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PRODUCTION OF PILOCARPINE IN CALLUS OF JABORANDI (*PILOCARPUS MICROPHYLLUS* STAPF)

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Summary

Jaborandi (*Pilocarpus microphyllus*) is the only known source of pilocarpine, and although this alkaloid is the only natural compound used to treat glaucoma, very little is known about its metabolism. Calluses obtained from petioles of *P. microphyllus* leaves were partially immersed in MS (Murashige and Skoog) liquid medium containing different pH levels (4.8, 5.8, and 6.8), nutrient concentration (MS normal basal medium concentration, absence of N, P, and K and three times normal concentrations), histidine and threonine (0.05, 0.15, and 0.75 m*M*), NaCl (25 and 75 m*M*) and polyethylene glycol (5 and 15%). Exposure to methyljasmonic acid (MJ) vapor was also investigated. The calluses were subjected to these conditions for 4 and 8 d under gentle agitation in the dark. Some calluses were also kept under continuous light. Pilocarpine was identified in the liquid medium by liquid chromatography–mass spectrometry/mass spectrometry. The alkaloid quantifications in the media and cells were carried out by high performance liquid chromatography (HPLC). The calluses maintained in the dark released the greatest quantities of pilocarpine into the medium. Methyljasmonate inhibited the release of pilocarpine in the medium. High pH (6.8), absence and excess of N, excess of P, and 0.75 m*M* of histidine and threonine induced the highest production of the alkaloid.

Key words: Pilocarpus microphyllus; jaborandi; alkaloid; stress.

Introduction

Pilocarpine is an imidazole alkaloid and, for a long time, it was the only substance used to treat glaucoma by causing a reduction in the intraocular pressure (Webster et al., 1993; Migdal, 2000). Consequently, its mechanism of action and pharmacological use has been studied extensively since the 1970s (Brenneke et al., 2004). A recent medical application for pilocarpine has been to treat xerostomia because it stimulates the sweat glands and lachrymal glands (Davies et al., 2001).

Representatives of the genus *Pilocarpus* (*Rutaceae*) are the only known source of pilocarpine and the leaves of *Pilocarpus microphyllus* (jaborandi) contain the highest concentration of this alkaloid (Pinheiro, 1997). *Pilocarpus* species are found exclusively in South America, mainly in northern Brazilian states (Pinheiro, 1997). Because of its commercial value, this species was exploited without control and by the 1990s it was considered an endangered species in Brazil (Pinheiro, 2002).

Several alkaloids have physiological effects on animals and, in many cases, they have pharmacological importance. Consequently, several studies have been carried out with different classes of alkaloids with a view to determining the mechanism of control of their biosynthetic pathways and thereby increasing their production in the intact plant as well as in cell cultures (Facchini, 2001; Hashimoto and Yamada, 2001; Endt et al., 2002). Such studies showed that abiotic factors such as light, salt, and osmotic stresses, as well as jasmonic acid and derivatives, and nutritional alterations may intensify the production of alkaloids (Baricevic et al., 1999; Godoy-Hernandez et al., 2000; Van der Fits et al., 2000; Li and Liu, 2003).

The route of pilocarpine biosynthesis in jaborandi is not known (Dewick, 1997) and so far there is only one report on the control of its content, studied in leaves of jaborandi seedlings (Avancini et al., 2003). Here, seedlings exposed to methyljasmonic acid (MJ) and salicylic acid led to a higher alkaloid content.

The aim of this investigation was to study the production of pilocarpine in callus of *P. microphyllus* exposed to different situations in order to create a model system to elucidate the biosynthesis of this alkaloid. Surprisingly, we observed that for most of the situations tested, pilocarpine was massively released into the medium, making the production of the alkaloid in bioreactors a possibility.

Materials and Methods

Callus induction. Petioles of *P. microphyllus* seedlings maintained in greenhouse conditions were treated with a saturated solution of calcium hypochlorite for 40 min, thoroughly washed with distilled sterile water, and then cultured in Petri dishes with MS (Murashige and Skoog, 1962) medium (0.6% agar, 3% sucrose, pH 5.8) containing 5.77 μ*M* 2,4-dichlorophenoxyacetic acid (2,4-D), and kept for 30 d in the dark at 25°C.

Treatments. The friable calluses were multiplied every 40 d, for 4 mo. Yellowish calluses were selected by size (*c.* 1 cm diameter) and transferred to

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the same medium for 15 d. The 15 d subcultured calluses were used in the experiments. Selected calluses (approximately 500 mg) were transferred to glass tubes (25 × 80 mm, one callus per tube) containing 1 ml of MS liquid medium containing 3% sucrose and 5.77 μ*M* 2,4-D. With this volume, the calluses remained half-immersed in the medium, avoiding oxygen depletion. The medium was modified in order to produce the following treatments: (1) pH 4.8, 5.8, and 6.8; (2) absence, normal, or three times the concentration of N, P, and K in the MS basal medium; (3) 0.05, 0.15, and 0.75 m*M* threonine; (4) 0.05, 0.15, and 0.75 m*M* histidine; (5) 25 and 75 m*M* NaCl; (6) 5 and 15% polyethylene glycol (PEG). Exposure to MJ vapor was achieved by dropping 50 μl of concentrated solution (95%, Aldrich, St Louis, USA) onto sterile cotton wool, which was fixed to the tube wall but did not mix with the medium. In the treatment where N was absent from the medium, KNO_3 was replaced by KCl. Where K was absent, KNO_3 was replaced by NH_4NO_3, and in the treatment where P was omitted, KH_2PO_4 was replaced by KCl. Excess of N was obtained with NH_4NO_3, P with H_2PO_4, and K with KCl.

Calluses from all seven treatments were maintained in the dark at 25°C under 50 rpm agitation. An eighth treatment was carried out maintaining callus under continuous light (25 $\mu mol\ m^{-2}\ s^{-1}$). The calluses from all treatments were harvested after 4 and 8 d of cultivation. These collection times were adopted because preliminary experiments showed that calluses kept longer than 15 d at 75 m*M* NaCl and 15% PEG became brownish.

Pilocarpine extraction. The calluses (*c*. 500 mg each callus) were removed from the glass tubes, quickly blotted dry on Whatmann 3MM (Whatman, Brentford, UK), weighed, and then extracted for pilocarpine according to Avancini et al. (2003). The calluses were crushed in a mortar with 5–6 drops of 10% NH_4OH and transferred to plastic tubes. After 15 min, 5 ml of chloroform was added, and the mixture vigorously shaken and then centrifuged to recover the organic solvent. This was repeated three times. The organic fractions were pooled and twice extracted with 2 ml of 2% H_2SO_4. The acid fractions were pooled, and the pH brought to 12 with NH_4OH. After two extractions with 2 ml chloroform, the organic fractions were pooled and dried in a Speed-Vac. High performance liquid chromatography (HPLC) buffer with the pH adjusted to 5.0 was added to the dried extract and retained for analysis. The culture media were filtered using 0.45 μm filters and freeze-dried before addition of HPLC buffer.

Pilocarpine determination. Pilocarpine was determined in the extracts and media using HPLC coupled to a diode array detector (Shimadzu, Kyoto, Japan). The alkaloid was separated with a Supelcosil LC18 column (250 × 4.6 mm, 5 μm, Supelco, St Louis, USA) using as solvent a buffer containing 1.3% H_3PO_4, 0.3% triethylamine, and 12% methanol, pH 3.0 at a flow rate of 1 ml min^{-1}. The diode array detector was set to operate from 190 to 340 nm, and pilocarpine was determined at 212 nm.

Pilocarpine identification. Pilocarpine was identified in the callus extracts by its retention time, coinjection with pure pilocarpine (Sigma, St Louis, USA), and by comparison with the UV spectral data obtained from the diode array detector of the HPLC. Analyses of the medium revealed a large amount of a compound eluting with the same retention time as pilocarpine. To confirm its identity as pilocarpine, samples of the medium were analyzed in a capillary liquid chromatography system (CaPLC, Waters, Milford, USA) equipped with a capillary column C_{18} (0.32 × 1500 mm, 5 μm, Waters). Solvents (A) H_2O + 0.1% formic acid, and (B) acetonitrile + 0.1% formic acid, were used to form a gradient: 0 min, 0% B; 20–25 min, 20% B; 29–30 min, 100% B; run at a flow rate of 10 μl min^{-1}. Samples of 10 μl were injected manually. The compounds eluting from the column were analyzed by mass spectrometry using a Q-TOF–Micromass, positive ion detection mode. The scan range for this analysis was *m/z* 100–600. The samples were subsequently analyzed by liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS): the scan range for MS/MS was *m/z* 50–600. The LC–MS and LC–MS/MS interface conditions were held constant, and were as follows: capillary voltage = 300 V; cone voltage = 30 V; solvation temperature = 120°C.

Statistical analysis. One tube containing one callus represented one replicate. The experiments were distributed in a completely randomized design and each treatment was composed of three replicates. The data were analyzed by one-way ANOVA and means compared by the Tukey test ($P \leq 0.05$).

Results and Discussion

Friable calluses were obtained from petioles and 30 d after initiation they were subcultured every 40 d for 4 mo. Pilocarpine was determined in the 30 d callus as well as after the first and second subculturing. Possibly because of the presence of plant tissue, more pilocarpine (6 $\mu g\ g^{-1}$ fresh mass) was found in the 30 d callus, decreasing and remaining stable at 2 $\mu g\ g^{-1}$ fresh mass in the subsequent determinations.

Except for a few treatments, we found low pilocarpine levels in the calluses. During analysis of the liquid medium by HPLC, we found a compound with the same retention time as pilocarpine, whose identity was confirmed as pilocarpine by mass spectrometry analysis (Fig. 1).

Light had a negative influence on the biosynthesis of pilocarpine, since less alkaloid per flask (medium + cells) was produced with this treatment compared to controls maintained in the dark (Fig. 2). The production of pilocarpine in the light treatments was lowest at both harvest days and, curiously, at day 8 and in contrast to the dark treatment, most of the alkaloid was retained in the cells.

Light has a stimulatory effect on the biosynthesis of several secondary metabolites in plant cell suspensions (Hobbs and Yeoman, 1991). Photosynthesis contributes little to the carbon supply for *in vitro* cell cultures, since they are usually maintained under low irradiance levels. In most cases, sucrose is added to the medium as the main source of carbon. Therefore, the increase in secondary metabolites provoked by light exposure is unlikely to be a consequence of increased carbon assimilation, but rather a specific response mediated by light receptors, as shown for vindoline in *Catharantus roseus* (Aerts and De Luca, 1992). On the other hand, as found here for pilocarpine, the alkaloids anabasine, anatabine, and nornicotine diminished in *Nicotiana* cell suspensions grown in the light despite an increase in cell mass (Hobbs and Yeoman, 1991).

Methyljasmonic acid (MJ) in plants elicits the octadecanoid cascade leading to the expression of genes related to pest resistance (Farmer and Ryan, 1992; McConn et al., 1997) and resistance to diseases (Vijayan et al., 1998; Thomma et al., 2000). Other reports have shown that MJ may elicit the production of specific compounds of secondary metabolism (Aerts et al., 1994; Imanishi et al., 1998; Walker et al., 2002; Yu et al., 2002). The total amount (cells + medium) of pilocarpine produced in the MJ treatment was similar to the control calluses at both evaluation times (Fig. 2). However, while most of the alkaloid was released into the medium in the control treatment, calluses treated with MJ retained a significant amount of the alkaloid. In contrast, MJ induced an increase of pilocarpine in jaborandi seedlings grown in a greenhouse (Avancini et al., 2003). Gundlach et al. (1992) observed that the cell suspension cultures of 36 plant species were elicited to produce secondary metabolites by adding MJ (250 μ*M*) to the medium. Here, MJ also increased the content of pilocarpine in the cells but when the total amount of the alkaloid per flask (cells + medium) was considered, there was no difference between the control and MJ treatment (Fig. 2). The way the cells were exposed to MJ may perhaps explain these results.

Water and saline stresses are known to induce several changes in cell metabolism (Dixon and Paiva, 1995). PEG and NaCl were used to induce osmotic and salt stresses, respectively, in jaborandi callus and to verify their effects on pilocarpine production (Fig. 3). At day 4, callus stressed with 5% PEG and 25 m*M* NaCl produced approximately three times more pilocarpine than the control treatment (Fig. 3*A*, *C*). At day 8, calluses stressed with 5% PEG still induced an increase of pilocarpine in the cell mass (Fig. 3*B*). The calluses responded differently at higher concentrations. While 15%

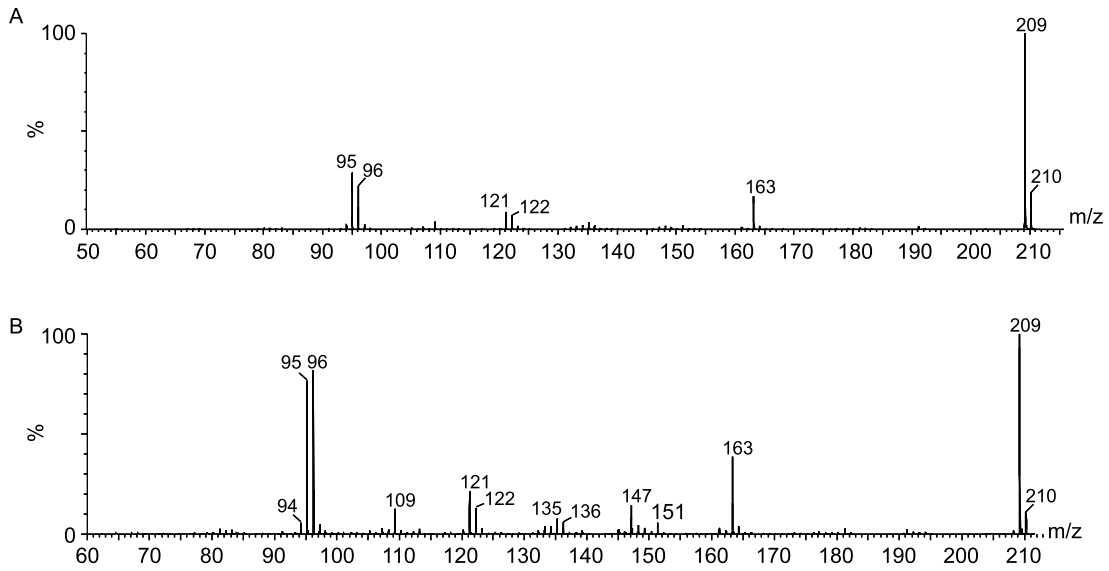

FIG. 1. MS/MS of (*A*) pure pilocarpine and (*B*) a sample of the culture medium taken 8 d after the histidine treatment.

PEG induced a 2-fold increase, there was a reduction of pilocarpine in the 75 m*M* NaCl at day 4 (Fig. 3*A*, *C*). On the other hand, at day 8, in the 75 m*M* NaCl treatment, pilocarpine returned to similar levels observed in the control treatment, while in the 15% PEG treatment, pilocarpine was lower than the control (Fig. 3*B*, *D*).

To verify the pH effect on pilocarpine production, the pH of the medium was adjusted to 4.8 and 6.8 (Fig. 4*A*, *B*). The control pH was 5.8, used in all other treatments. Pilocarpine concentration at pH 6.8 at day 8 showed the highest increase observed among all the treatments in this report (Fig. 4*B*). Considering the total amount of pilocarpine in the flask, there was a 4- to 5-fold increase, and most of the alkaloid was found in the medium.

The pH of culture medium normally changes during cell growth because nutrients are absorbed and the cells release organic products, and the buffering capacity of the medium is usually low (Torres et al., 1998). Some reports showed that the release of alkaloids to culture medium was dependent on pH. Godoy-Hernandez et al. (2000) observed that alkaloids were released from cells of *C. roseus* when the medium pH was higher than the cellular pH. Pitta-Alavarez and Giulietti (1999) also showed that low pH did not induce release of alkaloids in root cultures of *Brugmansia candida*.

Alkaloids are nitrogenous compounds and one may argue that the absence of nitrogen may decrease alkaloid contents in plants. However, this is not always the case (Waller and Nowacki, 1978). When jaborandi cells were grown in a medium without a source of N or at three times the normal (3 × N) concentration found in the MS medium, a similar increase of the total pilocarpine content was observed in the flasks at day 8 (Fig. 4*D*). At day 4, there was already a slight increase in the treatment 3 × N (Fig. 4*C*).

Saenz-Carbonell and Loyola-Vargas (1996) observed an increase in the tropane alkaloids of cell cultures of *Datura stramonium* when P was removed from the medium. Here, as for the N data, pilocarpine content was increased in the absence of P and at 3 × P in the medium, a change that could already be observed on day 4 (Fig. 4*E*, *F*). Also, with P omission, less pilocarpine was observed

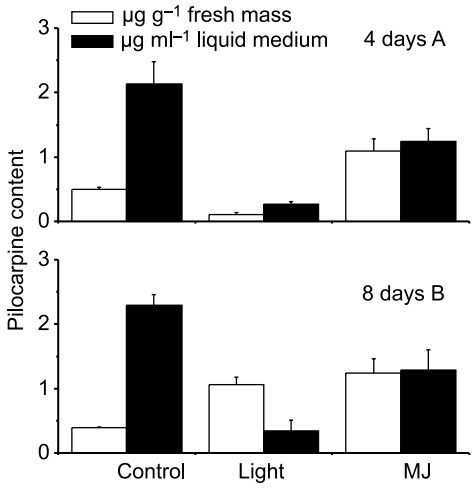

FIG. 2. Pilocarpine content in the callus of *P. microphyllus* and culture medium of the control (dark), light, and MJ treatments after (*A*) 4 and (*B*) 8 days of incubation. Data are means of three replicates, and *bars* indicate standard deviation.

than when N was omitted, suggesting that element recycling was more efficient for N.

Culture medium without or supplemented with K caused a significant reduction of pilocarpine (Fig. 4*G*, *H*). The importance of K for alkaloid biosynthesis was also reported by Khan and Harborne (1991), who observed that a 5-fold increase in K decreased the alkaloid concentration in *Atropa acuminata* plants.

Reactive oxygen species may severely damage cells and their levels are increased as a physiological response of plants to stress (Foyer et al., 1994). To combat the deleterious effects of reactive oxygen species, plants produce protective barriers such as antioxidant compounds and antioxidative enzymes (Foyer et al., 1994). Some reports have shown that alkaloids may be part of this

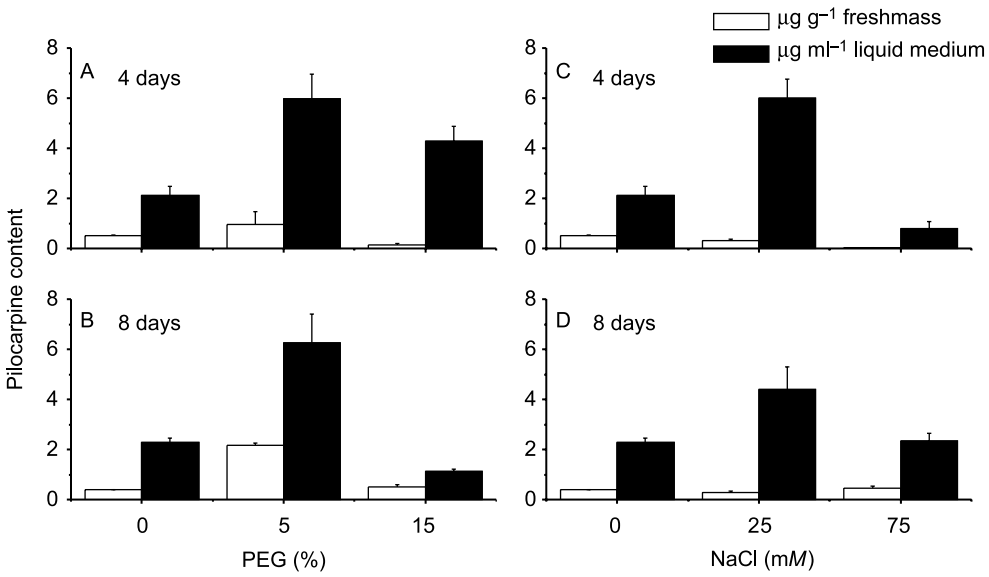

FIG. 3. Pilocarpine content in the callus of *P. microphyllus* and culture medium in the treatments with (*A*, *B*) PEG and (*C*, *D*) NaCl after (*A*, *C*) 4 and (*B*, *D*) 8 days of incubation. Data are means of three replicates and *bars* indicate standard deviation.

protection, displaying antioxidant potential for the scavenging of reactive oxygen species (Devasagayam et al., 1996: Gregianini et al., 2003; Schmeda-Hirschmann et al., 2003). Therefore, the increase of pilocarpine caused by treatments such as NaCl, PEG, 0 × N, and 0 × P might result from a protective role of this alkaloid against reactive oxygen species.

Pilocarpine appears to be derived from the imidazol ring of histidine and additional carbon may come from acetate or threonine

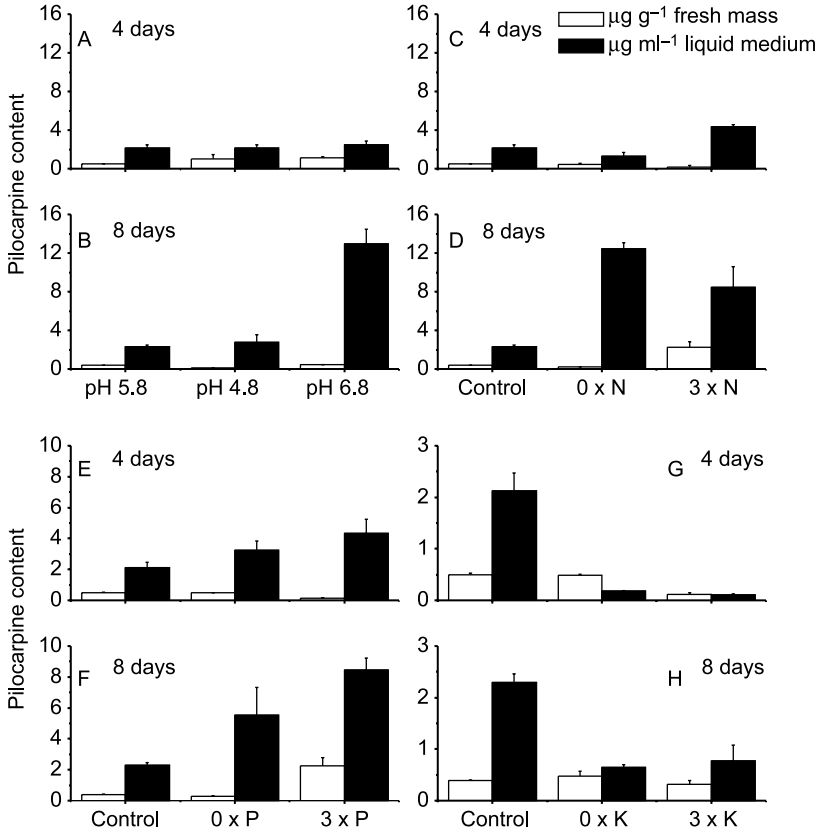

FIG. 4. Pilocarpine content in the callus of *P. microphyllus* and culture medium in the treatments with (*A*, *B*) different pH, (*C*, *D*) nitrogen supply, (*E*, *F*) phosphorus supply, and (*G*, *H*) potassium supply after (*A*, *C*, *E*, *G*) 4 and (*B*, *D*, *F*, *H*) 8 days of incubation. Data are means of three replicates, and *bars* indicate standard deviation.

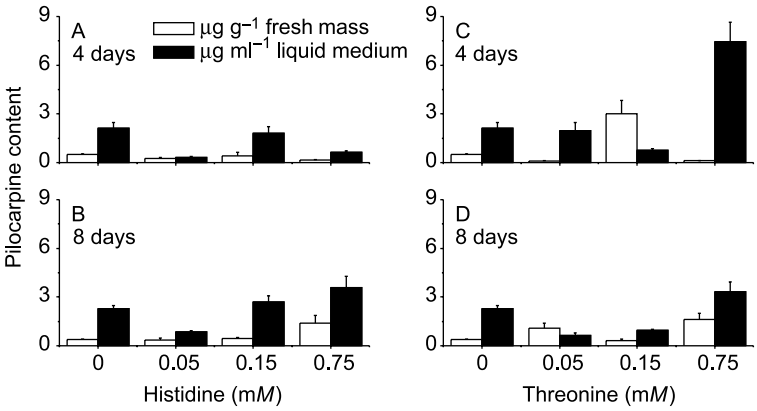

FIG. 5. Pilocarpine content in the callus of *P. microphyllus* and culture medium in the treatments with (*A*, B) histidine and (*C*, *D*) threonine after (*A*, *C*) 4 and (*B*, *D*) 8 days of incubation. Data are means of three replicates, and *bars* indicate standard deviation.

(Dewick, 1997), although this has not been proved. Preliminary experiments with ^{3}H-labeled threonine and histidine indicate that this could be the pilocarpine biosynthetic route in jaborandi (I.N. Abreu and P. Mazzafera, unpublished results). Therefore, jaborandi callus was grown with these amino acids (Fig. 5). In general, the production of pilocarpine was not strongly stimulated. Histidine caused a significant increase in the total alkaloid content in the flask only at 0.75 m*M* and at day 8 (Fig. 5*B*). Curiously, at day 4, this concentration induced a reduction of pilocarpine, and a concentration of 0.05 m*M* caused reduction on both days. The presence of threonine in the medium also led to changes in pilocarpine content (Fig. 5*C*, *D*), from reduction (0.05 and 0.15 m*M* at day 8) to significant increases (0.75 m*M* at day 4). At day 8, the level of pilocarpine using 0.75 m*M* threonine was still higher than the control, considering the total amount per flask.

There are several reports in the literature discussing the use of cell cultures for the production of compounds with pharmacological importance or other biological applications (Mulabagal and Tsay, 2004). The data presented in Figs. 1–5 showed that the highest pilocarpine production (callus + medium) was approximately $16\,\mu g\,g^{-1}$ fresh mass, while Avancini et al. (2003) found $350\,\mu g\,g^{-1}$ fresh mass, in young leaves of jaborandi. Despite this difference in the alkaloid content in favor of a commercial extraction of pilocarpine from the leaves, several tests on media optimization should be carried out before drawing a conclusion on the feasibility of producing pilocarpine by cell culture. Pilocarpine is extracted from jaborandi leaves with organic solvent in a costly process.

Among several technical aspects for the application of cell cultures to produce secondary metabolites on a commercial scale, the release of the compound in the medium seems to be crucial (Mulabagal and Tsay, 2004). Here, we have shown that pilocarpine production in cell suspension can be modulated, depending on the pH of the medium and the stress situations and, more importantly, that release of pilocarpine into the medium can be increased.

We are now in the process of establishing the biosynthetic route of this alkaloid, which will allow us to produce substrate for enzyme studies and protein isolation, opening up the possibility of developing molecular studies to increase pilocarpine production. Additionally, studies using bioreactors are being carried out. Besides offering a cleaner method for pilocarpine production (without the use of organic solvents), this approach would protect the jaborandi plant, an endangered species in Brazil, following years of intense, uncontrolled exploitation.

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