Glutamine enhances competence for organogenesis in pineapple leaves cultivated *in vitro*

Regina M. Hamasaki[1], Eduardo Purgatto[2] and Helenice Mercier[1]*

[1]Department of Botany, University of São Paulo, CP 11461, CEP 05422-970 São Paulo, SP, Brazil; [2]Department of Food and Experimental Nutrition,FCF, University of São Paulo, CP 11461, CEP 05422-970 São Paulo, SP, Brazil; *Corresponding author: hmercier@usp.br

Leaf bases of pineapple cultured on a shoot induction medium (SIM) produced protuberances followed by shoot-buds via direct organogenesis at a frequency of 46 %. When 8 mM glutamine (gln) was a supplement to SIM (SIM8gln), the regeneration rate increased to 70 %, thus suggesting that 8mM gln increased explant competence for organogenesis. Besides this, shoot vigor was strongly enhanced in SIM8gln. Other gln concentrations (16 or 32 mM) evoked a lower frequency of shoot-bud induction and number of regenerated shoots per explant when compared to SIM8gln. In this study, it was defined that explant organogenic commitment to form shoot-buds occurred in the first 7 days of culture on SIM8gln. Thereafter, endogenous indole-3-acetic acid (IAA) and cytokinin (4 types) measurements were carried out during this period, that is, during the induction phase of shoot-bud formation. The IAA content increased greatly until the 5th day in the leaf bases cultured on SIM8gln. No such change in IAA concentration was observed in the explants cultivated on SIM or in the presence of the highest gln concentration (32 mM), this being inhibitory to the organogenic process. The only natural cytokinin detected was isopentenyladenine. An increase of 50 % in the level of this phytohormone occurred in leaf bases cultured on SIM8gln at the 5th day, when compared to SIM or of 170% compared to SIM32gln. These results suggest that 8 mM gln favorably influenced the organogenic process through changes in IAA and iP concentrations in pineapple leaves.

Key words: *Ananas comosus*, amino acids, auxin, Bromeliaceae, cytokinins, phytohormones, shoot regeneration.

Glutamina aumenta a competência à organogênese das folhas de abacaxizeiro cultivadas *in vitro*: Numa freqüência de 46 %, bases foliares de abacaxizeiro cultivadas em meio indutor de regeneração de eixos caulinares (SIM) produziram, diretamente, protuberâncias que se desenvolveram, mais tarde, em gemas caulinares. Quando se adicionaram 8 mM de glutamina (gln) ao SIM (SIM8gln), a taxa de regeneração aumentou para 70 %, sugerindo que 8 mM de gln favoreceu a aquisição de competência à organogênese dos explantes foliares. Além disso, o vigor dos eixos caulinares surgidos foi incrementado quando as bases foliares foram cultivadas em SIM8gln. Outras concentrações de gln (16 ou 32 mM) geraram menores taxas de regeneração, além de menor número de eixos caulinares por explante, quando comparados com SIM8gln. Neste estudo, definiu-se que a determinação organogenética dos explantes para formar gemas caulinares foi alcançada nos primeiros 7 dias de cultivo em SIM8gln. Durante esse período, isto é, no decorrer da fase de indução da formação dos eixos caulinares, quantificaram-se as concentrações endógenas do ácido indolil-3-acético (AIA) e de quatro tipos de citocininas. O conteúdo de AIA das bases foliares cultivadas em SIM8gln aumentou fortemente até o quinto dia. Ao contrário, nenhuma alteração na concentração de AIA se observou nos explantes cultivados em SIM ou na presença de 32 mM de gln (SIM32gln). Essa mais alta concentração de gln foi inibitória ao processo organogenético. A única citocinina endógena detectada foi a isopenteniladenina (iP). Comparando-se a quantidade de iP nas bases foliares cultivadas em SIM8gln no quinto dia com os valores obtidos em SIM e SIM32gln, verificaram-se aumentos de 50 % e 170 % respectivamente. Esses resultados sugerem que 8 mM de gln teve uma influência positiva sobre o processo organogenético, mediada por alterações nas concentrações de AIA e iP ocorridas nas bases foliares de abacaxizeiro.

Palavras-chave: *Ananas comosus*, aminoácidos, auxina, Bromeliaceae, citocininas, fitormônios, regeneração de eixos caulinares.

INTRODUCTION

The formation of new shoots can be induced in tissue culture from vegetative tissue by supplying the appropriate nutrients and plant growth regulators (Cary et al., 2002). Skoog and Miller (1957) were the first researchers to show that the relative levels of the hormones cytokinin and auxin in the growth medium direct the developmental fate of the regenerating tissues. High concentrations of cytokinin relative to auxin levels induced the formation of shoots, while the inverse balance induced root production.

Christianson and Warnick (1985), studying the organogenesis events in *Convovulous* leaf explants in tissue culture, divided shoot organogenesis into three phases: acquisition of competence, organogenetic induction, and morphological differentiation and development. During the first phase, cells gain competence to respond to morphogenetic stimuli. In the induction phase, the cytokinin/auxin balance specifies the developmental fate of the regenerating tissue and new shoots or roots become determined, so that if the tissue is transferred to a medium without growth regulators, shoot or root formation would continue. In the third phase, the newly determined structure differentiates and begins to develop independently.

In vitro organogenesis has been classified as to whether the process is direct or indirect (Hicks, 1994). In the direct type, explants are competent to respond to inductive growth regulators, and do not require callus formation before shoot or root induction as is the case in shoot regeneration from pineapple leaf base (Mercier et al., 2003; Firoozabady and Moy, 2004). The cultivation of leaf pineapple explants in the Shoot Induction Medium (SIM), containing 2.0 mg.L⁻¹ of benzyladenine (BA) and 1.0mg.L⁻¹ of α-naphthaleneacetc acid (NAA), produced protuberances (or nodular globular structures) prior to shoot-bud formation (Mercier et al., 2003). These authors found an increase of endogenous auxin and cytokinin (CK) levels in the leaf explants cultivated on SIM in comparison to the growth regulator-free basal medium. By 15 days on SIM, 40 **%** of the explants produced protuberances and then shoot-buds.

Glutamine (gln) is frequently employed in the culture medium as an organic nitrogen source (Franklin and Dixon, 1994). Many papers have shown that the use of exogenous glutamine can be beneficial for *in vitro* culture, increasing the regeneration rate and biomass of the explants (Franklin et al., 1991; Shetty et al., 1992; Ogita et al., 2001; Sudarsana Rao et al., 2001; Vasudevan et al., 2004). In bromeliads, gln was an excellent nitrogen source to improve gain of dry shoot mass

of plantlets cultivated *in vitro* (Mercier and Kerbauy, 1998). Besides this, these authors showed that gln had a strong influence on shoot hormonal contents (IAA and CKs).

In this study, the glutamine effect on the promotion of shoot-bud organogenesis from leaf base explants in pineapple cultivated on a shoot induction medium (SIM) was evaluated. The period when the leaf explants become committed to form shoot-buds using media transfer experiments was described. In addition, the endogenous levels of the phytohormones indole-3-acetic acid (IAA) and four types of cytokinins (CKs) were quantified during the induction period.

MATERIAL AND METHODS

*Leaf explants***:** *In vitro* plants of *Ananas comosus* var. Smooth Cayenne were obtained from node segments as described earlier in Souza et al. (2003). Plants around 8cm high were taken and transferred to a liquid Murashige and Skoog (1962) (MS) medium, containing 0.5 mg.L⁻¹ pyridoxin-HCl and nicotinic acid, 4.0 mg.L⁻¹ glycine, 100 mg.L⁻¹ myoinositol and 0.5 mg.L⁻¹ NAA and BA, for 20 days. Then, the fourth and fifth leaves (counted from the top) were isolated using a scalpel to avoid leaf base damage. The explants comprised approximately 1 cm of the basal portion of the leaves.

Shoot regeneration assays: The regeneration procedure used in this study was based on results obtained earlier (Mercier et al., 2003). Leaf explants were cultured on a s̲hoot i̲nduction m̲edium (SIM) containing Knudson (1946) formula macronutrients, micronutrients of MS, 2 mg.L⁻¹ phytagel and growth regulators: 1.0 mg.L⁻¹ NAA and 2.0 mg.L⁻¹ BA. Different concentrations of L-glutamine were added to SIM: 8 (SIM8gln), 16 (SIM16gln) or 32 (SIM32gln) mM. Explants were inoculated into 125 mL Erlenmeyer flasks containing 15 mL medium, to form 10 replicate flasks each containing 5 explants, and kept in a growth room at 25 °C ± 2 °C with a photoperiod of 16 h and a fluorescent light intensity of 40 μmol.m⁻².s⁻¹. The regeneration rate (proportion of leaf bases with protuberances or shoot-buds) was calculated for each treatment at 30 days.

Commitment experiment: To study the influence of SIM8gln on explant determination for shoot formation, we tested the ability of leaf bases to continue on the course of organ devel-opment even when transferred to a non-inductive medium. Leaf explants were exposed to a SIM8gln medium for 0 (con-trol), 1, 3, 5, 7 and 15 days. They were then transferred to a

growth regulator+gln-free medium (basal medium) for the completion of a total of 30 days. An additional control with explants cultured on SIM8gln for 30 days was employed. All treatments were performed with ten replicates containing five explants, this being repeated twice.

Analysis of endogenous phytohormones: IAA and CKs: The levels of IAA and CKs of leaf explants cultured on SIM8gln were quantified. As a control, SIM and SIM32gln (which inhibited regeneration) treatments were chosen, the same hormones being measured. For analyses, 20 leaf bases (approximately 1 g fresh mass - FM) from each treatment were collected after 0, 1, 3, 5 and 7 days of *in vitro* culture, and then frozen in liquid nitrogen and maintained at -20°C. The previously frozen samples were macerated with liquid nitrogen, and 10 mL of cold 80 % (v/v) aqueous methanol were added, containing 146 μM butylhydroxytoluene to prevent oxidation. Each macerate was supplemented with tritiated radiolabelled standards ([^{3}H]IAA, Amersham® and [^{3}H]Z, Isotope Laboratory® with a specific activity of 9.25×10^3 Bq and 1.85×10^7 Bq, respectively) for recovery estimation after purification. Also, 550 ηg of [$^{13}C_6$]IAA (Cambridge Isotopes) was added as an internal standard for GC-MS-SIM quantification. After 60 h of constant shaking at 4°C in the dark, the extracts were filtered (0.45 and 0.2 μm mesh), passed through a Sep-Pack C_{18} cartridge, and eluted with 80 % (v/v) aqueous methanol. The resulting eluate was evaporated under a vacuum, and then diluted with 200 μL of 0.2 % formic acid. The hormones were then separated over 80 min by high performance liquid chromatography (HPLC), using a reverse phase, semi-preparative, C18 column (Waters®, Prep Nova-Pak, 6 μm, 7.8 x 300 mm), at a flow rate of 3 mL.min^{-1} with a methanol/acid water gradient. The gradient consisted of 5 % methanol (0-15 min), 30 % methanol (16-50 min) and 45 % methanol (51-80 min). In all cases acid water was added to the methanol to reach a correspondent 100 %. Absorbance of the effluent was monitored at 270 nm. Fractions were collected at thirty-second intervals and reduced to dryness in a speed vacuum concentrator.

IAA quantification was carried out by GC-MS-SIM according to Chen et al. (1988) adapted by Purgatto et al. (2002). HPLC fractions correspondent to IAA were methylated with 1 mL etheric diazomethane for 5 min and reduced to dryness at room temperature. The methylated extracts were redissolved in 100 μL ethyl acetate and analyzed in a gas chromatograph Hewlett-Packard® 6890 connected to a mass-selective detector model 5973. The column used for separation was an HP-1701 (30 m, I.D. 0.25 mm, I.T. 0.5 μm), He being the carrier gas, with a flux of 4 mL.min^{-1}. The injected volume for each sample was 5 μL. Ions with a mass/charge (m/z) ratio between 130 and 136 (endogenous IAA), and between 136 and 195 (internal standard), were monitored. The endogenous IAA concentration was obtained by comparing the peak areas of the chromatograms extracted in m/z 130-136 and 136-195.

The levels of isopentenyladenine (iP), isopentenyladenine riboside (iPR), zeatin (Z) and zeatin riboside (ZR) were measured using an enzyme-linked immunosorbent assay (ELISA) (Mercier and Endres, 1999).

CK fractions were redissolved in 2 mL ultrapure water containing 2.53 mM NaN_3 and then used for the ELISA method, which employed polyclonal antibodies against iPR and ZR. The hormone level was measured four times per sample, the results being corrected for recovery. Calculations were made by reference to a calibration curve established on each microtitration plate with a fourth-order polynomial regression obtained from four experimental standard curves (Peres et al., 1997).

Statistical analyses: Analysis of variance (ANOVA) and mean separations were carried out using Ducan's Multiple Range Test with significance determined at 5 % level (Callegari-Jacques, 2003).

RESULTS

Organogenic induction and shoot development event: In general, after 7 days in culture, the leaf explants presented a swelling on the adaxial surface, in the basal region. At 2 weeks of *in vitro* culture, protuberances and shoot-buds in the swollen portion were observed, preceding the appearance of shoots, which emerged at 30 days. Maximum frequency (70 %) of shoot-buds was recorded in explants inoculated in SIM8gln in comparison to SIM (46 %). Other glutamine concentrations evoked lower frequency of shoot-bud induction when compared to SIM8gln. The explants cultured with 32 mM gln resulted in the lowest (23 %) regeneration frequency (figure 1). The mean number of shoots developed from explants was 9 either in SIM8gln or in SIM, and 3 in SIM16gln or SIM32gln. Shoots formed from explants cultivated on SIM8gln showed better development and vigor compared to leaves cultivated on SIM (figure 2). On the other hand, explants cultivated on SIM32gln died after 30 days.

Shoot-bud initiation did not occur either in the basal medium itself or in the absence of BA or NAA (data not shown).

Explants were also assayed for their commitment to form shoot-buds by their ability to continue on the course of
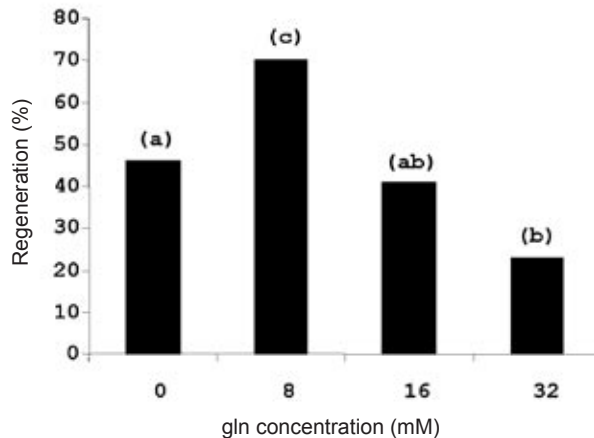

Figure 1. Regeneration ratio of pineapple leaf explants cultivated for 30 days in SIM supplemented with different concentrations of glutamine (0, 8, 16 or 32 mM). Different letters indicate that the means are significantly different according to Duncan's Multiple Range Test at 5 % level.
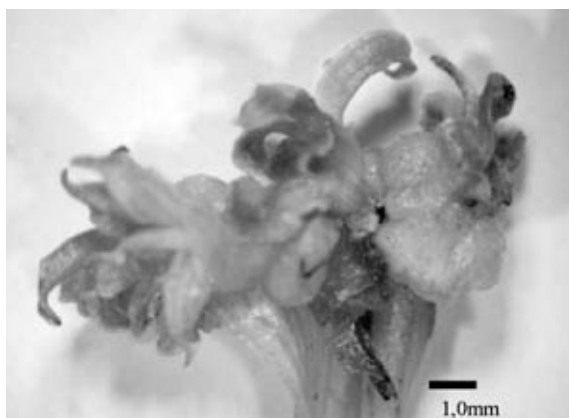

Figure 2. Shoots arising from pineapple leaf explants after 75 days of culture on SIM8gln or SIM.

shoot development even when transferred from SIM8gln to a non-inductive medium (without growth regulators and gln). At approximately 1-3 days on SIM8gln, explants became progressively growth regulator+gln-independent to form shoots, and after approximately 5-7 days on SIM8gln the leaf bases became committed to produced shoot-buds (table 1). Therefore explants were induced and became determined to form shoots several days before these structures actually appeared.

IAA content during the induction phase: Our results revealed a transient increase of IAA concentrations under SIM8gln condition (the best treatment to produce shoot-buds) during the induction period (figure 3). The IAA peak occurred at the 5th day (180 pmol.g^{-1} FM). In contrast, IAA levels of the explants cultivated on control treatments (SIM and SIM32gln) presented practically no difference during the investigated 7-day period.

CKs content during the induction phase: iP was the only natural cytokinin measured. Z, ZR and iPR were never detected. The contents of iP are shown in figure 3. From the 3rd day of *in vitro* culture, the iP level increased for all treatments. The highest concentration of iP was found in explants cultivated for 5 days on SIM8gln (314.2 pmol.g^{-1} FM). In the controls (SIM and SIM32mM), the peak of iP coincided in time (5th day) with the results obtained in SIM8gln, although concentrations were respectively 50 % or 170 % lower.

Table 1. Effect of SIM8gln on the determination of leaf base explants for shoot-bud organogenesis. Explants were initially cultured for different durations on SIM8gln, and subsequently transferred to a growth regulator + gln free medium (Basal Medium*) until the end of the experiment on the 30th day. Times zero and 30 days were control treatments.

Time exposition to culture medium (days)			Regeneration ratio after 30 days (%)
SIM8gln	Transference	BM*	
Zero		30	Zero
1		29	28
3		27	44
5		25	60
7		21	64
15		15	60
30		Zero	64

DISCUSSION

De novo shoot organogenesis in pineapple leaf bases proceeds directly from precursor cells in the explant itself, and does not involve callus formation. SIM evoked adventitious shoot-bud formation, the frequency of culture response being enhanced in its combination with 8 mM gln (SIM8gln). Besides this, vigor of the formed shoots was increased in the presence of this gln concentration. On the other hand, at 32 mM of gln, there was a strong reduction in the frequency of regeneration, showing that this concentration was inhibitory to pineapple leaves. The mechanisms of amino acid influence on *in vitro* organogenesis are poorly understood, although it is common to use media supplemented with amino acids or hydrolyzed proteins to promote explant proliferation. Gamborg (1970) explained the positive influence of gln on the growth rate on the basis that gln provided a readily available source of nitrogen. Glutamine and glutamate are known to be the main endogenous amino acids involved in plant metabolism, providing nitrogen for the biosynthesis of

Figure 3. Endogenous levels of IAA (A) and iP (B) in pineapple leaf explants cultured on SIM, SIM8gln or SIM32gln after 0, 1, 3, 5 and 7 days. Vertical bars indicate standard error (± SE).

amino acids, nucleic acids and other N-compounds (Coruzzi and Last, 2000).

The early events during the development of an adventitious shoot meristem are still largely unknown (Teo et al., 2001). In the case of a directly regenerating pineapple system, there are few studies focusing on the endogenous hormonal contents associated with shoot formation (Mercier et al., 2003). The minimum time required for pineapple leaf cells to be determined to form shoot-buds was 7 days (time induction) on SIM8gln. During this period, cultured pineapple explants produced substantial amounts of IAA and iP, reaching maximum quantities on the 5th day. In comparison to the control treatments (SIM and SIM32gln), the IAA level showed a strong increase in the first 5 days of culture (8 times higher at the 5th day in comparison to SIM). For cytokinin, explants cultured on SIM8gln showed an increase of 50 % in iP concentration at the 5th day in relation to results obtained in SIM. This increase was more pronounced when compared to SIM32gln. These results suggest that 8 mM gln affected endogenous hormonal concentration during the induction phase in pineapple explants. However, how gln acts on phytohormone metabolism remains to be defined. In the case of the melon (*Cucumis melo*), proline was the beneficial amino acid that stimulated *in vitro* shoot organogenesis from cotyledon explants (Milazzo et al., 1998). These authors hypothesized that proline promoted shoot formation through the activation of purine and aromatic metabolism via the stimulation of the pentose phosphate pathway, which may regulate endogenous CK and auxin biosynthesis respectively.

Higher endogenous IAA levels have been shown in different species/explants as being associated with an increased embryogenic response. In carrot cells, exogenous 2,4-D stimulates the accumulation of large amounts of endogenous IAA (Michalczuk et al., 1992a,b). This result was associated with an increase in the embryogenic competence of carrot cells. In alfalfa (*Medicago sativa*) leaf protoplasts cultured in the presence of 2,4-D, endogenous IAA levels increased considerably during the first 2-3 days of culture, being correlated with the reactivation of cell division (Pasternak et al., 2002). Based on the observations of others, we can speculate that the several-fold increase in IAA level detected during the induction phase of pineapple explants cultured on SIM8gln could increase the competence of basal leaf tissue for shoot organogenesis. Our results showed that the regeneration ratio changed significantly from 46 % in SIM to 70 % in SIM8gln.
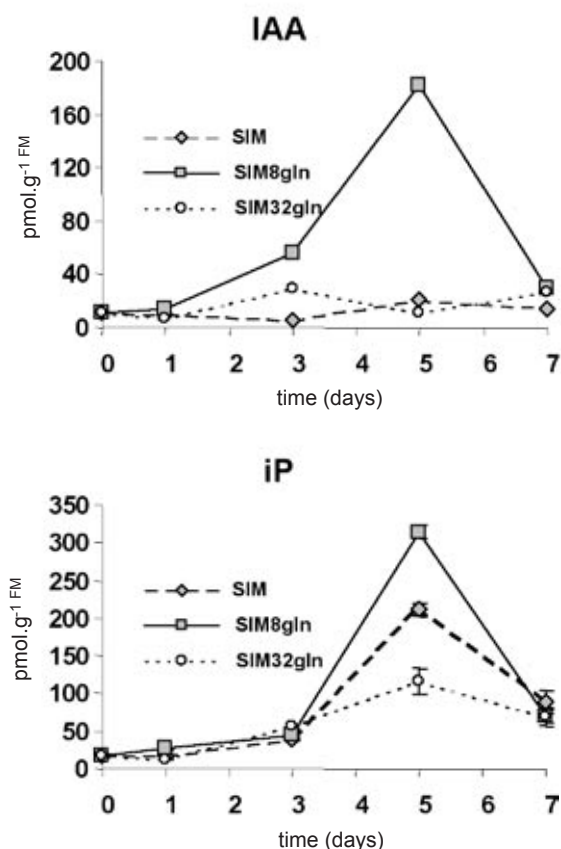
Higher endogenous CK levels appear to have important roles in shoot development *in planta*, thus confirming observations made in tissue culture (Howell et al., 2003). Cary et al. (2001) showed that shoot commitment occurred in *Arabidopsis* root explants midway through the 14-day incubation period (during the induction phase) on a cytokinin-rich SIM. Genes involved in shoot apical meristem formation, such as *SHOOTMERISTEMLESS* and *CLAVATA1* were up regulated at about the time of shoot commitment while *WUSCHEL* was up regulated somewhat earlier (Cary et al., 2002). In related studies of shoot formation via direct organogenesis from internodal stem segments in *Brassica oleracea*, Teo et al. (2001) found that *Brostm*, a *Brassica* homologue of *Arabidopsis SHOOTMERISTEMLESS* gene, expressed rapidly in response to cytokinin and not to auxin. Thus, it appears that the transient peak of iP, detected at the 5th day in basal leaf explants, could be an endogenous signal for shoot organogenesis in pineapple, since it was observed in this study that the presence of 8 mM gln in SIM increased the endogenous concentration of iP by 50 % at that time when compared to SIM. Besides, the lowest iP content (5th day), measured in the explants cultured on SIM32gln, correlated with the lowest frequency of organogenesis.

In conclusion, the present study showed that gln increases explant responsiveness to SIM, and that the induction phase occurs within the first week of culture when 8 mM of gln is added to SIM. Our results indicate that 8 mM gln acts indirectly increasing endogenous IAA and iP levels during the induction period. The timing of IAA and iP peaks correlate well with the time of determination of organogenic response of pineapple leaves.

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