Scientific Article

Effect of glutamine and arginine on growth of *Hibiscus moscheutos* "*in vitro*" (1)

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ABSTRACT

Nitrogen is the most essential element for plant growth and development. Amino acids, serving as the main organic nitrogen source in tissue culture media, provide for shoot and root elongation. Glutamine has been widely used in tissue culture for dedifferentiation and re-differentiation processes. Experiments were conducted to assess the effects of glutamine in comparison to some commonly used plant growth regulators (PGRs) on the growth of *Hibiscus moscheutos* propagated via tissue culture. An initial study suggested that 10 mg L^{-1} glutamine in MS basal medium was optimal for shoot elongation. At this optimal rate, glutamine showed superiority over other PGRs. No difference was found between glutamine treatments and the control in a later study. When comparing glutamine with arginine, shoots cultured on media with arginine displayed slightly greater growth. With arginine containing two extra nitrogen groups in its molecular structure, the higher percentage of nitrogen may have resulted in improved growth.

Keywords: ornamental plants, tissue culture, propagation

RESUMO

Efeito de glutamina e arginina no crescimento de *Hibiscus moscheutos "in vitro"*

O nitrogênio é o elemento mais essencial para o crescimento e desenvolvimento das plantas. Os aminoácidos, que servem como principal fonte de nitrogênio orgânico nos meios de cultura de tecidos proporcionam o alongamento da parte aérea e da raiz. A glutamina tem sido amplamente utilizada na cultura de tecidos para processos de desdiferenciação e re-diferenciação. Foram realizados experimentos para avaliar os efeitos da glutamina em comparação com alguns reguladores de crescimento de plantas (PGRs) comumente usados no crescimento de moscheutos de Hibiscus propagados via cultura de tecidos. Um estudo inicial sugeriu que 10 mg L^{-1} de glutamina em meio basal MS era ideal para o alongamento da parte aérea. Nesta taxa ótima, a glutamina mostrou superioridade sobre os outros PGRs. Nenhuma diferença foi encontrada entre os tratamentos com glutamina e o controle em um estudo posterior. Ao comparar glutamina com arginina, os brotos cultivados em meio com arginina apresentaram um crescimento ligeiramente maior. Com arginina contendo dois grupos extras de nitrogênio em sua estrutura molecular, a maior porcentagem de nitrogênio pode ter resultado em crescimento melhorado.

Palavras-chave: plantas ornamentais, cultura de tecidos, propagação.

1. INTRODUCTION

Nitrogen supports plant growth and development in its various forms: ammonia, nitrate and nitrogen gas as inorganic sources, and amino acids as organic nitrogen supplements. Of these, both nitrogen absorbed from the atmosphere and nitrate absorbed from soil and water need to be reduced to ammonia before being transported into metabolic pathways (Canovas et al., 1998). Amino acids in the glutamine/glutamate family (glutamine, glutamate, proline, arginine) initiate and accelerate ammonia and nitrite entering into organic nitrogen metabolism. Under catalysis of glutamine synthetase, glutamine is reduced to glutamate, and can be further modified into proline or arginine (Horton et al., 2006; Shahsavari, 2011). These amino acids also function as nitrogen storage sites (Flaig and Mohr, 1992; Okumoto et al., 2016), as intermedia to incorporate ammonia into amino acids (Horton et al., 2006; Okumoto et al., 2016), and eventually as building blocks of proteins or nucleotides, such as purine and pyrimidine (Horton et al., 2006). Because of this, amino acids serve as an efficient nitrogen source through direct synthesis of proteins (Efzueni Rozali et al., 2014), and provide an efficient pathway for nitrogen assimilation during long distance metabolic transportation (Okumoto et al., 2016).

In Murashige and Skoog basal media, inorganic nitrogen is supplied in sufficient quantities in both the ammonia and nitrate forms (Murashige and Skoog, 1962). Therefore, performance and growth of cultured plant tissues is thought to be limited by the nitrogen uptake and assimilation efficiency within the tissues. Supplementing tissue culture basal media with glutamine enables higher nitrogen intake due to an increase of both nitrogen sources and assimilation ability (Okumoto et al., 2016). Glutamine has been used in tissue culture media in both the dedifferentiation and re-differentiation processes (Habib et al., 2015): Callus induction (Cai et al., 2013), shoot differentiation (Perveen and Mansuri, 2015), faster rooting (Liu et al., 2015; Toppo et al., 2012) and somatic embryogenesis (Yapo et al., 2011).

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Besides glutamine, other amino acids in the glutamine family, specifically proline and arginine, are also beneficial to plant tissue growth and have been used for shoot and root induction (Toppo et al., 2012). As a nitrogen source for plants, arginine ($C_6H_{14}N_4O_2$) contains 32.2% nitrogen, which is greater than the 19.2% nitrogen content in glutamine ($C_5H_{10}N_2O_3$) (Winter et al., 2015). Hibbs et al. (1987) also stated a possible L-arginine deiminase activity that transfers the two extra imino groups from L-arginine structure into nitrite, which is easier for plants to assimilate.

The goal of the present study was to investigate glutamine and arginine as organic nitrogen sources for their effects on enhancing axillary shoot multiplication and elongation. Specific objectives were to: (1) compare the effects of glutamine with those of some plant growth regulators (PGRs) conventionally used for axillary shoot production and elongation, (2) identify an optimal rate for glutamine for shoot elongation, (3) compare the effect of shoot growth and root initiation specifically between glutamine and gibberellic acid, and (4) determine whether glutamine or arginine was the preferred amino acid as soluble nitrogen source to support shoot elongation.

2. MATERIAL AND METHODS

Plant material

A purple foliage selection of *Hibiscus moscheutos*, Hib 2014-113, from the breeding program of Dr. John M. Ruter, University of Georgia, GA, was used in this study. Studies were conducted in the Horticulture Tissue Culture laboratory at the University of Georgia Athens Campus. Healthy, vigorous Hib 2014-113 *in vitro* plants were selected when they were under culture in MS medium (Murashige and Skoog, 1962). Two-node shoots were cut from mid-stem of the plants and leaves on the lower node were removed before shoots were transferred into culture treatments.

All media was prepared using filter sterilization for any PGRs or amino acids, one day before the treatments. MS basal medium was prepared from powder mix (Sigma-Aldrich®, St. Louis, MO) and was supplemented with 30 g sucrose and gelled with 7 g agar (Fisher Scientific®, Hampton, NH). The pH of the tissue culture medium was adjusted to 5.7. MS medium was pre-autoclaved and placed in a 68 °C water bath while PGRs or amino acids were being filter sterilized. To ensure sterile conditions, pure powder of PGRs or amino acids was first completely dissolved in sterile distilled water and then the solution was sterilized through 0.2 mm pore-size filter (Whatman® sterile PVDF, Maidstone, UK) using a sterile syringe. Sterile PGRs or amino acids were then proportionally added to warm sterile MS basal media.

After shoots were transferred into tissue culture treatments, they were placed on shelves in a culture room with a constant 20 °C temperature and 16-h photoperiod under 98 µmol m^{-2} s^{-1} fluorescent light (measured with Li-250A light meter, Li-Cor®, Lincoln, NE) (F40T12/DX, Philips® Lightning, Amsterdam, the Netherlands).

Study I: Comparisons of BAP, GA_3, TDZ and L-glutamine

Two-node shoots of similar length and vigor were selected. They were then transferred in four treatment groups of MS media with 1.5 mg L^{-1} BAP (6-Benzylaminopurine), 0.35 mg L^{-1} GA_3 (Gibberellic acid), 10 mg L^{-1} L-glutamine, or 0.22 mg L^{-1} TDZ (Thidiazuron). Additionally, a control group with no PGRs or glutamine for a comparative study was prepared. There were 10 replicates per treatment with one shoot per replicate. Each shoot was transferred into MS gel media perpendicular to the surface of the gel in 25 x 150 mm glass test tube (Pyrex®, Corning, NY). Test tubes were then capped and sealed with Parafilm tape (Parafilm M®, Neenah, WI). New shoot elongation was measured weekly for seven weeks. Leaf necrosis of each plant was visually rated at the end of the study. The rating was designed as a 0 to 3 numbering system. Shoots with no necrosis were annotated as "0", shoots with light leaf necrosis were annotated as "1", shoots with medium necrosis were annotated as "2", and shoots with severe leaf necrosis were annotated as "3".

Study II: Comparisons of different concentrations of L-glutamine

Two-node shoots of similar length and vigor were prepared for the following study. Shoots were transferred in gelled MS media supplemented with 0-, 0.01-, 0.05, 0.1- or 0.5- g L^{-1} glutamine. Each treatment group contained 10 replicates. The length of new shoot growth was measured from outside the test tubes weekly. The entire study was terminated when the first tissue-cultured plant of any treatment reached the top of test tube cap. To take measurements after this stage of growth would provide to inaccurate results.

Study III: Comparisons between GA_3 and L-glutamine

Vigorous two-node shoots were used in this study. Two rates of GA_3, 2 mg L^{-1} and 4 mg L^{-1} and two rates of glutamine, 5 mg L^{-1} and 10 mg L^{-1} were added to MS basal medium. Adding an additional control group with no amino acid supplements, shoots were transferred into media of these five treatment groups in a method described in Study I. Each treatment unit contained 10 replicates. The length of new shoot growth was measured weekly for seven weeks. Binomial data was also taken on the presence or absence of roots at the termination of this study.

Study IV: Comparisons between L-glutamine and L-arginine

In this study, except for the control group, to the MS media in each treatment group was added 10 mg L^{-1} glutamine, 10 mg L^{-1} arginine, or a combination of both. Healthy two-node shoots were transferred into these four treatments using the method described in Study I. There were 10 replicates per treatment. The length of new shoot elongation was assessed (from outside of the test tubes) weekly until the first tissue-cultured plant reached the top

of the test tube cap at week seven, at which point the study was terminated. Plants were inspected weekly to determine if rooting had occurred. The number of weeks it took, post treatment, for the first root to emerge was recorded as rooting data. Shoots that had not rooted before termination were not included in the data analysis.

Data analysis

Data for each study was collected weekly and analyzed using R statistical software (R, 2015). The statistical significance of different treatment groups was assessed in ANOVA and means comparisons were made using Tukey's honest significance test (HSD Test) (Tukey, 1949) or Dunnett's test (Dunnett, 1964). Means (±Standard Deviation) are presented.

3. RESULTS AND DISCUSSION

Study I: Comparisons of BAP, GA_3, TDZ, L-glutamine

After seven weeks of culture, some two-node shoots expanded leaves, elongated stems and grew up to the top of the test tubes. Glutamine treatments (13.9 ± 2.2 cm) and GA_3 treatments (12.0 ± 4.4 cm) displayed significantly greater shoot elongation than other treatment groups (Table 1).

Table 1. Two-node shoots of *Hibiscus moscheutos* were cultured in MS basal medium containing 1.5 mg L^{-1} BAP, 0.35 mg L^{-1} GA_3, 10 mg L^{-1} L-Glutamine or 0.22 mg L^{-1} TDZ. Overall shoot growth was measured after culturing for seven weeks. A Tukey's HSD test was performed at 95% significance level on the overall shoot elongation and foliage necrosis. Foliage necrosis was rated in a numbering system from 0 (no necrosis) to 3 (severe necrosis).

Treatment	Shoot Elongation (±SD) (cm)	Plant Necrosis * (±SD)
Control	9.5 (±3.4) bc	2.6 (±0.7) a
BAP	8.3 (±2.8) bc	1.7 (±0.5) bc
GA_3	12.0 (±4.1) ab	1.4 (±0.5) c
L-Glutamine	13.9 (±2.2) a	1.5 (±0.5) bc
TDZ	7.1 (±1.2) c	2.2 (±0.6) ab

TDZ did not stimulate, but inhibited, shoot growth when overall shoot elongation was compared to the control group. BAP promoted shoot differentiation at an early stage of culture and largely encouraged axillary shoot proliferation, but overall impact on length of shoot elongation was minor. BAP, glutamine and GA_3 performed the best in preventing leaf necrosis after seven weeks of culture. Shoots in the control group and the TDZ-treated group showed significant leaf yellowing and abscission (Table 1).

Study II: Comparisons of different concentrations of L-glutamine

Different concentrations of glutamine were added to the culture to allow for examination of their effect on shoot growth over the seven-week period. At week seven, shoots growing in media with 0.01 g L^{-1} glutamine showed the greatest elongation at 5.8 ± 2.8 cm (Table 2).

While there was no significant differences between the control group and 0.05 g L^{-1}, 0.1 g L^{-1} or 0.5 g L^{-1} glutamine treated groups, shoots cultured in 0.01 g L^{-1} glutamine-amended medium were significantly longer than those of the control group (p=0.003).

Study III: Comparisons between GA_3 and L-glutamine

Two-node shoots in this study showed slow or no growth initially, but most of them resumed growth at week six or seven and developed rapidly in the last two weeks. The experiment was terminated after week seven because some of the shoots reached the top of the test tubes. The means of shoot elongation in the GA_3 treatments (1.27 ± 0.88 cm, 1.41 ± 0.96 cm, for 2 mg $L^{-1}$and 4 mg L^{-1}, respectively) were lower than those of other groups; however, there were no significant differences between the GA_3 treatments and glutamine treatments or the control due to great variability among replicates within treatments. Shoots treated with GA_3 did not initiate rooting at any concentration. Fifty percent of the plants in the control group developed roots, while 50 mg L^{-1} glutamine promoted 90% rooting among treated plants, which was the highest of all treatments (Table 3).

Table 2. Two-node shoots of *Hibiscus moscheutos* were cultured in MS basal medium supplemented with 0-, 0.01-, 0.05-, 0.1-, or 0.5 g L^{-1} glutamine. Length of shoot growth (±Standard Deviation) was measured at seven weeks before the study was terminated. Dunnett's test was performed to look at the difference between each glutamine concentration and the control group.

Glutamine Concentration (g L^{-1})	Shoot Elongation (±SD) (cm)
0	4.2 (±1.6)
0.01	5.8 (±2.8)
0.05	4.2 (±3.6)
0.1	4.7 (±4.7)
0.5	4.4 (±3.6)
Dunnett's Test	**Significance**
0 vs 0.01 g L^{-1}	**
0 vs 0.05 g L^{-1}	NS
0 vs 0.1 g L^{-1}	NS
0 vs 0.5 g L^{-1}	NS

**- significant at p≤0.01, NS-not significant.

Table 3. Two-node shoots of *Hibiscus moscheutos* were cultured in MS basal medium supplemented with 10 mg L^{-1} glutamine, 50 mg L^{-1} glutamine, 2 mg L^{-1} GA_3 or 4 mg L^{-1} GA_3. Treatment groups wasa considered as categorical factors for a one-way ANOVA. Length of shoot elongation was measured (±Standard Deviation) after culturing for seven weeks. Rooting percentage of each treatment was analyzed in Tukey's HSD test.

Treatment	Shoot Elongation (±SD) (cm)	Rooting Percentage (%)
Control	4.8 (±5.1)	50 (±52.7) b
Glutamine (10 mg L^{-1})	5.0 (±3.9)	40 (±51.6) b
Glutamine (50 mg L^{-1})	5.7 (±5.6)	90 (±31.6) a
GA_3 (2 mg L^{-1})	1.3 (±0.9)	0 (±0) b
GA_3 (4 mg L^{-1})	1.4 (±1.0)	0 (±0) b
Analysis of variance		
Treatment	NS	*

*-significant at p≤0.05, NS-not significant.

Study IV: Comparisons between L-glutamine and L-arginine

Shoots in different treatment groups developed varying lengths of new growth at varying speeds. This study was terminated when some shoots reached the tops of sealed test tubes. Although shoots with the greatest elongation were observed in the treatment containing both glutamine and arginine, there was no significant difference among treatments (p=0.73) (Table 4). Observations from the last two weeks of this study, however, revealed a greater increase in shoot elongation for the treatment containing glutamine plus arginine (p=0.002) (Table 5 and Figure 1). Within the seventh week, shoots in the glutamine plus arginine treatment developed greater elongation (7.6 ± 3.1 cm) than those in other treatments, and shoots in the glutamine treatment (2.7 ± 6.1 cm) developed the least elongation among all treatments. No significant difference was found between any treatments for when the first root emerged (Table 4).

Table 4. Two-node shoots of *Hibiscus moscheutos* were cultured in MS basal medium supplemented with 10 mg L^{-1} glutamine, 10 mg L^{-1} arginine, 10 mg L^{-1} glutamine plus 10 mg L^{-1} arginine, or in plain MS media. Overall shoot elongation (±Standard Deviation) was measured after seven weeks. Rooting week was the number of weeks after the initial treatment when the first root was observed on individual tissue-cultured plants. No significant treatment effect was found from Tukey's HSD test, on either shoot growth or root initiation.

Amino Acids	Shoot Elongation (±SD) (cm)	Rooting Week (±SD)
Control	11.2 (±3.7)	3.5 (±1.0)
Glutamine	11.8 (±6.1)	4.2 (±1.2)
Arginine	14.0 (±3.5)	4.0 (±0.7)
Glutamine & Arginine	14.9 (±3.1)	4.2 (±0.7)
Analysis of variance		
Treatment	NS	NS

NS-not significant

Table 5. Two-node shoots of *Hibiscus moscheutos* were cultured in MS basal media supplemented with 10 mg L^{-1} glutamine, 10 mg L^{-1} arginine, 10 mg L^{-1} glutamine plus 10 mg L^{-1} arginine, or in plain MS media. The study was terminated after seven weeks. Shoot elongation within the seventh week (±Standard Deviation) was compared among treatments.

Amino Acids	Shoot Elongation (±SD) (cm)
Control	3.6 (±3.7) bc
Glutamine	2.7 (±6.1) c
Arginine	5.1 (±3.5) ab
Glutamine & Arginine	7.6 (±3.1) a
Analysis of variance	
Treatment	**

**- significant at p≤0.01

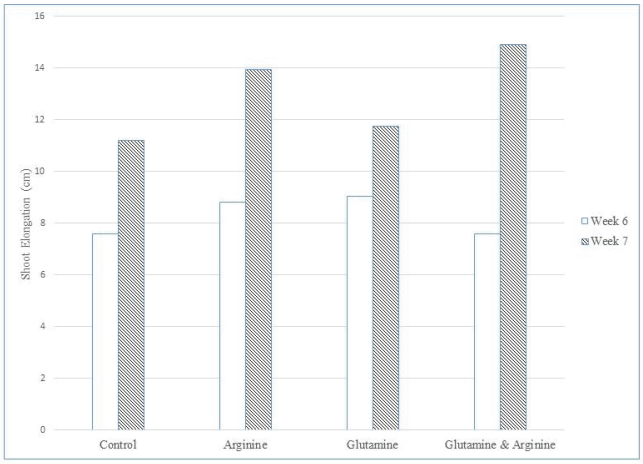

Figure 1. *Hibiscus moscheutos* shoots were cultured in MS medium amended with 10 mg L^{-1} glutamine, 10 mg L^{-1} arginine or a combination of both. Shoot elongation was observed weekly for seven weeks. This chart showed the new shoot growth in last two weeks of the study.

Although it is evident that glutamine accelerates plant development, inconsistent performance was found among our experiments. Glutamine was found effective in promoting shoot elongation at a rate of 10 mg L^{-1} and in preventing leaf necrosis. As a nitrogen provider, the effect of glutamine might be dependent on the demand of certain plant species. More trials could be conducted to articulate the effect of glutamine on tissue-cultured *H. moscheutos*. Including BAP in the tissue culture media was determined to be ineffective for promoting shoot elongation, however it was very effective at inducing shoot proliferation, thus it can be used for the purpose of promoting the number of axillary shoots.

One positive result is that glutamine displayed a greater effect on shoot elongation than GA_3. GA_3 has long been recognized as an effective PGR for promoting shoot elongation, especially in tissue culture (Curtis and Cross, 1954). Shoots cultured in 0.35 mg L^{-1} GA_3 displayed significant shoot growth; while GA_3 showed degrees of inhibition on stem elongation at levels of 2 mg L^{-1} and 4 mg L^{-1}. A general reference for an effective concentration at which to apply GA_3 was 5 mg L^{-1}; however, the rate can be species dependent (Nickell, 1958). While a low concentration of GA_3 can be stimulating to stem elongation; a high concentration of GA_3 can be inhibitory on shoot elongation and root development.

Treatment with arginine, as well as a mixture of arginine and glutamine, resulted in greater shoot elongation in the last two weeks of tissue culture. Of all 21 major amino acids, arginine has the highest nitrogen to carbon ratio (Winter et al., 2015). Due to its unique double-imino structure, arginine has an extra nitrogen atom per molecule, and these imino structure could be transformed into nitrite for assimilation (Hibbs et al., 1987). The extra nitrogen groups may have given arginine the potential to transform into soluble nitrogen and provide nitrogen at later stages of the study when growth was rapid.

CONCLUSIONS

Glutamine was found to be superior to other plant growth regulators for shoot growth of *Hibiscus moscheutos* when produced using tissue culture practices. Arginine, which contains a higher percentage of nitrogen compared to glutamine, also improved the growth of shoots cultured on solid media. The combination of both amino acids used together has the potential to further increase shoot elongation *in vitro*.

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AUTHORS CONTRIBUTIONS

Z.L.G. 0000-0001-6535-4844: responsible for the implementation, conduct of the research, and writing of the article; **J.M.R.** 0000-0002-8845-6760: assisted with the preparation of the study, correction of the article, and final draft of the article for submission.

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