SAM, the stimulatory effects of ethylene synthesis biosynthetic inhibitors on growth might be due to enhanced polyamine production (Martin-Tanguy, 2001). Inclusion of spermine did not inhibit germination of *C. ensiformis*, and had very similar effects to those of AVG in stimulating both root and shoot growth of germinated seedling to lengths greater than those of the controls (Fig 6A and Fig 6B, respectively). According to Locke et al. (2000) this is probably due to inhibition of ethylene biosynthesis as reported by Apelbaum et al. (1981).

The effect of polyamine may be directed at the step for converting ACC to ethylene (AdaGC and Yang, 1979), therefore polyamines may interact with the plant membranes in a way that inhibits ethylene evolution by a physical mechanism (Cheng et al., 2012). It has already been shown that putrescine and spermidine alter membrane fluidity of *Phaseolus vulgaris* L. (Roberts et al., 1986). Our results are in accordance

with those of Locke et al. (2000) that when adding putrescine and spermine to the medium, stimulated root and shoot growth to levels above those of control.

As indicated in the work of Pang et al. (2010), MGBG in the medium competitively inhibits the enzyme SAM decarboxylase and, therefore should reduce spermidine and spermine contents that result in slightly reduced shoot and root length. However, our work showed the opposite, where *C. ensiformis* seedlings under the influence of MGBG presented a normal development (Fig. 6H). Thus, considering that MGBG decreasing seedling growth and because putrescine biosynthesis is not affected by this compound, complete growth inhibition would not be predicted (Locke et al., 2000).

PUT, SPD, and SPM play different roles in the morphogenesis of different plant species (Zhu and Chen, 2005). The addition of PAs to the culture medium has been widely applied to assess the function of PAs in cell growth and differentiation (Martin-Tanguy, 2001). The present work showed that exogenously added SPM decreases levels of ethylene and increased *C.ensiformis* seedling growth. This PA has been reported to improve shoot growth in orchids from the Dendrobium genus (Kumari and George, 2011). Addition of SPM increased total Put levels, leading to its production instead of ET.

In treatments that there ethylene accumulation of cadaverine was increased in *C. ensiformis*. The same result was noticed in seedlings of *Pisum sativum*. The elevated level of cadaverine resulted from an increase in lysine decarboxylase activity in the tissue exposed to ethylene, where the cadaverine was observed in enlogation zone tissue. This consequent accumulation of cadaverine in ethylene-treated plants, is of a compensatory nature as a response to the inhibition of arginine and *S*-adenosylmethionine decarboxylase activity provoked by ethylene (Icekson et al 1986).

Addition of SPM increased the PUT/(SPD + SPM) ratio (Figs.13A, 13B and 13C); and this result corroborates with the antagonist relationship reported for these groups of PAs (Shoeb et al., 2001). Increase in the PUT/(SPD + SPM) ratio coincides with the treatments where *C. ensiformis* shoot development was greatest. The same is observed in the study of Astarita et al. (2003), where the increased PUT/(SPD + SPM) ratio resulting in taller seedlings of *Araucaria angustiofolia*.

The addition of MGBG to the medium reduced PA levels, except Put, decreasing ET production and stimulating *C. ensiformis* seedling growth. This suggests that MGBG may influence the expression of key genes involved in ET synthesis (Pang et al., 2010).

For the treatments with ACC no increase was observed in PA levels inhibiting *C. ensiformis* growth. In this treatment, the Put concentration was reduced in the shoots because of reduced competition with ethylene biosynthesis. Similar behavior in polyamine levels were found by Dias et al. (2010).

An increase in the PUT concentration was observed in *C. ensiformis* treated with STS, possibly as a result of decreased SPM biosynthesis caused by the increased ethylene biosynthesis. Roustan et al. (1990) observed that the addition of silver nitrate promoted an increase in ADC (arginine decarboxylase) activity, leading to an increase in endogenous polyamine levels in *Daucus carota* embryogenic cultures. Biondi et al. (1998) studied tobacco subepidermis layers and observed that when ethylene action was blocked by STS, organized growth was inhibited but was not undifferentiated proliferative growth.

It was ascertained that neither the absolute ethylene and polyamine levels nor the competition between them were the defining factor of the growth response. The PAs may play an important role in homeostasis, preventing ethylene action and regulating sensitivity to ethylene. Studies investigating the effects of polyamines on the expression of genes related to ethylene perception during cell differentiation are of fundamental importance for a complete understanding of these processes in *C. ensiformis.*

The results presented in *Canavalia ensiformis* highlight novel interactions between ethylene, polyamines and plant growth. Firstly, exogenous polyamines stimulate seedling root and shoot growth in *C. ensiformis*. Thus, polyamines can substitute ethylene in the promotion of growth, and ethylene scavengers such as mercury perchlorate, ethylene inhibitors like AVG and membranes system can be useful improving seedling growth in *Canavalia ensiformis*. This is because the presence of ethylene modifies the growth of *Canavalia ensiformis* seedlings, reaching complete inhibition.

## CONCLUSÕES

*Canavalia ensiformis* apresentou alta sensibilidade aos níveis de etileno, cessando o desenvolvimento após a germinação.

Os tratamentos com inibidor de etileno, aminoetoxivinilglicina (AVG), perclorato de mercúrio (PM), sistema de membranas, poliaminas e inibidor de poliaminas (MGBG) acarretaram crescimento da planta, coincidindo com elevado teor

de poliaminas, principalmente, espermina e putrescina, e alta razão entre putrescina e espermidina + espermina.

O tratamento com o ácido 1-aminociclopropano-1-carboxílico (ACC), precursor de etileno, apresentou características de baixo crescimento em *C. ensiformis*.

Poliaminas exógenas, perclorato de mercúrio e o sistema de membranas apresentaram efeito estimulador do crescimento na parte aérea e raízes de *C. ensiformis*. Assim, esses compostos podem ser utilizados para controlar o crescimento *in vitro* em *C. ensiformis*.

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**APÊNDICE**

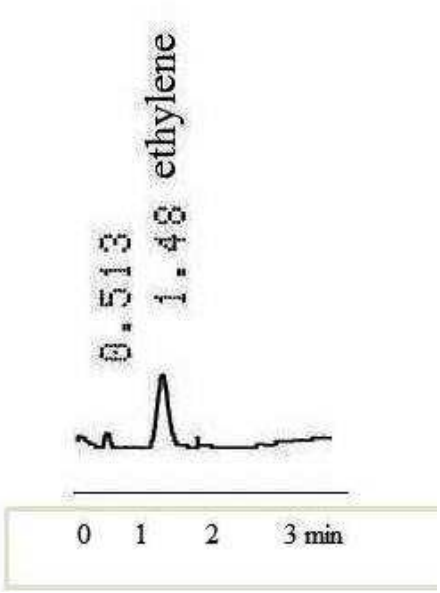


**Figura 14 –** Cromatograma da atmosfera do grupo controle (1 mL) aos sete dias de cultivo de plântulas de *Canavalia ensiformis*, com pico de etileno no tempo de retenção de 1,48 minutos.

| dias | área do padrao | massa (g) | MS | massa (g) | MGBG | massa (g) | ACC | massa em (g) | STS |
|---|---|---|---|---|---|---|---|---|---|
| **3** | 1473 | | 0 | | 0 | 1,87 | 462,4 | | |
| **6** | 1068 | | 0 | 4,37 | 129,6 | 1,87 | 273,8 | | |
| **7** | 900 | 3,88 | 103 | 8,75 | 94,4 | 3,42 | 378,8 | 8,4 | 90 |
| **8** | 1015 | 3,9 | 102,4 | 8,75 | 375,4 | 3,32 | 327,8 | 8,78 | 176 |
| **9** | 1090 | 3,87 | 180,2 | 8,75 | 704 | 3,3 | 278 | 8,8 | 146 |
| **13** | 1261,5 | 3,87 | 475,4 | 8,75 | 169 | 5,04 | 97,6 | 8,8 | 147 |
| **14** | 861 | 3,75 | 491,2 | 7,84 | 169 | 2,06 | 100 | 7 | 457 |
| **15** | 1274,5 | 3,49 | 1154,4 | 8,75 | 169 | 3,12 | 111 | 5,1 | 900 |
| **16** | 1100 | 3,82 | 1060,6 | 8,75 | 367,2 | 3,12 | 169,6 | 5,1 | 800 |
| **19** | 1141 | 3,75 | 371,4 | 8,75 | 317 | 3,12 | 268,6 | 5,1 | 712 |
| **20** | 1237 | 3,12 | 283,2 | 7,5 | 277,8 | 3,12 | 134,2 | 5,1 | 730 |
| **21** | 1120 | 3,12 | 278,6 | 7,5 | 278,4 | 3,12 | 129,4 | 5 | 750 |
| **22** | 1440 | 3,12 | 264,6 | 7,5 | 278,4 | 3,12 | 176 | 5 | 888 |

**Tabela 3** – Média das áreas dos picos de etileno em plântulas de *Canavalia ensiformis* durante 22 dias e massa fresca das plântulas.

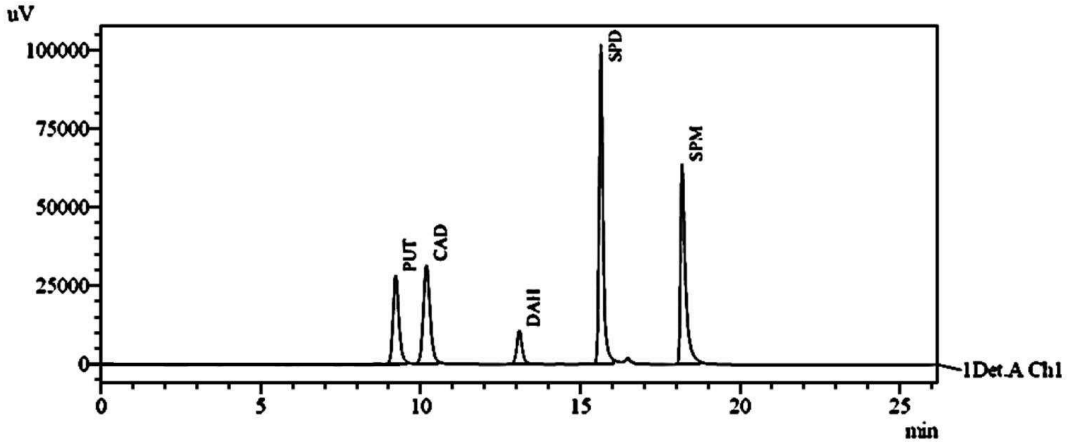


**Figura 15 –** Cromatograma de mistura de padrões de poliaminas putrescina (Put), cadaverina (Cad), espermidina (SPD), espermina (SPM) e padrão interno 1,7-diaminoheptano (DAH 20 µL) na concentração de 1 nmol por 40 µL de amostra. As poliaminas foram analisadas em HPLC numa coluna C18 de 5 µm (Shimadzu Shin-Pack CLC ODS), utilizando-se gradiente acetonitrila como fase móvel. O fluxo foi de 1 mL $min^{-1}$ a 40 ºC e o detector de fluorescência foi calibrado para 340 nm (excitação) e 510 nm (emissão).

**Dados: área do pico (uV)**

| **[ ] nmol/40 uL da Amostra** | **88,15 Put** | **102,18 Cad** | **DAH** | **145,20 Spd** | **202,30 Spm** | **MW** |
|---|---|---|---|---|---|---|
| 1 | 180082 | 222085 | 169202 | 308856 | 152237 | |
| 2,5 | 355789 | 460564 | 120976 | 860974 | 677232 | |
| 5 | 493402 | 653977 | 217250 | 876685 | 713904 | |
| 25 | 2872837 | 3703866 | 136197 | 5539878 | 5935038 | |
| 50 | 4950805 | 6309382 | 130717 | 10040962 | 10662558 | |
| **R2** | **0,9938** | **0,9999** | | **0,9961** | **0,9946** | |
| **Máximo:** | | **217250** | | | | |

**Dados: área do pico (uV) corrigida por DAH***

| [ ] nmol/40 uL da Amostra | Put | Cad | Spd | Spm |
|---|---|---|---|---|
| 1 | 231220 | 285150 | 396561 | 195467 |
| 2,5 | 638930 | 827086 | 1546146 | 1216180 |
| 5 | 493402 | 653977 | 876685 | 713904 |
| 25 | 4582508 | 5908096 | 8836747 | 9467073 |
| 50 | 8228175 | 10486113 | 16687952 | 17721036 |
| **R2** | **0,9931** | **0,9925** | **0,9933** | **0,9937** |

*** Área do pico poliamina/Área do pico DAH * Àrea máxima do pico de DAH**

**Tabela 4 –** Cálculo da curva padrão de poliaminas corrigidas por padrão interno de DAH

| | | PUT | | CAD | | SPD | | SPM | | DAH | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | F | C | F | C | F | C | F | C | F | C |
| **ACC** | shoot | 161082 | 259515 | 95390 | 48615 | 525947,67 | 840284 | 124490,3333 | 252217 | 97435,66667 | 224465,6667 |
| | root | 33840,66667 | 42074 | 2784 | 1343 | 153989,67 | 196761 | 25256,66667 | 44174,66667 | 125026 | 212924,6667 |
| **CG** | shoot | 348653 | 330747,3333 | 157305,67 | 24533 | 928304,33 | 863084 | 463260,3333 | 310464,3333 | 130681 | 239932,6667 |
| | root | 102335 | 147763,6667 | 91286,667 | 35441,33333 | 500200 | 676308,6667 | 145486,6667 | 224995,6667 | 118926 | 206435,3333 |
| **MGBG** | shoot | 784525,3333 | 785776,5 | 88360,667 | 12454,5 | 748490,33 | 779908,5 | 182236 | 171063 | 125371,6667 | 220739,5 |
| | root | 104509,6667 | 157203 | 88360,667 | 39174,66667 | 336650 | 429357,3333 | 66613,66667 | 91710,66667 | 127103 | 179094 |
| **STS** | shoot | 161591 | 282500 | 25861 | 32113 | 765372,33 | 1319989 | 202818,6667 | 367578 | 194233 | 226950,6667 |
| | root | 243822,3333 | 287323,3333 | 94215,667 | 12742 | 802371 | 994472 | 163524 | 238833,3333 | 126638,6667 | 230474,6667 |
| **AVG** | shoot | 254070,3333 | 318370 | 48174,667 | 24571 | 474677 | 743616,3333 | 123619 | 197001,6667 | 184739,6667 | 209327 |
| | root | 169974 | 195471,3333 | 20929 | 5671 | 491055,67 | 609598,3333 | 86300 | 126348,6667 | 124846,6667 | 233486 |
| **MP** | shoot | 473747 | 729368,6667 | 19340 | 17210,33333 | 472512,33 | 879812,3333 | 83887 | 158455,3333 | 213742,6667 | 225066 |
| | root | 215754,6667 | 257591 | 33395,333 | 11462,33333 | 378878 | 594849 | 82447,33333 | 134625 | 177275,6667 | 219535,3333 |
| **1M** | shoot | 212243,6667 | 271849,6667 | 24484 | 12723 | 415949,67 | 716952,6667 | 88641,66667 | 125493 | 178592 | 335208,6667 |
| | root | 70075,66667 | 75642,33333 | 15866,667 | 6564,666667 | 194862 | 276976,3333 | 52740,66667 | 81129 | 209938,6667 | 218913,3333 |
| **2M** | shoot | 457213 | 604302 | 53754,333 | 53142,66667 | 575509,33 | 987335,3333 | 117242,3333 | 228935 | 182743,3333 | 242037 |
| | root | 28283,66667 | 28920,66667 | 6813,3333 | 2458,333333 | 89430,333 | 131612,3333 | 15240 | 32510,66667 | 189258,6667 | 234616,3333 |
| **SPM** | shoot | 1351290,667 | 1915314 | 66955,333 | 72666 | 544533 | 1083294,333 | 105253 | 180125,3333 | 167823 | 243100,3333 |
| | root | 58047 | 111622,5 | 8411,6667 | 3311 | 73679,667 | 153522,5 | 112692,3333 | 307575,5 | 212555 | 247627,5 |

**F = Free polyamines; C = Conjugated polyamines**

**Table 5 -** Peak area means (uV) of polyamines chromatograms of *Canavalia ensiformis* shoots and roots in following treatments: ACC (1-aminocyclopropane-1-carboxylic acid), CG (control group), MGBG (methylglyoxal-bis(guanylhydrazone)), STS (silver thiosulfate), AVG (aminoethoxyvinylglycine), MP (mercury perchlorate), 1M (mercury perchlorate), 2M (two membranes), SPM (spermine).