Sustainability of Jaborandi in the eastern Brazilian Amazon

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\textbf{A B S T R A C T}

The genus \textit{Pilocarpus} (Rutaceae) includes the only known species able to synthesize pilocarpine, an imidazole alkaloid largely employed in glaucoma and xerostomia treatments. \textit{Pilocarpus microphyllus} Stapf ex Wardleworth has a broad geographical distribution in the Northeast Brazilian territory, produces a substantial amount of pilocarpine and constitutes the only known natural source of this metabolite. Intensive extractivism and deforestation over recent decades have led to substantial declines in yields, plant populations and genetic diversity of \textit{P. microphyllus}. Currently, it is recognized as a threatened species, and the few remaining areas harboring large populations (and potentially high genetic diversity) experience substantial farming and mining pressures. In addition, extractivism in these areas is still occurring by local communities because of the high market demand for pilocarpine. Conservation programs and sustainable management are urgently needed to maintain the long-term viability of this species in natural areas. Here, we highlighted the socioeconomic importance of this species, showing the massive reduction in the harvesting of raw materials and the consequences for the local people. We identified new potential areas of natural occurrence, including locations beyond the Brazilian borders, using species distribution modeling. We showed well-synchronized patterns of vegetative and reproductive phenophases, which may facilitate the management of this species. Finally, we emphasized the importance of cultivating \textit{P. microphyllus} in a sustainable and productive way, involving the local communities and companies as a conservation strategy.

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\section*{Introduction}

Jaborandi is the common name for \textit{Pilocarpus} species, which comes from the Tupi-Guarani language \textit{yu-mbor-endi}, meaning “what causes slobbering”. Traditional knowledge related to jaborandi plants is assumed to be one of the most precious inheritances of Amazonian indigenous culture, where this plant is historically used for medicinal purposes. Users have observed that infusions of jaborandi leaves have the property of stimulating the production of sweat and salivation, and they are applied in shamanic rituals for fever treatment, stomatitis and as an antidote for poisons and toxins (Holmstedt et al., 1979). This knowledge spread through the generations and cultures, resulting in the wide use of this plant in popular medicine by native people and the people who came from abroad after the XVI century (see the short description of \textit{Pilocarpus} records in Fig. 1).

The stimulation effects observed derivate from the imidazole alkaloid pilocarpine. The active ingredient extracted from jaborandi leaves acts as a cholinergic parasympathomimetic agent, i.e., it stimulates secretions in sweat, lachrymal and salivary glands (Fox et al., 2016; Sidhu, 2014). Since its isolation (Gerrard, 1875; Hardy, 1875) and the discovery of its power to reduce intraocular pressure (Sneader, 2005), pilocarpine has constituted the base of several medicaments, especially those applied in glaucoma treatment. More recently, it has also been used to reduce xerostomia induced by head and neck radiation therapy and for the treatment of Sjogren’s syndrome (dry mouth) (Gornitsky et al., 2004; Sidhu, 2014).
The genus *Pilocarpus* (Rutaceae) is the only natural and economically viable source of pilocarpine currently known (Abreu et al., 2007). Despite the occurrence of pilocarpine in several *Pilocarpus* species, it is essentially obtained from *Pilocarpus microphyllus* Stapf ex Wurdeworth because of the high pilocarpine concentration in the leaves of this species (Pinheiro, 2002) and due to its supposedly broad geographic distribution (Kastra, 1982). The habitat loss resulting from Amazon deforestation and the uncontrolled extractivism of jaborandi leaves (especially in the 1970s and 1980s) led to a depletion of the plant population, with an estimated reduction of natural populations by approximately 50% (Martineili and Moraes, 2013). Therefore, since 1992, *P. microphyllus* has been included on the List of Endangered Brazilian Plant Species (IBAMA n° 37, 1992), along with two other *Pilocarpus* species (*P. jaborandi* Holmes and *P. trachylophus* Holmes). More recently, another *Pilocarpus* species (*P. alatus* C. J. Joseph ex Skorupa) was also added to the Brazilian red list, since its populations have also undergone a significant reduction (Martineili and Moraes, 2013). In addition, jaborandi leaf harvesting under natural conditions constitutes an important income source for communities living in poor regions of Brazil. Here, we compile our understanding of the genus *Pilocarpus*, including knowledge gaps related to its natural occurrence, genetic variability, pilocarpine concentrations, pilocarpine synthesis pathway and domestication/cultivation, as well as socioeconomic aspects of the species *P. microphyllus*.

**Pilocarpus microphyllus**: plant description, occurrence and potential distribution

The genus *Pilocarpus* was firstly described by Martin Vahl in 1797 (Vahl, 1797) from the species *Pilocarpus racemosus* Vahl. Despite its importance as a widely used medicinal plant, most complete taxonomic studies of this genus were only latterly performed by Kastra (1982) and Skorupa (1996), who identified 16 *Pilocarpus* species. Currently, we found 17 described and recognized species in the repository *The Plant List* (www.theplantlist.org) and 43 scientific plant names proposed for the *Pilocarpus* genus, involving synonyms and potentially new species (Supplemental Table 1).

These species present a widespread distribution in Latin America, occurring from southern Central America (Mexico) to southern South America. The north and northwest portions of South America contain a large part of these species, especially in the Brazilian territory, which harbors 13 species, with 11 being endemic to Brazil (Skorupa, 2000). As a consequence, this part of the continent is recognized as the center of genetic diversity of *Pilocarpus* species (Oliveira, 2007). *Pilocarpus microphyllus* is a tree reaching 1–6 m in height, with a terminal raceme 15–40 cm in length as the most common inflorescence type (Fig. 2). Leaves are composite and glabrous, and the leaflets vary in shape and color, are commonly elliptical, exhibit opposite lateral insertion, are 1.5–6 x 1–3.5 cm and sessile and have an emarginated apex (Skorupa, 2000). We can observe substantial morphological variation in the leaf traits of different accessions growing under the same environmental conditions (Fig. 3), suggesting potentially large genetic variability in this species, at least for some traits (Moura et al., 2005b).

Described to naturally occur mainly in the northern region of Brazil (comprising the states of Pará, Maranhão and Piauí) and some locations in Suriname (Santos and Moreno, 2004; Skorupa, 1996), the potential distribution of *P. microphyllus* may be larger than currently observed. For the present study, we designed a potential distribution model considering the public data available from speciesLink (http://www.splink.org.br/) and the Global Biodiversity Information Facility (GBIF, http://www.gbif.org/). In the construction of this model, we used the eight least correlated bioclimatic layers with 30 arc-seconds obtained from the Worldclim variables (Hijmans et al., 2005) and the Maxent algorithm (Phillips et al., 2006). The ROC-AUC (area under receiver-operating curve), whose values near 1.0 indicate good results (Fielding and Bell, 1997), was calculated. Additionally, to describe the main environmental features where *P. microphyllus* was recorded, we used the same dataset of occurrence points to calculate the frequency distribution considering three topo-climatic variables: altitude, annual mean temperature and annual precipitation using ArcGIS (Esri Inc.). As expected, the model shows that Piauí, Maranhão, and Pará contain the most representative jaborandi areas (Fig. 4). It also shows...
that northern areas of Ceará and Tocantins are suitable for the occurrence of this species. Additionally, the model suggests that some areas in the south of French Guiana, Suriname and Guyana as well as the Brazilian states of Amazonas, Roraima and Amapá are also suitable for the occurrence of *P. microphyllus*. For some of these locations (Guyana, Amazonas, Roraima, Amapá, Tocantins and Ceará), there is no record of *P. microphyllus* so far, and these areas should be checked through field studies. Particularly in the Brazilian territory, these areas have little biodiversity data available.

Results from the model suggest that *P. microphyllus* has a great probability of occurrence in regions with low altitudes (0–100 m), annual precipitation of approximately 1300–1700 mm, and a mean annual temperature between 25.5 and 27 °C (Supplemental Fig. 1). These results confirm previous studies (Santos and Moreno, 2004; Skorupa, 1996) suggesting that *P. microphyllus* is mainly found in the lowlands of Amazon Tropical Moist Forest, despite reported occurrences of this species in environmental conditions such as those in the Caatinga Dry Forest in the Northeast region of Brazil. Overall, our results suggest that *P. microphyllus* can be found growing outside the limits of the three Brazilian states (Maranhão, Pará and Piauí), supporting the hypothesis that *P. microphyllus* is not a Brazilian endemic species (Martinelli and Moraes, 2013). In addition, the occurrence of this species in the northwestern portion of the species potential distribution map needs confirmation through field surveys.

The two National Reserves highlighted in Fig. 4, Carajás and Tapirapé-Aquiri, likely contain the largest remaining *P. microphyllus* populations and may act as natural reserves of genetic diversity for this species. Both of these areas have undergone intensive deforestation pressure inside and outside their limits, causing a consequent threat to several species, such as *P. microphyllus*. Outside the reserves, cattle farms have expanded into large areas that currently reach the reserve borders (Souza-Filho et al., 2016). Inside, mining is also expanding into the areas where *P. microphyllus* frequently occurs, i.e., the borders of iron crust inselbergs in the Amazon Forest, called Canga ecosystems (Canga is a Brazilian...
common name used to designate the ecosystems associated with the superficial ironstone (Skirycz et al., 2014)). Because local people predominantly collect leaves of *P. microphyllus* in these areas, especially in the Carajás National Reserve, management programs and reforestation with this species are highly recommended.

**Current status and prospects of genetic diversity analyses in *Pilocarpus* species**

Karyotype analyses have provided several tools for plant breeding programs that can be applied in different phases of the process to develop new cultivars from accessions of a germplasm bank (Singh, 2003; Sybenga, 1992). However, as generally observed for most of the angiosperm families with recognized economic importance (Guerra, 2008), only a few groups in the Rutaceae have cytogenetic data available, as is the case of Citrus and related genera (see, for instance, Carvalho et al., 2005; Mendes et al., 2011; Silva et al., 2015). For *Pilocarpus* species, however, the chromosome numbers of only eight species (out of the 17 spp. in the genus) are available in the literature (*P. giganteus, P. grandiflorus, P. microphyllus, P. pauciflorus, P. sulcatus and *P. trachyophlus*, with 2n = 44, and the two putative tetraploids *P. carajaensis* and *P. spicatus*, with 2n = 88) (Skorupa, 2000). There is a lack of information on the divergence/similarity levels among karyotypes, which may be important in better directing interspecific hybridizations in jaborandi breeding programs (see Chen et al., 2003).

In addition, there are few studies addressing the genetic diversity of jaborandi (see Moura et al., 2005a,b; Sandhu et al., 2006; Rocha et al., 2014), which is a noteworthy fact, considering the importance of the species. However, two out of the four published analyses (Rocha et al., 2014; Sandhu et al., 2006) sampled a very limited number of genotypes because their primary interest was to find molecular markers rather than conduct a more detailed characterization of natural populations or compile germplasm banks. Sandhu et al. (2006) attempted to describe RAPD (Random Amplified Polymorphic DNA) markers associated with contrasting levels of jaborandi populations. However, Rocha et al. (2014) tested the applicability of 48 ISSR (Inter-Simple Sequence Repeat) primers for genetic diversity analyses among jaborandi populations and reported some promising markers with high levels of polymorphism indexes.

On the other hand, a considerably larger sample was used in a study with two aims, one to evaluate the discriminatory power of RAPD markers (Moura et al., 2005a) and the other to use morphological leaf traits to access the genetic diversity among different populations of jaborandi (Moura et al., 2005b). Although moderate genetic variability among the tested accessions was observed in both studies, the authors could not observe geographic structure among the collection plots in addition to observing higher molecular variance within (75.84%) than among populations (26.16%) in the RAPD assay (Moura et al., 2005a). Despite the studies recognizing the existence of enough diversity in natural populations for genetic breeding programs, there is not enough knowledge on the genetic diversity of the species to assure successful programs.

Considering the current conservation status of wild jaborandi populations, the development and application of molecular biology tools to find genetic markers is mandatory. In contrast to the dominant nature of both RAPD and ISSR markers, i.e., there is not information on both alleles of a given locus, co-dominant marker systems (which assess both alleles) offer a better view of the genotypes by providing the assessment of a wider range of polymorphism (see Weising et al., 2005; Nybom et al., 2014). Favorable by recent advances in sequencing technologies, the development of either single- or multi-locus co-dominant markers, such as the traditional and widely used SSR (or Simple Sequence Repeats, or microsatellites) and SNP (Single Nucleotide Polymorphisms) marker systems, respectively, has been significantly faster and more cost-effective over the last decade (Metzker, 2010; Nybom et al., 2014). Therefore, future investments in jaborandi breeding programs should be focused on developing such co-dominant markers for the species by planning large-scale genotyping of both natural populations and cultivated accessions in order to direct the first steps of the more efficient genetic improvement of cultivars with traits of interest, such as higher jaborande concentration.

**Phenology of *P. microphyllus***

Most of the plant phenology studies conducted in the tropics have been carried out at the community level, addressing specific life events, such as flowering and fructification (Croat, 1969; Frankie et al., 1974; Haugasen and Peres, 2005; Morellato and Leitão-Filho, 1996; Muniz, 2008). This was the case for *P. microphyllus* until recently, when (Muniz, 2008) determined the timing of flowering (April to May) and fructification (May to August) for this species in Northeast Brazil. These phenophases, shared with the other species also growing in the same forest understory, occur at the end of the wet season, contrasting with other species occupying the forest canopy (Croat, 1969; Muniz, 2008).

We recently performed a more comprehensive phenological study of *P. microphyllus* by monitoring vegetative (leaf fall and leafing) and reproductive (flower bud, flowering, fructification) events of 414 plants growing in the Carajás Natural Reserve (North Brazil) over 27 months (February 2014 to May 2016). In this study, the activity and intensity of each event were monitored monthly, following the methods proposed by (Bencke and Morellato, 2002) and (Fournier, 1974). We observed leaf fall in a large part of the plants over almost the entire period of evaluation (Fig. 5a). However,
because the intensity of this event remained constantly at low values, *P. microphyllus* can be classified as an evergreen species (Rivera et al., 2002) in the studied site. Canopy renovation by leafing occurred continuously, with a peak of activity and intensity of this phenophase between September and January (Fig. 5b), a period coinciding with the transition of the dry to wet season in the Carajás National Reserve (Supplemental Fig. 2), aligning the leaf-growing period with the wet season. Because leaves are predominantly harvested in the forest during the dry season (June–September), most of these leaves have completed their growth, and the period of leafing can be avoided, which is essential in plant canopy recovery and leaf productivity.

The flowering (flower budding and flower anthesis) of *P. microphyllus* plants occurred during several months of the year, suggesting a possible annual pattern of long duration (Newstrom et al., 1994). Flower buds initially appeared in December, having maximal activity and intensity between February and April (Fig. 5c). We observed flower anthesis almost simultaneously (one month later) following a similar trend as described for the flower buds (Fig. 5d). The overall lower activity and intensity of this phenophase suggests possible leaf bud abortion, reducing the number of developed flowers or the duration over which the flowers remain open. Fructification also showed a regular annual pattern, starting in February, with a peak between May and June, and ending by July/August (Fig. 5e), when the dry period occurs in the region, which may favor seed dispersal because *P. microphyllus* has dry fruits with explosive dehiscence.

The earlier occurrence of reproductive events (flowering and fructification) we observed at the studied site compared to those described by Muniz (2008) is consistent with the suggestion that water availability drives most of the phenological patterns in the tropics (Fenner, 1998). While at both sites (500 km distant from each other), flowering occurred in the middle of the wet season followed by fructification, which ends when precipitation declines, precipitation at the site where we performed our study started earlier (September–October) when compared to the Muniz site (November) (Supplemental Fig. 2). We could expect phenological patterns that are not synchronized with the changing of the seasons because *P. microphyllus* grows in the tropics. In these areas, it is common to observe continuous, sub-annual, annual, or supra-annual events (Newstrom et al., 1994; Sarmiento and Monasterio, 1983) that are normally associated with the diversity of pressures (biotic and abiotic) affecting these communities (Fenner, 1998). Nonetheless, the number of studies observing synchronism between plant events even when climatic seasonality is not obvious is increasing (Borchert et al., 2005; Caldeira et al., 2008; Menzel et al., 2006; Newstrom et al., 1994). In addition, observations of *P. microphyllus* phenological patterns in populations occurring in areas other than those that have been already studied will shed some light in regard to the behavior of such species.
Alkaloids, coumarins, terpenoids and flavonoids

Other metabolites with potential applications in the pharmaceutical and cosmetic industries are also found in leaves of *Pilocarpus* spp., including coumarins, terpenoids, flavonoids and other alkaloids (an extensive compilation of these metabolites and their activities can be found in Santos and Moreno (2004)). Overall, pilosine, pilosinine, pilocarpidine, N,N-dimethyl-triptamine,
dictamine, etc., are also alkaloids synthesized by *Pilocarpus* species. Some of them have well-known biological activities, such as N,N-dimethyl-triptamine, the most abundant alkaloid of *P. organensis* Occhioni & Rizzini (= *P. pauciflorus* A. St.-Hil.), which acts as a hallucinogen (Balsam and Voigtlander, 1978), and dictamine, common in *P. grandiflorus* and having antifungal effects (Souza et al., 2002). A large range of terpenes (approximately 75 types, including monoterpenes, diterpenes, triterpenes, sesquiterpenes, polypropenols and steroids) can be found distributed among different species of *Pilocarpus*. Playing an essential role in pollination (especially for insect/animal attraction–volatile oils), terpenes also exhibit antimicrobial, antibacterial and antihelminthic activities (Moon et al., 1999; Santos et al., 1997; Singh and Singh, 2003; Villaseñor et al., 2002). Approximately 27 coumarins have been isolated from *Pilocarpus* species. A wide range of uses are expected from this group of metabolites, including promising results in the treatment of some diseases (Mafezoli et al., 2000; Santos and Moreno, 2004) in addition to insecticidal (Calcagno et al., 2002), antifungal and bactericidal activities (Guerreiro et al., 2001). Finally, of the five flavonoids identified in one out of seventeen *Pilocarpus* species (*P. trachylophu*s), four of them were found in the leaves (Bertrand et al., 2001) and one in the roots (Andrade-Neto et al., 1996).

Screening for other phytochemical products and developing economically viable extraction techniques are ways to detect new active ingredients, improving the valuation and conservation possibilities of such species, especially those that are threatened. In this way, synthetic procedures to obtain pilocarpine have been developed (Horne et al., 1993), but high costs make such production economically unviable when faced with the market for natural pilocarpine (Sawaya et al., 2010). Associated with the increased demand for products with more sustainable sources, the extraction of pilocarpine from *Pilocarpus* leaves will apparently continue and requires further investigations to enable sustainable harvesting in nature and the identification of cultivation conditions to produce high biomass and pilocarpine yields.

**Socioeconomic aspects of *P. microphyllus* extractivism**

Brazil is the only known country commercially supplying pilocarpine (CNI, 2014), probably because of the natural occurrence and abundance of the species *P. microphyllus*. As a result of market demand, the harvesting of jaborandi leaves in nature was maximized between 1950 and 1990 (Berlinck, 2012), reaching its peak around the 1970s, when the state of Maranhão (major producer) alone provided more than 2500 ton year⁻¹ of dried leaves (Fig. 6). A significant leaf yield reduction in these areas began in the early 1990s, when it was partially buffered by increasing harvesting in new areas, such as in the Carajás mountain range in the state of Pará (Grabher, 2015). The intense harvesting in these areas without a management program compromised the sustainability of this activity especially for the major *P. microphyllus* populations growing inside national forests (Carajás and Tapirapé-Aquiri), where environmental agencies started controlling and organizing the extractivism. Additionally, the establishment of mining operations in these areas also affected the mobility of leaf collectors and the availability of plants, since *P. microphyllus* usually occurs close to the mining areas in these National Forests.

Motivated by the reduction in the raw material from nature and the need to supply pilocarpine demand, the Merck company, which holds a pilocarpine extraction patent and was the major pilocarpine supplier until recently, initiated a jaborandi domestication program in 1989–1990 by selecting accessions and establishing a jaborandi plantation with more than 3 million plants (300 ha) in Maranhão. Currently, this farm has approximately 15 million jaborandi plants. It is owned by Centroflora Group, a Brazilian company devoted to extracting and processing compounds from jaborandi (and other species), especially those associated with the pharmaceutical and cosmetic industries. However, similar operations have not yet been established in other locations where *P. microphyllus* occurs.

The jaborandi market has played an important economic role by creating job opportunities and providing important income by connecting collectors to the pharmaceutical industry. It has been suggested that jaborandi extractivism corresponded to major revenue for thousands of families in the Brazilian Northeast region during the peak of extraction (1970–1980), estimated to reach up to 25 and 1.2 thousand families in Maranhão and Pará, respectively (Hommá, 2003). Despite the market fluctuations and the reduction in areas where it naturally occurs, jaborandi is still being exploited, mainly in wild areas. Overall, these activities remain economically feasible because the pilocarpine price has experienced a significant increase (up to 36%) in the last two decades, when the pilocarpine supply capacity declined while its demand did not (IBGE, 2014). Moreover, pilocarpine is currently the eighth most exported pharmaceutical substance from Brazil, reaching around
U$ 6.8 million year\(^{-1}\) (Grabher, 2015). Increasing the number of cultivated areas and establishing natural reserves to maintain genetic diversity and population viability may sustain this activity and preserve the threatened *Pilocarpus* species.

Conservation programs and management plans have been established for some species in the Carajás National Reserve (Pará, Brazil). More recently, a group involving the mining company Vale S. A. and institutions such as the environmental agencies IBAMA (Brazilian Institute of Environment and Renewable Natural Resources) and ICMBio (Chico Mendes Institute for Biodiversity Conservation) began promoting a series of conservation and social activities in this National Forest and surrounding communities. In regard to *P. microphyllus* activities, it includes (i) the establishment of a germplasm bank and rescue accesses, involving the reintroduction of thousands plants in areas designated for conservation and sustainable uses; (ii) a series of studies aiming to map populations inside the Carajás National Reserve, determine their growth dynamics, genetic diversity, and pilocarpine content and select genotypes for cultivation purposes considering efficient propagation protocols and nutritional requirements to maximize yield (pilocarpine and biomass); and (iii) helping collectors organize a cooperative (CoEx-Carajás) and perform sustainable management of *P. microphyllus* and other native species to generate income.

**Cultivation of *P. microphyllus***

Several approaches have been used in an attempt to improve the yield of crops and other plant species. In contrast to most common crops (maize, rice, wheat, etc.), for which environmental conditions favorable for growth and biomass production are translated into increased yield, improving the yield of medicinal plants is not necessarily linked to biomass accumulation. Most of the products extracted from these plants (secondary metabolites) often require some degree of stressful conditions to trigger and/or boost the biosynthetic process (Akula and Ravishankar, 2011; Avancini et al., 2003; Zhao et al., 2005). As an example, when *P. microphyllus* plant leaves were treated with methyl-jasmonate or salicylic acid, pilocarpine content increased up to 4-fold when compared to the control (null treatment) (Avancini et al., 2003). However, when the same *Pilocarpus* species is cultivated as a crop, with soil preparation, adequate nutrients and water supply via irrigation, the pilocarpine content of leaves can be reduced to approximately 0.5%, while we found a mean of 1.2% in the 91 accessions we have collected so far.

Techniques for cultivating *P. microphyllus* remain incomplete and have not yielded maximal productivity because of the few and fragmented studies conducted so far. A large part of these studies involved metabolite identification (Abreu et al., 2007; Santos and Moreno, 2004; Sawaya et al., 2011), taxonomy (Kaastra, 1982; Skorupa, 2003, 1996), and in vitro cell culture (Abreu et al., 2005; Sabá et al., 2002). Some studies provided information regarding propagation by seeds (Calil et al., 2008), micropropagation (Sabá et al., 2002) and potential relationships between low nutrients and pilocarpine (Avancini et al., 2003), but none have addressed genotype selection, nutrient requirements or culture management.

**Propagation of *P. microphyllus***

The propagation of *Pilocarpus* plants is commonly carried out by producing seedlings from recently collected seeds, allowing germination to reach up to 85% and 96% for *P. pennatifolius* (Calil et al., 2008) and *P. microphyllus* (Meneses et al., 2007), respectively. This method can be used because species of the *Pilocarpus* genus can produce a substantial number of viable seeds and because some species may have continuous flowering/fructification (Lameira et al., 2014; Skorupa, 2000). This is also common in some *P. microphyllus* accessions when they are growing in a germplasm bank, where these plants normally receive supplemental fertilization. However, a peak in fructification and seed dispersal have been observed for this species, occurring between May and July under natural conditions and for most of the accessions collected so far (Muniz, 2008; Rocha et al., 2011). Despite the abundance of seeds during this period, *Pilocarpus* seedling production may be laborious because of a significant reduction in the viability of stored seeds. It has been suggested that the seeds of *P. pennatifolius* have a longevity of less than one year (Calil et al., 2008). Our preliminary results showed similar trends with *P. microphyllus* seeds stored for one year in a seed chamber (low temperature and air humidity). We found significant seedling recovery after pericarp removal and almost null germination when the seeds were maintained within the pericarp even after gibberellic acid was added to the culture media (Supplemental Fig. 4). Overall, these results suggest that *Pilocarpus* propagation requires recently collected seeds and the establishment of germplasm banks as nurseries and not as seed banks.

Other techniques such as clonal propagation via stem cuttings or in vitro cell culture have not yet been applied to produce *Pilocarpus* seedlings, likely due to the limited information available or the high costs associated with them. In the only assay found using auxin (indole butyric acid, IBA) addition to promote root system development in shoot segments, few plants were recovered (Marques and Costa, 1994). In regard of explant development in media culture (Murashige and Skoog, 1962), apical explants were more efficient in shoot emission than nodal explants, having maximal growth when 6.66 mM of cytokinin (6-benzilaminopurine) was added to the media (Sabá et al., 2002). The nonexistent adapted protocols to produce a large number of selected genotypes are highly recommended to standardize high producing areas. However, this works against conservation programs, as high genetic variability is recommended to maintain viable populations.

**Field plantations**

Field planting is normally carried out at the beginning of the wet season with seedlings that are at least six months old (Marques and Costa, 1994). In the unique Jabonandí commercial plantation at Vegeflora’s farm, seedlings are currently planted into the field at high densities, reaching 50,000 plants per hectare. Part of this field receives supplemental irrigation and fertilization, where leaf harvesting can begin after 3 years. In these areas, it is possible to produce up to 3000 kg of dried leaves over 4 or 5 harvests throughout the year when these plants achieve maximal productivity (Costa, 2012). As there are no specific recommendations in regard to nutritional requirements for growing *Pilocarpus* plants, all technical management is based on information from cultivated plants and may not provide ideal conditions for vegetative growth and pilocarpine production.

Apart from the high biomass yield, the pilocarpine content found in cultivated plants (0.5–0.6%) is largely inferior to that obtained from plants in natural areas (mean of 1.2%), and this remains an unsolved puzzle. The few results obtained from *P. microphyllus* plants suggest that a well-balanced nutrient supply is essential for maintaining pilocarpine production (Avancini et al., 2003). They also suggest that potassium (K) omission could increase pilocarpine content by 10x in young plants (five months old), as it does to caffeine in coffee seedlings. On the other hand, it has also been suggested that soil iron availability (Fe) may positively affect the pilocarpine content (Furtini Neto, personal information). This is
particularly supported by recent (and current) observations of high pilocarpine concentrations in leaves of P. microphyllus accessions collected close to the iron ore mining areas in the Carajás National Reserve (data analysis in progress).

The application of phytohormones methyl-jasmonate and salicylic acid may be another possibility to increase pilocarpine biosynthesis. These molecules are usually associated with secondary metabolite accumulation in plant tissue, and Avancini et al. (2003) showed that pilocarpine production increased by 4× in P. microphyllus plants after methyl-jasmonate and salicylic acid application. Because the effects of the application of such phytohormones may vary considerably in dose-, time- and species-dependent ways (as they did in the [Avancini et al., 2003, study]), a series of investigations still needs to be performed aiming to determine an adapted protocol for P. microphyllus.

In vitro cell culture

The development of in vitro cell culture techniques devoted to producing specific plant compounds has received special attention because it offers the advantage of easy control of the environmental conditions. Techniques and protocols have been developed for a diversity of plant species, including Pilocarpus spp. A wide range of pilocarpine yields were observed when cells in a suspension culture (callus in a bioreactor) were fed with various concentrations of nutrients in the medium (nitrogen, phosphorus, potassium, histidine, and threonine) and in response to potential environmental stressors such as PEG (polyethylene glycol), NaCl and pH variations, as well (Abreu et al., 2005; Andreazza et al., 2009). Although there has been progress, pilocarpine production via in vitro cell culture remains economically univiable due to its high cost and low productivity. The limited information concerning the pilocarpine biosynthetic pathway remains one of the major difficulties in increasing its production.

Concluding remarks

Pilocarpus is a well-known plant genus, especially because of the medicinal importance of pilocarpine. As one of the most extracted and exported pharmaceutical constituents from Brazil, pilocarpine is an important source of income for many families depending on the extractivism of P. microphyllus leaves. Because of the absence of sustainable management for this natural resource, four (out of 17) of the Pilocarpus species (P. microphyllus included) were placed on Brazilian threatened plant species lists. Almost three decades later, limited progress has been made in terms of the continuous reduction of genetic diversity and population size of these species, causing the necessity for conservation programs.

Since the demand for natural pilocarpine remains high because there is no viable synthetic process and no efficient substitute active ingredient, sustainable management in natural areas and the cultivation of P. microphyllus are potential avenues for the conservation of this species. The establishment of a germplasm bank is one alternative that can be used in an effort toward preservation and as a source of genetic material for breeding. We suggest that conservation programs should pay attention to the development of adaptive management that will allow local families to cultivate this species. Such efforts will be expected to reduce the demand for the current unsustainable P. microphyllus harvesting in natural conditions. To do so, a sequence of multidisciplinary studies will be necessary, including those focused on genotype selection, propagation, the maximization of biomass and pilocarpine production, and the identification of nutritional requirements and environmental conditions that will improve pilocarpine yield.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.pecon.2017.08.002.

References


