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Induction of pilocarpine formation in jaborandi leaves by salicylic acid and methyljasmonate

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Abstract

Jaborandi seedlings were subjected to different treatments in order to study the induction of pilocarpine in the leaves. In addition four extraction methods were assessed to extract the alkaloid from dried leaves. The highest yielding extraction and recovery was observed when dried leaves were first treated with base and then extracted with chloroform. Salt stress (NaCl), wounding, hypoxia, and N and K omission of the nutrient soln caused reductions in pilocarpine contents. Whereas complete nutrient soln and P omission maintained normal levels of the alkaloid. Salicylic acid and methyljasmonate induced a 4-fold increase of pilocarpine, but this increase was dependent on the concentration and time after exposure.

Keywords: *Pilocarpus jaborandi*; Rutaceae; Pilocarpine; Methyl jasmonate; Mineral nutrition; Wound; Salicylic acid; Salt stress

1. Introduction

Jaborandi is the common name given to different species of the *Pilocarpus* genus. Ten species of Jaborandi are distributed in the Brazilian territory, all of them having varied concentrations of pilocarpine in the leaves (Kaastra, 1982; Pinheiro, 1997). However, *P. microphyllus* and *P. jaborandi* contain the highest content of this alkaloid and they are found mainly in the Brazilian states of Pará, Maranhão, Piauí, Pernambuco and Ceará (Pinheiro, 1997).

Over the period from 1975 to 1994 Maranhão state was responsible for almost 95% of the Brazilian production of *P. microphyllus* dry leaves (Marques and Costa, 1994; Pinheiro, 1997) and the price was approximately U$ 4.00/kg (Vieira, 1999). This price paid by pharmaceutical industries prompted the widespread participation of the local people in harvesting the leaves from the shrubs growing in the forests. As a consequence of this intense gathering, jaborandi was included in the Brazilian list of endangered plant species (Pinheiro, 1997, 2002).

Jaborandi leaves are the only known source of pilocarpine, an imidazole alkaloid probably derived from histidine (Dewick, 1997). The most important pharmaceutical application of pilocarpine is in reducing the intraocular pressure in the treatment of glaucoma (Migdal, 2000; Webster et al., 1993). It is also a salivation and perspiration stimulant and recently has been prescribed for the treatment of xerostomia, which is the reduction of saliva production, very common in patients subjected to radiotherapy (Davies et al., 2001; Wynn, 1996).

In spite of its pharmaceutical importance, only a few reports have been published on the pilocarpine content in jaborandi (Andrade-Neto et al., 1996). However, nothing is known about the control of its concentration in the leaves. Following a general pattern for alkaloids, the highest content of pilocarpine in jaborandi is found in young tissues (Pinheiro, 1997).

Alkaloid concentrations in plants may respond to biotic and abiotic treatments, such as temperature variations (Frischknecht and Baumann, 1985), methyljasmonate (Aerts et al., 1994), osmotic stress (Godoy-Hernández and Loyola-Vargas, 1991), abscisic acid

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(Saenz et al., 1993) and nutritional stress (Mazzafera, 1999). However, in most cases, such studies used cell suspension cultures and only a few were carried out with intact plants. Therefore, the aim of the present study was to investigate the induction of pilocarpine formation in leaves of jaborandi seedlings in order to establish a model system for further studies on pilocarpine biosynthesis and biodegradation.

2. Results and discussion

Among the four extraction methods evaluated, the third method yielded the greatest amount of pilocarpine (Table 1). This same method showed the best recovery when a known amount of pure pilocarpine was added to dried leaves, and consequently it was used in all other pilocarpine determinations.

Nutrient supply had a marked negative effect on pilocarpine accumulation in the leaves of jaborandi seedlings (Fig. 1A). Nitrogen omission usually causes a decrease in the alkaloid content in several plants, probably because alkaloids are N containing compounds (Waller and Nowacki, 1978). However, in the case of other nutrients the response may change from plant to plant. Caffeine increased in leaves of coffee seedlings under K omission but increased with P (Mazzafera, 1999). On the other hand, lupinus alkaloids varied inconsistently when the plants were grown in the greenhouse or in the field and depending on the variety used or the locality the field trial was carried out. (Gremigni et al., 2001). K omission had a more pronounced negative effect on pilocarpine content than N, inducing a 10-fold reduction when compared with the content of plants receiving the complete nutrient soln (Fig. 1A). Apart from these results, our data showed that balanced nutrition might favour the accumulation of pilocarpine in leaves of jaborandi.

Salt stress, wounding and hypoxia drastically decreased pilocarpine in jaborandi leaves (Fig. 1B). The alkaloid reduction in salt treated plants was dependent on salt concentration and length of the exposure. The most pronounced negative effect on pilocarpine content was observed with wounding and 75 mM NaCl. Salt also induced a decrease of alkaloids in *Atropa belladona* (Ali, 2000). We are not aware of any report on the effect of hypoxia and alkaloid concentration in plants.

Table 1
Comparison of four methods for the extraction of pilocarpine from dried leaves of jaborandi

Extraction method	Pilocarpine content (μg/mg)	Recovery of total pilocarpine (μg)
1	0.12±0.04	4.07+0.70
2	0.11±0.03	3.57+1.40
3	2.31±0.15	104.25±2.55
4	0.70±0.34	56.62±9.24

Fifty milligrams of dried leaves were used for extraction. To study the recovery, 100 μg of pure pilocarpine was added to 50 mg of dried leaves with extraction following (see Experimental).

Salicylic acid (SA) and jasmonic acid and its ester, methyljasmonate (MJ), have been extensively studied because of their involvement in the resistance of plants against diseases and insect attack in plants. Farmer and Ryan (1992) proposed that wounds caused by insects or microbial attack may lead to a cascade of responses in plants whose signals were compounds generated by a lipid-based signal transduction system. These compounds, jasmonic acid and MJ are responsible for the induction of defense proteins. Exposure to synthetic jasmonic acid also showed the same induction. Interested in the insect/microbe-plant interactions, Zenk and coworkers (Blechert et al., 1995; Dittrich et al., 1992; Gundlach et al., 1992; Mueller et al., 1993) used fungal cell wall elicitors to study whether jasmonate compounds were involved in the accumulation of defense substances (phytoalexins) in the supension cultures of several plant species. They observed accumulation of various types of secondary metabolites, including alkaloids, in response to exposure to jasmonate. Several other reports also showed an increase in alkaloids due to MJ exposure (Aerts et al., 1994; Baldwin et al., 1994; Ignatov et al., 1996; Waldhauser and Baumann, 1996). In the case of herbivory by insects it is suggested that disruption of plant cells causes a rapid release of linolenic acid, initiating the octadecanoic-based pathway, which results in the synthesis of the jasmonate family of compounds.

SA has been more investigated regarding its role as a component of a signal transduction pathway in disease resistance, being considered the main signal molecule involved in the systemic acquired resistance of plants to pathogens (Hammerschmidt, 1999; Raskin, 1992). This precedes the development of necrotic lesions (hypersensitive response) limiting the development of the disease.

A few studies were carried out to investigate the effect of SA on the accumulation of secondary compounds in plant tissues. Hypericin accumulation was induced by jasmonic acid but not SA or fungal cell wall elicitor (Walker et al., 2002). Alkaloids accumulated in hairy root cultures of *Brugmansis candida* (Lee et al., 2001) and *Atropa belladona* (Pitta-Alvarez et al., 2000) in response to SA exposure. However, *Catharanthus roseus* cell cultures accumulated more indole alkaloids with MJ but not with 2,4-dichlorophenoxyacetic acid, SA or abscisic acid (El-Sayed and Verpoorte, 2002). Therefore, the increase of alkaloids in plants due to SA and MJ treatment varies considerably, depending on plant species, the way the plant is grown, and the concentration of the inducer.

While wounding had a strong depressive effect on pilocarpine content (Fig. 1C), MJ (Fig. 2A) and SA (Fig. 2B) induced an approximately 4-fold increase. The increase due to MJ and SA were dependent on the dose and time. The increase in pilocarpine content was

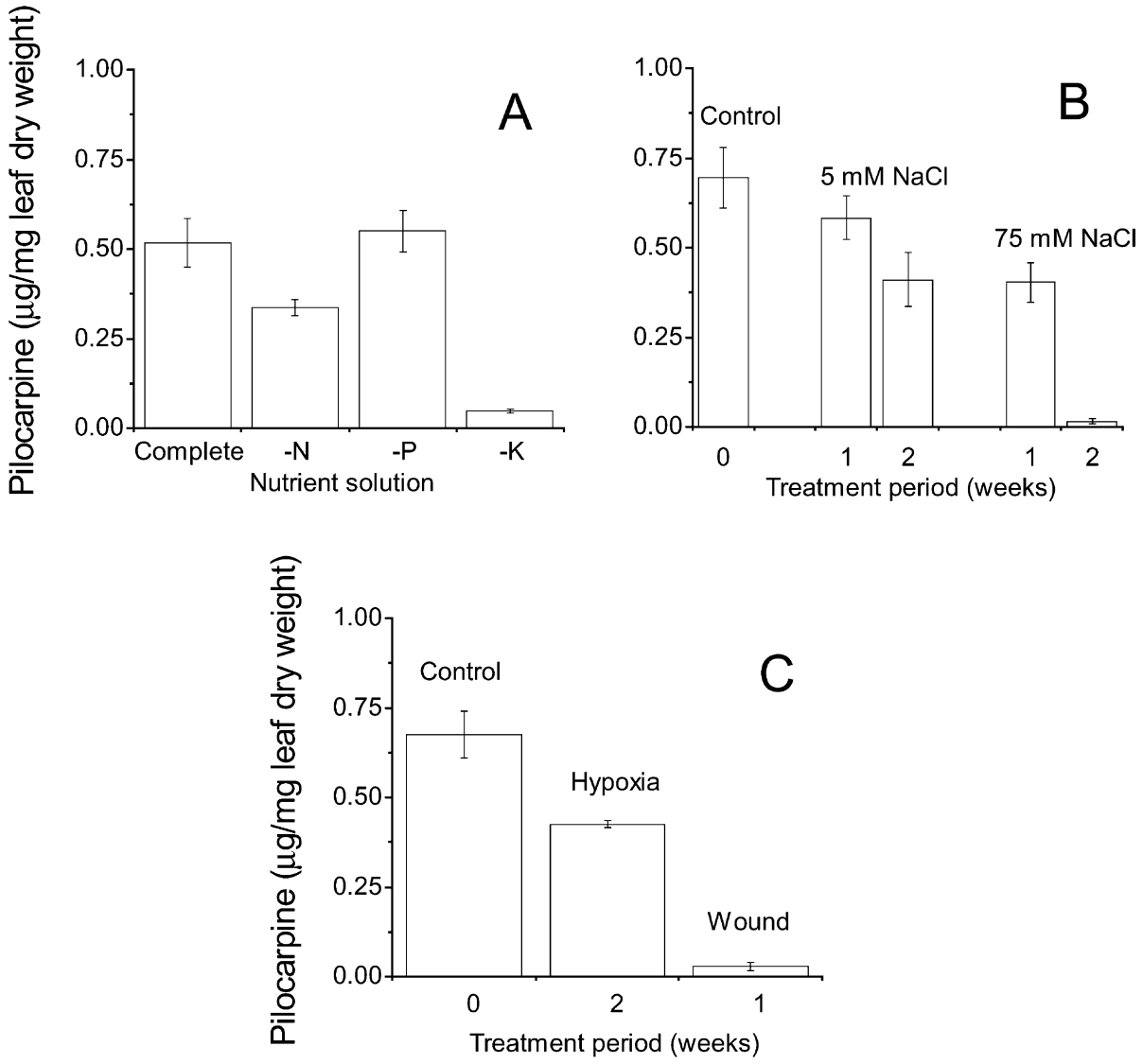

Fig. 1. Pilocarpine content in jaborandi leaves treated with different nutrient solns (A), with NaCl (B) and subjected to hypoxia and wounding (C).

observed 5 days after the plastic bags containing the MJ had been removed from the seedlings. For SA the increase was quicker with 25 mM spraying, but it was higher with 5 mM (Fig. 2B).

3. Conclusions

The present data show that a balanced nutrient supply is essential to maintain the content of pilocarpine in jaborandi. More importantly, it showed that the phytohormones MJ and SA led to a significant increase in the alkaloid. In addition to the lack of information on pilocarpine metabolism in jaborandi, tissue culture of this species is complicated and the growth of the calli is very slow (Abreu and Mazzafera, data not published). Therefore, seedlings might be used instead as a model to study the biosynthesis of pilocarpine and its regulation.

4. Experimental

4.1. Plant material

Seeds of *Pilocarpus microphyllus* Stapf ex Holm. were kindly supplied by Merck Company, Maranhão State, Brazil, and germinated in sand. When seedlings reached approximately 5 cm (five months old) they were transferred to 0.5 l pots containing sand and each pot received complete nutrient (50 ml) soln twice a week (Hoagland and Arnon, 1950).

4.2. Extraction procedures

Four methods were assessed for the extraction of pilocarpine from dried leaves obtained from a local Herbal Pharmacy. In the first protocol leaves (50 mg) were extracted with 80% aq EtOH (3 ml) in cap sealed

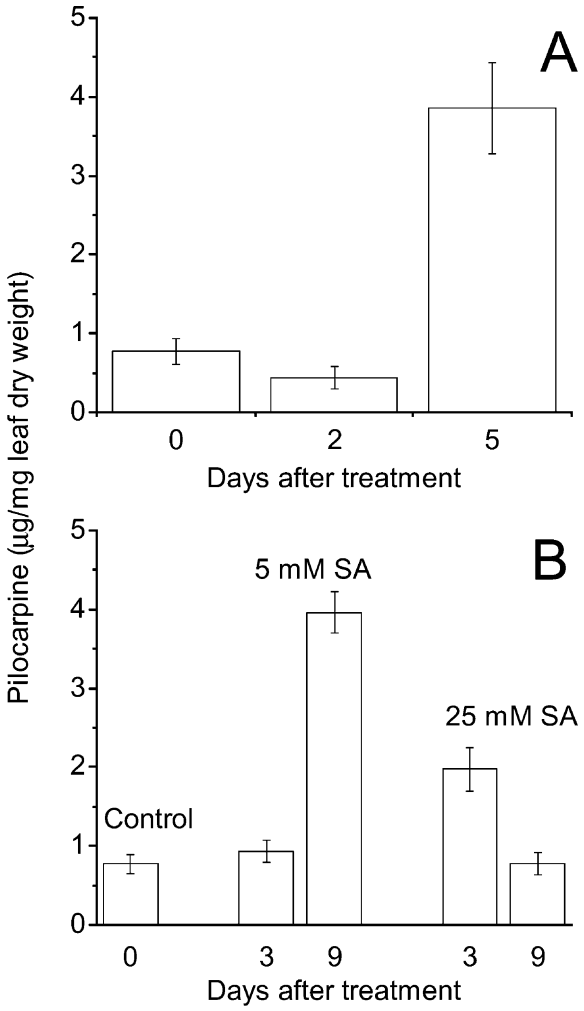

Fig. 2. Pilocarpine content in jaborandi leaves treated with methyljasmonate (A) and salicylic acid (B).

tubes maintained in a boiling water bath for 30 min, with occasional agitation. After cooling at room temperature the extract was clarified with Carrez reagent (100 µl of reagent 1 and 100 µl reagent 2) (Ky et al., 1997). The tube was centrifuged and the supernatant was recovered and kept at −70 °C until analysis. The difference between protocol 1 and 2 was that in the second, the extraction was carried out with 80% aq-EtOH overnight at room temperature and continuous shaking. In the third protocol dried leaves (50 mg) were wetted with 10% NH_4OH (2 drops) and after 15 min extraction was carried out with $CHCl_3$ (0.3 ml) with vigorous shaking for 1 min. The $CHCl_3$ was recovered by centrifugation and the precipitate re-extracted with $CHCl_3$. The $CHCl_3$ fractions were pooled and extracted with 2% H_2SO_4 (0.3 ml × 2). The pooled acid extracts were neutralised to pH 12 with NH_4OH and extracted twice with 0.3 ml $CHCl_3$ (0.3 ml×2). The organic fractions were pooled, dried in Speed-Vac and dissolved in HPLC buffer. In the fourth protocol, dried leaves (50 mg) were extracted with 80% EtOH (0.5 ml) for 30 min and vigorous shaking. The material was re-extracted and the ethanolic fractions pooled and dried in Speed-vac. EtOAc (0.3 ml) was added to the tubes and partitioned with 0.1 M HCl (0.3 ml × 2). The acid fractions were pooled, basified with 2.5 M Na_2CO_3 (50 ml), and extracted with $CHCl_3$ (0.3 ml × 2). The fractions were pooled, dried and dissolved in the HPLC solvent (see below).

In order to check the efficiency of the protocols pure pilocarpine (100 µg) was added to 50 mg samples and they were extracted as specified for each protocol. Four replicates were used for each protocol.

4.3. Pilocarpine analyses

Pilocarpine was determined in the extracts by reversed phase HPLC analysis. The alkaloid was separated in a Supelcosil LC18 column (4 × 250 mm, 5 µm, Supelco) using for elution 13.5 ml H_3PO_4 and 3 ml triethylamine in 850 ml MilliQ H_2O (pH 3 with NaOH) with further addition of 112 ml MetOH (Fan et al., 1996). The flow rate was maintained at 1 ml/min and the detection was monitored with a UV monitor operating at 212 nm.

4.4. Induction treatments

The induction treatments were carried out over a 45-day period (day 0–45). Jaborandi seedlings were subjected to the following stresses: 1—Salt stress: initiated at day 0. The pots containing the plants received aq. solns containing 5 or 75 mM NaCl every second day. During the intervening days these plants and the control plants received nutrient soln. Salt treatment was carried out for one or two weeks; 2—Wounding: initiated at day 7. Some leaflets of the compound leaves were wounded with an haemostat and analysed for pilocarpine one week later (Constabel and Ryan, 1998; Mazzafera and Robinson, 2000); 3—Hypoxia: wounding: initiated at day 7. The pots were immersed in other larger pots containing water. The water was changed every 3 days. Leaves were collected after two weeks of treatment; 4—Wounding: initiated at day 14. Salicylic acid: aq. solns (5 and 25 mM) were sprayed on the leaves that were collected for analysis after 3 and 9 days; 5—Methyljasmonate: initiated at day 14. The whole pot was placed inside a plastic bag which had inside a cotton wetted with 50 µl of MJ. After 24 h the bag was opened and a new application of jasmonate was made. After 24 h the bags were removed and the leaves collected after 3 and 9 days; 6—Nutrient stress: nutrient solns were prepared without the nutrients N, K and P and used to irrigate the plants (Hoagland and Arnon, 1950). The control plants received complete nutrient soln. Leaves of control plants were collected for analysis 45 days after the beginning of the treatments.

In all treatments the leaves were collected and frozen in liquid N, and then freeze-dried. They were maintained at −20 °C until analysis.

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