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GROWTH CURVE AND BIOCHEMICAL ANALYSES OF CALLUS OF IPÊ-BRANCO (*Tabebuia roseo alba* (Ridl.) Sand.)

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ABSTRACT

The understanding of biochemical changes that occur during the callus growth and development can provide support to the *in vitro* cultivation process. In addition, the substances present in these structures such as secondary metabolites can also be determined. With these premises in mind, the objective of this work was to determine the growth curve of callus of ipê-branco and to biochemically analyze them through the determination of the contents of total soluble sugars, reducing sugars, total soluble proteins and amino acids. Leaf segments of ipê-branco were inoculated onto MS medium supplemented with sucrose (30 g L^{-1}), agar (6 g L^{-1}) and 1 mg L^{-1} 2,4-D associated to 2 mg L^{-1} kinetin. The material was kept in the dark for 90 days. Each 15 days the material was taken out to be analyzed. The growth curve of the fresh material of the callus formed from leaf explants of ipê-branco presents sigmoid growth, with five distinct phases. The transfer of the calli to a new culture medium should be done between 60th and 75th days of cultivation. Maximum contents of total soluble sugars and reducing sugars were observed on the 45th day of cultivation. The contents of total soluble proteins were higher on the inoculation day, showing a decrease on the following days. Maximum contents of amino acids were observed on the 15th day of cultivation.
Keywords: MS medium, leaf explants, sugars, amino acids, proteins

RESUMO

O entendimento das mudanças bioquímicas que ocorrem durante o crescimento e desenvolvimento dos calos pode fornecer subsídios ao processo de estabelecimento in vitro, permitir a determinação de substâncias presentes nestas estruturas como metabólitos secundários. Com o objetivo de determinar a curva de crescimento de calos de ipê-branco e analisá-los bioquimicamente, por meio da determinação de açúcares solúveis totais, de açúcares redutores, proteínas solveis totais e de aminoácidos, segmentos foliares de ipê-branco foram inoculados em meio MS suplementados com sacarose (30gL-1), ágar (6gL-1) e com 1mg. L-1 de 2,4-D associado a 2mg. L-1 de cinetina. O material foi mantido no escuro por 90 dias. A coleta do material foi feita ao cada 15 dias para as análises. A curva de crescimento de matéria fresca de calos formados a partir de explantes foliares de ipê-branco apresenta crescimento sigmóide, com cinco fases distintas. A repicagem dos calos para um novo meio de cultura deve ser realizada entre 60° e 75° dias cultivo. Teores máximos para açúcares solúveis totais e açúcares redutores foram observados no 45° dia de cultivo. As proteínas solúveis totais apresentaram maiores teores no dia da inoculação, reduzindo em seguida. Teores máximos de aminoácidos foram observados no 15° dia de cultivo.

Palavras-chave: MS; explantes foliares; açúcares; aminoácidos; proteínas.

INTRODUCTION

The callus consists of a mass of cells which proliferate disorderly, with a certain degree of organization, from where the differentiated organs and tissues originate (Pierik, 1990). Similarly to the superior plants, callus presents a typical growth pattern, which is characterized by distinct phases. The periods where these phases occur are determined in the study of callus growth and each phase is characterized by events that are peculiar to each one of them.

From the study that indicates the phases in which fundamental processes to the kinetic study of callus growth occur, either the exact moment of transferring the callus to the new medium or the possibility of its use in cell suspensions can be established (Azevedo, 2003).

According to Santos et al. (2003), the growth phases of callus culture have been characterized by cell growth, whose parameters include fresh matter weight of the cell, number of cells, total cellular protein, metabolic activities and concentration of nutrients in the culture medium. According to George (1993), the different periods which compose the callus growth are the lag phase, log or exponential phase, linear phase, deceleration phase and stationary phase.

The determination of the growth curve and the biochemical analyses were already performed by other authors (Serra et al., 2000; Mesquita et al., 2002; Azevedo, 2003; Soares, 2003; Lima et al., 2007), by studying mainly woody plants. The establishment of this curve allows the study and the knowledge of the biochemical alterations that occur during the development of the calli. These can help improve the knowledge of the morphogenetic process which occurs during the *in vitro* cultivation, as well as allowing the identification of substances present in these structures.

In accordance to Phan et al. (1987), compared to the original explants, callus can present distinct biochemical composition and nutritional requirements. The quantitative and qualitative determinations of the macro and micromolecules, which constitute the plant tissues, can help with the determination of different life phases of a vegetable, according to the increase or decrease of the essential compounds (Santos et al., 2003).

The determination of the carbohydrate level in callus reveals the readily available source to the plant growth and has several applications in physiological studies (Passos, 1996). It also allows the determination of the growth phase or cellular development that provides a higher production of specific cellular compounds (Serra, 1999). Sugars, which act as a storage of energy, as an important component of the mechanical support tissues or also as suppliers of carbon skeletons for organic compounds synthesized by the cells (Serra et al., 2000). The determination of the sugar levels has application in several physiological studies because it reveals reserve levels promptly available to the growth (Passos, 1996). In the *in vitro* cultivation the sugars are added to the culture medium as energy source for the development of the explants. These tissues present a much reduced photosynthetic rate becoming practically heterotrophic (Azevedo, 2003).

The proteins can have enzymatic and structural functions. Physiological and biochemical studies involving their quantification, localization and determination of the *in vitro* enzymatic activity should be encouraged (Passos, 1996).

Amino acids have been used as organic nitrogen source in in vitro cultures of several species to enhance somatic embryogenesis and regeneration (Skokut et al., 1985; Claparols et al., 1993; Rao et al., 1995; Hamasaki et al., 2005; Grewel et al., 2006). It has been suggested that positive effect of organic nitrogen, in comparison to that of inorganic sources is associated to enhanced mobility of the former at a lower energy cost than the later (Kim and Moon, 2007).

The understanding of the biochemical changes that occur during the growth and the development of calli can provide the determination of the support for the *in vitro* establishment, in addition to allowing the identification of the secondary metabolites (Lima et al, 2007). Callus and cell culture could not only be an alternate continuo source of proteins, but could also be an useful and important model system to study their regulation and biosynthesis (PODDER et al., 1993) and in biochemistry studies (SERRA et al., 2000).

The scope of this study was to determine the growth curve of the calli originated from leaf explants of ipê-branco and to perform biochemical analyses, by determining the contents of protein, amino acid, total soluble sugar and reducing sugar.

MATERIAL AND METHODS

Establishment of growth curve for calli of ipê-branco

Young leaf segments excised from mother plants of ipê-branco maintained in growth room were used as explants for the obtainment of callus growth curve.

Leaf segments with approximately 1.0 cm^2 were inoculated into test tubes containing MS medium (Murashige and Skoog, 1962) supplemented with 1 mg L^{-1} 2,4-D (2,4-Dichlorophenoxyacetic acid) and 2 mg L^{-1} kinetin, 30 g L^{-1} sucrose and solidified with 6 g L^{-1} agar, with pH adjusted to 5.8, before autoclaving at 120 °C for 20 min. The explants were kept in a growth room at 25$\pm$2 °C, in the dark.

The procedure for the determination of the growth curve consisted of randomly withdrawing 20 explants from the test tubes and weighing them at 15-day intervals during 90 days of cultivation. The weights were measured starting at the first day of inoculation. A completely randomized design with 20 replications was used. The percentage of callus growth was determined according to an equation established by Lameira et al. (1996).

Biochemical analyses of calli of ipê-branco

The calli designated to the biochemical analyses were formed from leaf explants originated from mother plants of ipê-branco. They were inoculated onto MS medium supplemented with 1.0 mg L^{-1} 2,4-D and 2.0 mg L^{-1} of kinetin and maintained in a growth room at 25$\pm$2 °C, in the dark.

For the biochemical analyses, the calli were collected at 15-day

intervals, during a cultivation period of 90 days. The weights were taken starting at the first day of incubation. For each repetition 500 mg of callus was used, with a total of two repetitions, which were stored in a freezer at the temperature of -80 °C afterwards.

The procedure to get callus crude extract was based on the methodology described by Lemos et al. (1999). Each sample of 500 mg of callus was homogenized in graal homogenizer with liquid nitrogen and then centrifuged at 1000 g during 10 min in a cooling centrifuge at the temperature of 4 °C. The supernatant was separated from the pellet and placed in a 1.5 mL eppendorf and stored in a freezer at -4 °C to be later used in the quantification of the proteins, amino acids, reducing sugars and total soluble sugars.

The content of total soluble sugars was determined according to the methodology described by Yemm and Willis (1954). To aliquots of 4 µL of the crude extract, 996 µL of distilled water and 2.0 mL of anthrone reagent (20 mg anthrone, 0.5 mL distilled water and 10 mL H_2SO_4 concentrated) were added. After agitation the samples were heated at 100 °C for 5 min. They were read in the spectrophotometer at 620 nm. The values were expressed in milligrams of glucose per gram of fresh matter (mg glucose g^{-1} FM), based on the standard curve obtained from different concentrations of glucose.

The determination of the reducing sugars was done using the methodology described by Miller (1959), using the 3,5-dinitrosalicylic acid (DNS). To aliquots of 40µL of the crude extract, 1.1 mL distilled water and 1.0 mL DNS reagent were added. The mixture thus formed was homogenized in an agitator and heated in a water-bath at the temperature of 100 °C for 5 min, and later was cooled at room temperature. The samples were read in the spectrophotometer at 540 nm. The values were expressed in milligrams of glucose per gram of fresh matter (mg glucose g^{-1} FM), based on the standard curve obtained from different concentrations of glucose.

The method described by Stein and Moore (1948) for the determination of the amino acid contents was used. To aliquots of 20 µL crude extract, 998 µL of distilled water was added. In addition, 1.7 mL of the following reagents was added: 0.2 M sodium citrate buffer, pH 5.0; 5% ninhydrin reagent in methyl cellosolve and 2% KCN in methyl cellosolve.

The mixtures were agitated and taken to a water-bath at 100 °C, during 20 min. To the spectrophotometer reading at 570 nm, 1.3 mL 60% ethanol (v/v) was added. The values were expressed in milligrams of amino acids per gram of fresh matter (mg amino acids g^{-1} FM), based on the standard curve obtained from different concentrations of glycine.

Total proteins were quantified according to the method developed by Bradford (1976), which is based on the principle of protein-dye binding. Afterwards, the samples were homogenized in an agitator and a spectrophotometer reading was performed at 595 nm.

The values were expressed in milligrams of protein per gram of fresh matter (mg protein g^{-1} FM), based on the standard curve obtained from different concentrations of bovine serum albumin (BSA).

RESULTS AND DISCUSSION

Establishment of growth curve of Ipê-branco callus

Figure 1 shows the general aspect of the callus formation in leaf segments of ipê-branco inoculated on MS medium in the presence of 1 mg L^{-1} 2,4-D and 2 mg L^{-1} kinetin, from the inoculation day, until the 90th day of cultivation.

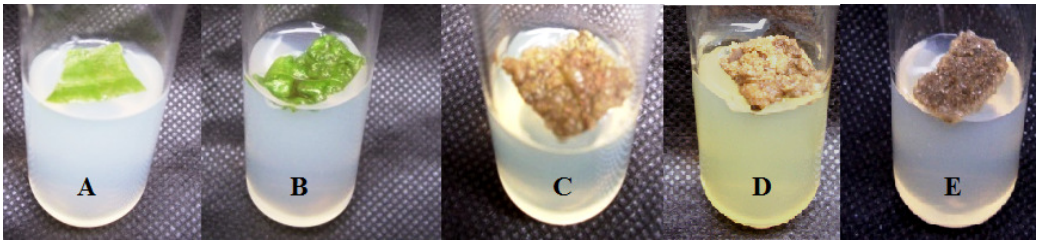

FIGURE 1. General aspect of calli of ipê-branco formed from leaf segments inoculated *in vitro* onto MS medium supplemented with 1 mg L^{-1} 2,4-D and 2 mg L^{-1} kinetin. A- inoculation, B - 15 days, C - 45 days, D - 75 days and E - 90 days.

The growth curve of ipê-branco calli presented a sigmoidal-type pattern where the five growth phases can be distinguished (Figure 2). This type of sigmoidal growth curve has been already observed in other species of woody plants such as lychee (Mesquita et al., 2002), Brazil nut (Serra, 2000), copaíba (Azevedo, 2003), coffee plant (Santos et al., 2003), and "sangra d'água" (Lima et al., 2007).

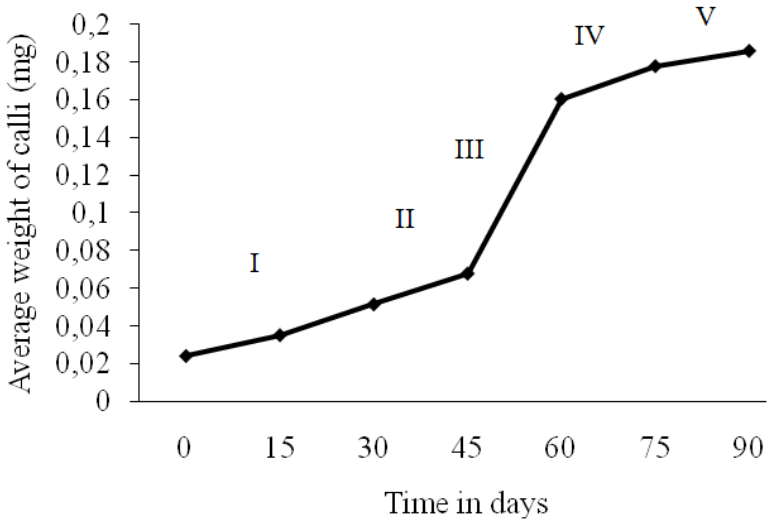

FIGURE 2. Growth curve of calli of ipê-branco formed from leaf segments. I – lag phase, II – exponential phase, III – linear growth phase, IV – deceleration phase and V – stationary phase.

The lag phase, in which the cells of the explants prepare themselves for the division, occurred up to the 30th day from inoculation, representing 43% of the *in*

vitro growth. Serra et al. (2000), while evaluating the growth of calli obtained based on the leaf segments of Brazil nut (*Bertholletia excelsa*), also observed an accumulation of fresh matter until the 30th day after the inoculation.

The exponential growth phase, period in which maximum division of cells is observed, occurred between the 30th and 45th days after the inoculation, with observed growth of 36%.

The lag phase can be considered an energy producing phase and the exponential phase a biosynthesis phase (Shimizu, et al., 1977). Santos et al. (2003), in their study of the callus growth, observed that the occurrence of the exponential growth phase occurred between the 42th and 77th days of cultivation in leaf explants of coffee (*Coffea arabica*, cultivar Rubi).

The period of linear growth, in which the calli diminish their division and increase their cellular area, was observed between the 45th and 60th days of inoculation, representing 57% of the *in vitro* growth. Even thought the linear growth period of ipê-branco calli had been reached with approximately two months of inoculation, other species can reach this phase more quickly, especially when secondary explants are used as these tissues show more homogeneity. Soares (2003), when studying the growth curve of calli induced in leaf explants of "ingá" (*Inga vera*), observed the occurrence of the linear phase between the 30th and the 60th days after the inoculation. Lima et al. (2007) observed that the linear phase of the growth curve of leaf explants of "sangra-d'água" (*Croton urucurana*) occurred only between the 70th and 98th days of cultivation.

The interval of growth deceleration was observed between the 60th and 75th days from inoculation, with only 8% of growth. According to Smith (1992), it is in this phase that the calli should be transferred mainly because of the lowering of the nutrients, agar dryness or accumulation of the toxic substances in the culture medium. The results indicated that the transfer of the callus derived from leaves of ipê-branco should be executed in the beginning of the deceleration phase, which means on the 60th day of cultivation. Lima et al. (2007), in their study of the growth curve of sangra d'água (*Croton urucurana*), also recommend that the transfer should be done close to the 70th day after the inoculation. The callus growth reached the stationary phase between the 75th and 90th days of inoculation, with 3% of growth being observed in this period.

The results presented by the growth curve of calli of ipê-branco based on leaf segments indicate that their growth is slow, possibly being associated to the occurrence of a cellular cycle which is also slow (Alberts et al., 1997).

Biochemical analyses of callus of ipê-branco

The total soluble sugar (TSS) content was high on the inoculation day, followed by a sudden fall and posterior synthesis, reaching higher levels on the 45th day of cultivation. After this period, there was a decrease of the content again, as shown in Figure 3. The content of TSS on the inoculation day can be related, according to Lima et al. (2007), to the sugars contained in the leaves of the mother plant, from which the explants are derived. The

initial sugar can be later consumed to supply the metabolic demand of the growing calli. In the stationary phase, the reduction of TSS indicates that this increase is related to the energy used, as well as the depletion of the culture medium. Lima et al. (2007) found maximum content of total soluble sugars on the day of inoculation and on the 21th day, which corresponds to the lag phase of the growth curve of calli of foliar explants of sangra-d'água (*Croton urucurana*). Azevedo (2003) observed maximum TSS on the 84th day after the inoculation of leaf explants of copaíba (*Copaifera langsdorffii*). Nogueira (2003) observed that the maximum content of TSS occurred on the 70th day of cultivation, which coincided with the period indicated for the transfer of the callus of murici-pequeno (*Byrsonima intermedia*).

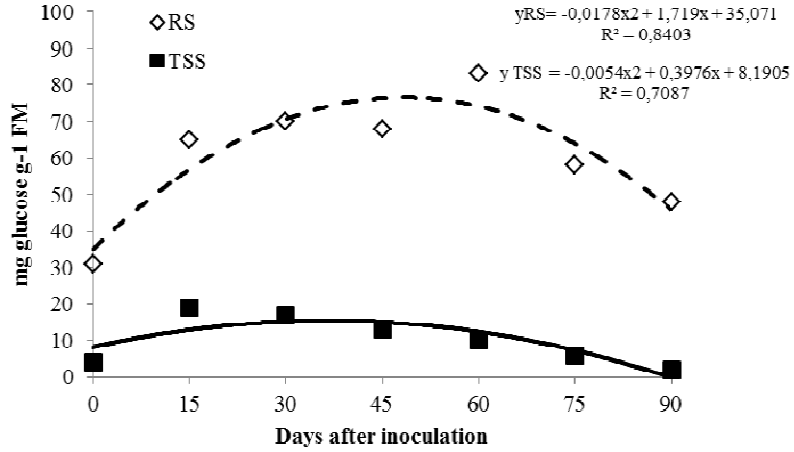

FIGURE 3. Contents of total soluble sugars (TSS) and reducing sugars (RS) obtained from calli of ipê-branco formed in leaf segments inoculated *in vitro* onto MS medium supplemented with 1 mg L^{-1} 2,4-D and 2 mg L^{-1} kinetin.

Serra et al. (2000) obtained different results with callus formed from leaf segments of Brazil nut (*Bertholletia excelsa*). The contents of TSS were constantly reduced during the whole period of cultivation. Soares (2003) also observed that the contents of TSS remained relatively constant, a slight increase occurring on the 80th day after the inoculation in leaf explants of "ingá" (*Inga vera*).

For the content of reducing sugars, there was initially an accumulation and then ulteriorly a decrease after the 45th day of cultivation. On this day, the highest level of reducing sugars (RS) was observed (Figure 4). Relating the contents of RS to the growth curve, it can be perceived that these contents were decreasing in the linear growth phase. The reduction of the RS contents during callogenesis of Brazil nuts (*Bertholletia excelsa*), indicates that certain explants possibly show difficulties in absorbing the carbohydrate source present in the culture medium (Serra et al., 2000).

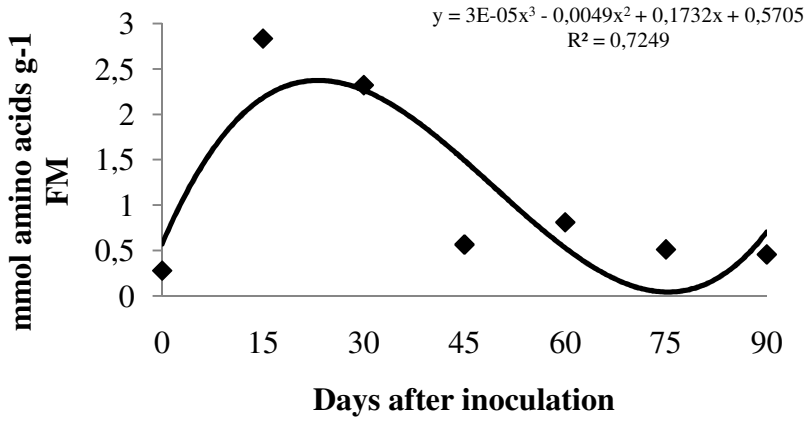

FIGURE 4. Contents of amino acids obtained from calli of ipê-branco formed in leaf segments inoculated *in vitro* onto MS medium supplemented with 1 mg L^{-1} 2,4-D and 2 mg L^{-1} kinetin, during 90 days of cultivation.

Lima et al. (2007) verified that the reducing sugars increased between the 35th and 77th days, being increased in the exponential phase in "sangra-d'água" (*Croton urucurana*) cultivation. Working with leaf explants of ingazeiro *(Inga vera subsp. affinis)*, Soares (2003) has found great variation in the RS contents in the period of 110 days of inoculation. Opposing results were verified by Nogueira (2003) who has evidenced decrease of the RS content along the period of leaf explants cultivation of "murici-pequeno" (*Byrsonima intermedia*).

The amino acid contents obtained from calli of leaf explants of ipê-branco showed an accumulation until the 15th day after the inoculation, with a reduction until the 45th day and a slight elevation occurred until the 60th day of cultivation (Figure 4).

These results are in accordance with the ones obtained by Paiva Neto et al. (1997) in callus of mora, where the maximum content of amino acids was observed on the 15th day after the inoculation. In callus originated from leaf segments of Brazil nut, Serra et al. (2000) also observed that the contents of amino acids increased until the 15th day of inoculation. These authors also observed that these contents coincided with the lag phase of the growth curve of the calli. Soares (2003) also observed that in leaf explants of "ingá" *(Inga vera),* the amino acid contents increased until the 20th day after the inoculation, presenting a fall and ulterior increase after the 40th day.

The initial increase and subsequent reduction in the amino acid contents was also detected by Sacchi et al. (1995), in callus of kiwi (*Actinidia deliciosa*). Relating the content of amino acids found in the calli of the leaf explants to the phases of the growth curve, it can be noticed that, opposite to what occurs to the protein content, initial increase of amino acids occurred during the lag phase. From this point on, the amino acid contents started to decrease until the 60th day, in the linear phase, when the accumulation of amino acids restarted to occur until the beginning of the stationary phase.

The elevation on the amino acid contents right in the beginning of the cultivation period can indicate that probably the synthesis of the amino acids occurred from the protein degradation (Serra et al., 2000). The amino acid content increased continually during the 30 days (Figure 4). This increase can be attributed to proteolysis, because the leaf protein concentration decreased sharply during this period (Figure 5). Amino acids when supplied at sufficient concentrations can be used for synthesis of protein (Dougall, 1966). Furthermore, the increase in the amino acid content can be occasioned by the considerable absorption of the ammonium ion and glycine from the culture medium. According to George et al. (1988), the presence of ammonia in the culture medium leads to an increase of amino acid and protein synthesis, which are produced using the energy liberated from the carbohydrate catabolism.

By analyzing the relation between the protein and amino acid contents, it was observed that on the 15th day after the inoculation, the relation was of 22.2 µm amino acids to 0.27 mg proteins. On the 60th day of cultivation the amino acid content was of 12,5 µmol and the proteins of 0.73 mg. The relation between the proteins and amino acids increased from 0.012 to 0.058, indicating an elevation in the biosynthesis of amino acids.

In Figure 5 it can be observed that the maximum contents of the protein were shown in the beginning of the cultivation period, followed by a drastic reduction from the 15th day of inoculation on. This decrease suggests that the calli possibly used the protein reserve of the explants on the day of the inoculation, originated from the mother plant. From the 30th day of inoculation on, an increase in the protein contents was observed, which remained about the same until the 60th day of cultivation, with ulterior decrease. Between the 45th and 60th days of cultivation, there was a small increase in the protein content followed by a decrease. This increase coincides with the linear growth phase, indicating that synthesis and consumption of these proteins occurred, since the highest rates of cellular division occur in this phase.

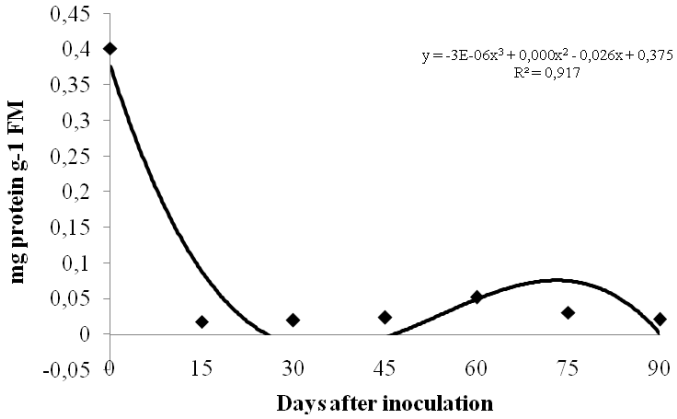

FIGURE 5. Contents of proteins soluble in water obtained from calli of ipê-branco based on leaf segments inoculated *in vitro* onto MS medium supplemented with 1 mg L^{-1} 2,4-D and 2 mg L^{-1} kinetin, during 90 days of cultivation.

In their study of calli of lychee (*Licthi chinensis*) originated from leaf explants, Mesquita et al. (2002) observed maximum content of proteins 42 days after the inoculation with 2 mg L^{-1} 2,4-D and on the 70th day of cultivation, for explants inoculated with 6 mg L^{-1} 2,4-D. Silva et al. (2005) observed that between the 0 and 12th day of culture there was a decrease in the protein content and increase in the protein levels between 12th and 16th day of culture could be related to mitotic activity during the exponential and linear growth phases in callus of *Glycine wightii.*

It was observed that in the lag phase a degradation of total soluble sugars initially occurred, generating a slight increase of the reducing sugars; the proteins were also degraded and as a result the amino acid content increased. In the exponential phase the synthesis of total soluble and reducing sugars occurred and the amino acids were consumed. In the linear growth phase, the total soluble and reducing sugars were degraded and the proteins and amino acids were synthesized. In the deceleration and stationary phases, total soluble sugars, proteins and amino acids were reduced, their consumption being initiated by the calli.

CONCLUSIONS

The growth curve of the fresh matter of calli formed from leaf explants of ipê-branco presents sigmoidal growth, with five distinct phases.

The transfer of the calli to a new culture medium should be done during the 60th and the 75th days of cultivation.

Maximum contents for total soluble and reducing sugars were observed on the 45th day of cultivation.

The total soluble proteins presented highest contents on the day of inoculation, showing a reduction afterwards.

REFERENCES

ALBERTS, B.; BRAY, D.; LEWIS, J.; RAFF, M.; ROBERTS, K.; WATSON, J.D. **Biologia molecular da célula**. Porto Alegre: Artes Médicas, 1997. 1294p.

AZEVEDO, K. de S. **Indução e análises bioquímicas de calos e aspectos as anatomia foliar de copaíba (Copaifera langsdorffii Desf.)**. 2003. 86 p. Dissertação (Mestrado em Fisiologia Vegetal) - Universidade Federal de Lavras, MG.

BRADFORD, M.M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. **Analytical Biochemistry**, New York, v. 72, p. 248-254. 1976.

CLAPAROLS, I; SANTOSA, M. A.; TORNE, M. J. Influence of some exogenous amino acids on the production of maize embryogenic callus and on endogenous amino acid content. **Plant Cell Tissue Organ Culture**, v. 34, p. 1-11, 1993.

DOUGALL, D. K. Biosynthesis of Protein Amino Acids in Plant Tissue Culture II Further Isotope Competition Experiments Using Protein Amino Acids. **Plant Physiology**, v. 41, p. 1411-1415, 1966.

GEORGE, E.F. **Plant propagation by tissue culture**: part I – the technology. 2.ed. Edington: Exergetics, 1993. 574p.

GEORGE, E.F.; PUTTOCK, J.M.; H.J. **Plant culture media: commentary and analysis.** England: Exegetics, 1988. 420p.

GREWEL D.; GILL R.; GOSAL, S. Role of cysteine in enhancing androgenesis and regeneration of indica rice (*Oryza sativa* L.). **Plant Growth Regulation**. v. 49, p. 43-47. 2006.

HAMASAKI, R. M.; PURGATTO, E.; MERCIER, H. Glutamine enhances competence for organogenesis in pine apple leaves cultivated *in vitro* **Brazilian Journal of Plant Physiolology.** v. 17, n. 4, p. 383-389. 2005.

KIM, Y. W.; MOON, H.K. Enhancement of somatic embryogenesis and plant regeneration in Japanese Larch (*Lerix leptolepis*). **Plant Cell Tissue Organ Culture**. v. 88, p. 241-245. 2007.

LAMEIRA, O. A. ; PINTO, José Eduardo Brasil Pereira ; ARRIGONII-BLANK, M. F.; PEREIRA, F. D. . Estabelecimento da curva de crescimento em calos de Erva-baleeira (Cordia Verbenacea L.). **Horticultura Brasileira**, Rio de Janeiro, v. 4, n. 1, p. 92-93, 1996.

LEMOS, G. B. ; DELÚ FILHO, Nelson ; OLIVEIRA, L. E. M. ; PURCINO, A. A. C. . Atividade das enzimas de assimilação do nitrogênio em plantas jovens de seringueira cultivadas com diferentes relações de nitrato e amônio. **Revista Brasileira de Fisiologia Vegetal**, Brasília/DF, v. 11, n. 2, p. 113-118, 1999.

LIMA, E. C.; PAIVA, R.; SOARES, F. P.; NOGUEIRA, R.C.; EMRICH, E. B.; NICIOLI, P. M. Avaliação bioquímica do desenvolvimento de calos *in vitro* a partir de segmentos foliares de Sangra d'água (*Croton urucurana* Baill.) **Magistra**, v. 19, n. 3, p. 184-190,2007.

LINDSEY Y.K.; YEOMAN, M.M. The relationship between growth rate, differentiation and alkakoid accumulation in cell culture. **Journal of Experimental Botany**, v. 34, n. 145, p. 1055-1065, 1983.

MESQUITA, A. C.; PAIVA, R.; SANTIAGO, E. J. A. DE; PAIVA[4] P. D. O., PAIVA; L. V. PAIVA; GOMES, G. A. C. Análises bioquímicas de calos obtidos de segentos foliares de lechieira (*Litchi chinensis* Sonn.) **Magistra**, Cruz das Almas - BA, v. 14, n. 1, 2002.

MILLER, GL. use o dinitrosalicylic acid reagent for determination of reducing sugar. **Analytical Biochemistry**, New York, v. 31, p. 426-428. 1959.

MURASHIGE, T.; SKOOG, F. A revised medium for rapid growth and bioassays with tobacco tissue culture. **Physiologia Plantarum**, Copenhagen, v. 15, n.3, p. 473-497, 1962.

NOÉ, W.; LANGEBARTELS, C.; SEITZ, H.U. Anthocyanin accumulation in PAL Searlet rose cell suspensions. **Journal of Experimental Botany,** v. 23, p. 75-91, 1980.

NOGUEIRA, R.C. **Propagação *in vitro* de murici-pequeno (*Byrsonima intermedia* A. Juss.).** 2003. 88 p. Dissertação (Mestrado em Fisiologia Vegetal) - Universidade Federal de Lavras, MG.

PAIVA NETO, V. B.; PAIVA, R.; GOMES, G. A. C.; PÓVOA, J. S. R. Comportamento *in vitro* de segmento nodal de moreira (*Chlorophora tinctoria* (L.) Gaudichaud). **Arquivos de Biologia e Tecnologia**, v. 40, n. 1, p. 135-141, 1997.

PASSOS, L.P. **Métodos analíticos e laboratoriais em fisiologia vegetal.** Coronel Pacheco: EMBRAPA-CNPGL, 1996. 233p.

PHAN, C.T.; DO, C.B.; HEGEDUS, P. Metabolic aspects of "*in vitro*" culture of plants; problems and applications, comparison of soluble contents, marker enzymes between explant and cell suspension culture. **Experimental Biology,** v. 46, n. 3, p. 58, 1987.

PIERIK, R.L.M. **Cultivo *in vitro* de Ias plantas superiores.** Martins Nijoff, 1990,

326 p.
PODDER, K. S.; D SILVA, I.; VAIDYANATHAN, C. S. Ribosome-inactivating proteins and agglutinins from callus and suspension cultures of Ricinus communis L. and *Abrus precatorius* L. **Plant Science**, Shannon, v. 94, p. 161-172, 1993.
RAO A. M.; SREE KP, KISHOR PBK. Enhanced plant regeneration in grain and sweet sorghum by asparagine, praline and cefotaxime. **Plant Cell Regulation**. v. 15, p. 72-75. 1995.
SACCHI, G.A.; MORGUTTI, S.; ABRUZZESE, A. Changes in some physiological and biochemical parameters during two subcultures in Kiwi *(Actinidia deliciosa)* callus. **Plant Science,** Berkeley, v. 106, n. 1, p. 107-113, Mar. 1995.
SANTOS, C. G. dos; PAIVA, R.; PAIVA; P. D. O.; PAIVA, E. Indução e análise bioquímica de calos obtidos de segmentos foliares de *Coffea arabica* L., cultivar RUBI. **Ciência e Agrotecnologia**, v. 27, n. 3, p.571-577, 2003.
SERRA, A.G.P; PAIVA, R.; PAIVA, P.D.O. Análises bioquímicas de calos formados de explantes foliares de castanheira-do-Brasil (*Bertholletia excelsa* H. B. K.) **Ciência e Agrotecnologia**, Lavras, v.24, n.4, p. 833-840, 2000.
SHIMIZU, T.; et al. Changes in metabolic levels during growth of *Acer pseudoplatanus* (sycamore) cells in batch suspension culture. **Physiologia Plantarum**, Copenhagen, v.40, p. 125-129, 1977.
SILVA, A. L. C. DA; CARUSO, C. S.; MOREIRA, R. A.; HORTA, A. C. G. Growth characteristics and dynamics of protein synthesis in callus cultures from G*lycine wightii* (Wight & Arn.) Verdc. **Ciência e Agrotecnologia**, Lavras, v. 29, n. 6, p. 1161-1166, 2005.
SKOKUT TA, T. A.; MANCHESTER J.; SCHAEFER J. Regeneration in alalfa tissue culture. **Plant Physiololy**. v.79, p. 579-583. 1985.
SOARES, G. A. **Aspectos do cultivo *in vitro* do ingazeiro (*Inga vera* Willd, subsp. *affinis* (DC.) T. D. Penn.)** 2003. 90 p. Dissertação (Mestrado em Fisiologia Vegetal) - Universidade Federal de Lavras, MG.
STEIN, W.; MOORE, S.A. A modified ninhydrin reagent for photometric determination of amino acids related compounds. **Journal of Biological Chemistry**, Bethesday, v.176.], p. 367-372, 1948.
YEMM, E.W.; WILLIS, A.J. The estimation of carbohydrates in plant extracts by anthrone. **The Biochemistry Journal**, London, p. 508-514, 1954.

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