Germination and soil seed bank traits of *Podocarpus angustifolius* (Podocarpaceae): an endemic tree species from Cuban rain forests

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Abstract: *Podocarpus angustifolius* is an endangered recalcitrant-seeded small tree, endemic to mountain rain forests in the central and Pinar del Río regions in Cuba. In this study, the germination patterns of *P. angustifolius* seeds were evaluated and the nature of the soil seed bank was determined. Using a weighted two-factor design, we analyzed the combined germination response to seed source (i.e. freshly matured seeds directly collected from trees *versus* seeds extracted from soil samples) and pretreatment (i.e. seed water-immersion for 48h at room temperature). Germination was delayed for four weeks ($\approx$30 days) in all cases, regardless of both factors analyzed. Moreover, nine additional days were necessary to achieve high germination values (in the case of fresh, pretreated seeds). These results overall may indicate the existence of a non-deep simple morphophysiological dormancy in *P. angustifolius* seeds. The water-immersion significantly enhanced seed germination, probably as a result of the hydration of recalcitrant seeds. Although germination of seeds extracted from soil samples was low, probably due to aging and pathogen effects throughout the time of burial, the study revealed the existence of a persistent soil seed bank (at least short-termed) of $\approx$42 viable seeds per m^2 in the upper 10cm of soil. Such a record is noteworthy since references to persistent soil seed banks in recalcitrant-seeded species are scarce in the literature. The population consequences derived from the formation of persistent soil seed banks in this endangered species are discussed. Rev. Biol. Trop. 59 (3): 1061-1069. Epub 2011 September 01.

Key words: endangered plant species, morphophysiological seed dormancy, persistent soil seed bank, pregerminative treatment, recalcitrant seeds.

Seed germination and seedling establishment are the most vulnerable phases in plants life cycles (Solbrig 1980), and information about them is especially important in understanding the distribution of rare plants. Schemske *et al*. (1994) stressed the importance of compiling information on the stages in the reproductive cycle of endemic, rare, and threatened plant species in order to enhance our understanding of their scarcity, and to design efficient conservation measures. So, basic information on dormancy and seed germination is very important for the development of conservation strategies of plant species (Ikeda & Itoh 2001, Navarro & Guitian 2003, Copete *et al*. 2005).

In the soil, water is the key resource triggering the physiological changes driving seed germination, but temperature and sometimes light/dark conditions also can be important (Nikolaeva 1977, Baskin & Baskin 1998). Many trees in tropical forests produce recalcitrant seeds with high water content (Chin *et al*. 1989), and if soil water conditions are appropriate, these seeds tend to germinate rapidly after being dispersed (Ng 1978). Typically, the

radicle emerges in a few days and germination is completed in a few weeks. Indeed, recalcitrant seeds which fail to germinate usually lose viability promptly (Whitmore 1989, Choinski 1990). However, seeds of some species are able to tolerate a certain degree of drought in the soil (i.e. semi-recalcitrant or minimally recalcitrant seeds). If water content of tissues is reduced, seeds can enter a quiescent stage until humidity in the soil recovers in the early rainy season (Vázquez *et al*. 1997).

Innate physiological factors and environmental conditions in the habitat determine the length that seeds remain viable in the soil prior to germination, die by physiological processes, or suffer attack from predators or pathogens (Schafer & Chilcote 1969, Simpson *et al*. 1989, Baskin & Baskin 1998). The soil seed bank is formed after viable seeds become buried in the soil and litter, or accumulate on the ground surface (Simpson *et al*. 1989). The number of seeds in the soil strongly depends on factors such as history, diversity, and the dynamics of the plant community covering the soil (Major & Pyott 1966, Brown & Oosterhuis 1981, Thompson 1992, Vázquez *et al*. 1997), and seeds are usually abundant in the soil under normal conditions (Simpson *et al*. 1989).

Soil seed banks of individual species vary significantly in length from transient to persistent types. In a transient seed bank, all seeds disappear from the soil before the next seed dispersal episode occurs, so there will be no seed reserve in the soil for a period of time. In the case of persistent seed banks, a significant fraction of seeds remains viable in the soil until the next seed dispersal event, so a plant population can always rely on a soil seed reserve (Thompson & Grime 1979, Thompson *et al*. 1997). Consequently, the ecological significance of soil seeds banks is transcendental since a persistent seed bank can contribute decisively to the resilience of plant populations (Baskin & Baskin 1978), even though no seeds are produced in a given year, and can even facilitate re-colonization after a local extinction (Milberg 1994). Obviously, such a point

becomes particularly important in the case of threatened plant species.

The goals of the present study were to determine the nature of the soil seed bank and germination responses of the endangered *Podocarpus angustifolius* Griseb., a recalcitrant-seeded tree endemic to mountain rain forests in the central and Pinar del Río regions in Cuba. We analyzed soil seed content and characterized key seed germination patterns. The general aim of the study was to enhance our knowledge of the reproductive traits of *P. angustifolius*, for which nearly no information is currently available, in order to contribute more effectively to its conservation.

MATERIAL AND METHODS

Study area and species: The study was carried out in the Ecological Reserve of Alturas de Banao, located in the Guamuhaya mountain range (Sancti Spiritu municipality, central Cuba). The 6 159ha reserve is managed by the State Department for Protection of Flora and Fauna in Banao. The Reserve has a floral richness of over 1 200 species, of which a fifth is endemic or belongs to a rare, threatened or endangered category. Vegetation forms highly contrasting communities in the reserve: semi-deciduous forests forming vegetation complexes on "mogotes" (hills of limestone with rounded, tower-like structure) and rocky outcrops; evergreen forests and herbaceous vegetation on rocks and fissures; and mountain rain forests, where *P. angustifolius* is found. Mountain rain forests in Cuba range from 800 to 1 800m altitude, where the annual rainfall varies from 1 700 to 3 000mm (Borhidi 1996). The lowest temperatures in the reserve are reached in January and February, when values range from 20.7° to 21.3°C. During July and August, temperatures reach the highest values around 25°C. The annual average air humidity is 82%, wind velocity 5.5km/h and the photoperiod 7.5 h/day (CITMA 2003). Moderately deep, mountain ferralitic soils are dominant in the area, accompanied by karstic outcrops. In the mountain rain forest, a dense leaf-dominated

litter commonly accumulates on the ground. The litter retains water and, in general, avoids highly variable humidity changes in the upper centimeters of the soil.

Vegetation is structured into two well-differentiated tree layers from 20-25m and 8-19m height, supporting a rich community of epiphytes. Arborescent ferns are also abundant. The highest tree layer is dominated by micro- and notofilous-leaf perennial species such as *Magnolia cubensis* Urb., *Laplacea angustifolia* Britt. et Wils., *Laplacea wrightii Griseb, Cyrilla racemiflora* L., *Ocotea cuneata* (Griseb.) Urb., *Ocotea floribunda* (Sw.) Mez, *Ocotea leucoxylon* (Sw.) Mez and *Myrsine coriacea* (Sw.) R. Br. ex Roem. & Schult. In the lowest tree layer, tree species such as *Clusia tetrastigma* Vesque*, Gomidesia lindeniana* Berg., *Garrya fadyenii* Hook*, Miconia punctata* (Desv) D. Don*, Alchornea latifolia Sw., Matayba domingensis* (DC) Radlf., and an arborescent fern, *Cyathea arborea* Smith., are dominant. Hygrophilous orchids and Bromeliaceae plants mainly form the epiphyte community, and ferns are of secondary importance. In the understory, terrestrial orchids and Melastomataceae shrubs are abundant (Borhidi 1996).

P. angustifolius belongs to the botanical family of Podocarpaceae (Coniferopsida). It is a small tree which reaches 8m in height and 30cm of normal trunk diameter. Leaves are lineal-lanceolate, 4-5cm wide. Microstrobili are arranged in clusters, whereas the ovules are isolated. The fruit has a red fleshy peduncle holding one 5-6mm diameter elliptic seed. Seeds are recalcitrant, a trait which is well represented in Podocarpaceae (Fountain *et al*. 1989, Schaefer 1990). Fruit ripening occurs from January to March and seed dispersal occurs around the middle to end of April. *P. angustifolius* is endemic to mountains in the Escambray and Pinar del Río regions. Populations have undergone a recent decline due to the abusive use of *P. angustifolius* wood for the production of ornamental elements and hardwood devices. Recently, *P. angustifolius* has been classified as is in danger of extinction by the IUCN (2009), so national Cuban law has protected it.

Plant nomenclature: Liogier (1962)

Soil seed bank: We selected a stand in a well conserved mountain forest in the Ecological Reserve of Alturas de Banao containing 16 *P. angustifolius* adult individuals with apparently healthy phenotype. Distance between *P. angustifolius* tree crowns ranged from 2m to 50cm, so branches from neighboring trees were interlaced in some cases. Eight 30cm x 30cm plots were placed at random in the stand. In early April 2007, just a few days before the beginning of *P. angustifolius* seed dispersal, plots were excavated at 10cm depth with a small garden spade and collected individually in labeled polyethylene bags, forming each one a soil sample.

In the laboratory, healthy *P. angustifolius* seeds (intact seed coat, dark color similar to that when ripened, and resistance to slight pressure with tweezers) were extracted by washing soil samples on a 0.5mm-mesh sieve. When extracted, four 25-seed lots were prepared at random. Seeds of each lot were placed on a distilled-water saturated filter paper sheet in a white plastic rectangular tray (21.2cm x 12.2cm) and incubated in the laboratory over 2 months at 20-25ºC, which closely resembled temperature conditions in the natural habitat. Filter paper was kept wet by periodic watering.

Checks for germination started five days after the first watering. Germinated seeds were counted and removed. Seeds were considered as germinated when cotyledons emerged. The presence of fungi on seeds was also registered.

Seeds directly collected from trees: During April 2007, freshly ripened seeds were collected directly from trees, using a ladder when necessary. Seeds from different trees where combined forming a unique seed lot. After storage for two days in the laboratory, seeds were incubated following an identical protocol to that described for seeds extracted from soil samples.

Rev. Biol. Trop. (Int. J. Trop. Biol. ISSN-0034-7744) Vol. 59 (3): 1061-1069, September 2011

1063

Pretreatments of seeds: One hundred seeds selected at random from both samples extracted from the soil and those directly collected from trees were submerged for 48h in distilled water at room temperature (20-25ºC). Immediately, they were incubated under identical conditions as described above.

To analyze the germination response of *P. angustifolius* seeds, a balanced factorial design including two factors was used. Factors and levels were as follows. Seed source, with two levels: (i) seeds extracted from soil samples and (ii) freshly ripened seeds directly collected from trees. Pretreatment: (i) seed water immersion for 48h and (ii) seeds non-submerged.

The effects of both factors were evaluated on two dependent variables: (i) final cumulative germination and (ii) rate of germination. The latter was computed by the formula proposed by Hartmann & Kester (1966):

$$VC = \frac{SG}{A_1 T_1 + A_2 T_2 + A_n T_n} * 100$$

where VC is the coefficient of germination rate; SG is the number of seeds germinated; A is the number of germinating seeds between two consecutive times; and T is the time elapsed between the beginning of the germination and the interval determined as the mean.

Two-way ANOVAs were conducted with the statistical package SPSS v. 17 (2008). Prior to analysis, the value of germination (in percentage) was square-root arcsine transformed in order to improve the fit of data to the normal distribution. Significance of interactions was explored by contrasting confidence intervals.

Data of cumulative seed germination were fitted to logistic-model curves with the program Curve Expert 1.3.

RESULTS

Germination response of *P. angustifolius* seeds: Germination began about 4 weeks after incubation, both in seeds extracted from soil samples and those collected from trees, regardless of the pretreatment (Fig. 1). Final
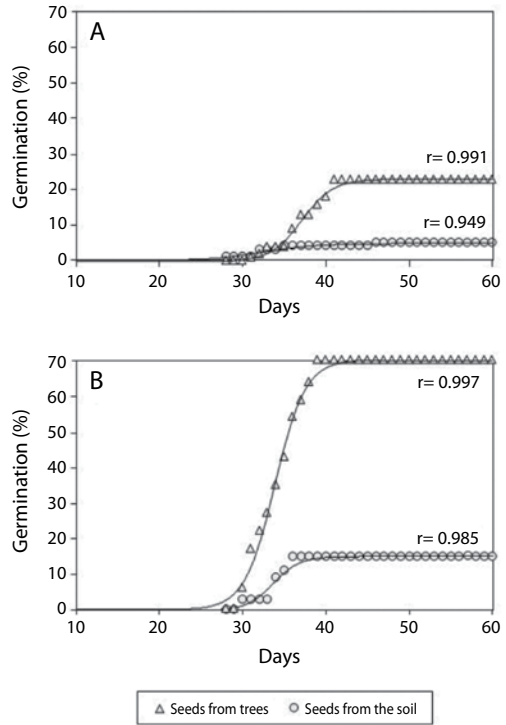

Fig. 1. Cumulative germination without (A) and with (B) pretreatment (seed water-immersion for 48h) of freshly matured seeds collected directly from trees (triangles) and seeds extracted from soil samples (circles). The logistic curve adjusted (r= correlation coefficient) is shown for each case.

cumulative germination was significantly affected by the two factors analyzed and their interaction (Table 1). Indeed, germination was higher in seeds collected from trees than in those from soil. Differences increased with the water-immersion seed pretreatment (Fig. 1). So, the lowest germination percentage was recorded in seeds extracted from the soil samples that were not pretreated (5%; Fig. 1A), whereas the highest value was achieved by pretreated freshly matured seeds collected from trees (70%; Fig. 1B). In addition, seeds coming from the soil, which were not exposed to water-immersion, suffered the attack of fungus *Rhizopus stolonifer* Ehrenb. (Ex Fr.) Lind, at the beginning of day 12 of incubation.

TABLE 1

Two-way ANOVA for final cumulative germination and germination rate

Final cumulative germination	df[a]	F[b]	p[c]
Source[d] (S)	1	366.19	<0.001
Pretreatment[e] (PT)	1	224.62	<0.001
S x PT	1	60.30	<0.001
Germination rate			
Source (S)	1	0.11	0.744
Pretreatment (PT)	1	22.11	<0.001
S x PT	1	0.08	0.786

[a] Degrees of freedom; [b] F ratio value; [c] Associated probability; [d] Source: source of seeds (directly collected from trees *versus* retrieved from soil samples); [e] Pretreatment: treatment of seed water-immersion for 48h prior to seed incubation (*versus* no immersion pretreatment). Interaction is also shown.

Germination rate was significantly affected by the pretreatment. Seed water-immersion accelerated germination. In contrast, seed source (soil *versus* tree) was irrelevant for germination rate, as no significant interaction was detected (Table 1). Maximum cumulative germination (i.e., asymptote of the logistic-model curve) without (Fig. 1A) and with (Fig. 1B) the germination pretreatment was achieved after 40 and 36-38 days, respectively.

Soil seed bank: The mean number of *P. angustifolius* seeds with healthy appearance that were extracted from soil samples was 25.50 (SE=1.67), corresponding to a density (number of seeds per surface unit at a given depth) of c.a. 283 seeds/m². If such a value is corrected by the maximum percentage of germination recorded in the incubation of seeds extracted from the soil (15% final cumulative germination in seeds pretreated; Fig. 1B), then we can estimate a viable seed reserve of c.a. 42 seeds/m² in the upper 10cm of soil.

DISCUSSION

Several findings in the present study strongly suggest that *P. angustifolius* seeds have non-deep, simple morphophysiological dormancy, according to the classification proposed by Baskin & Baskin (1998). Germination was delayed four weeks in all cases, regardless of seed source (soil seed bank versus direct seed collection from trees) and the pretreatment (water immersion versus non-immersion). Fors (1965) previously detected the same length of delay in seed germination of this species. Such a delay would be the result of the presence of a lineal underdeveloped embryo in *P. angustifolius* seeds (Osorio & Bonilla pers. observ.), a seed trait broadly found among members of the Podocarpaceae family (Baskin & Baskin 1998). This rudimentary embryo may need a period of 4 weeks to complete growth and reach the degree of maturation enabling it to germinate (Nikolaeva 1969, Baskin & Baskin 1998). Moreover, the fact that seeds need nine additional days to achieve a high value of germination after it is started may suggest the existence of an additional physiological dormancy mechanism in *P. angustifolius* (Baskin & Baskin 1998). Our findings are in accordance with those made by Ng (1978) and Ng & Asri (1979), who described the existence of morphological and morphophysiological dormancy in seeds of other *Podocarpus* species inhabiting tropical forests in Malaysia.

A noteworthy result of the study was the fact that seeds extracted from the soil immediately before the next seed dispersal event showed dormancy. Such a finding raises the following question: why did embryos of seeds at least one year old not complete their growth yet? The answer should be examined

Rev. Biol. Trop. (Int. J. Trop. Biol. ISSN-0034-7744) Vol. 59 (3): 1061-1069, September 2011

1065

in the potential factor triggering the embryonic development and thus responsible for overcoming morphological dormancy. The only marked environmental difference to what seeds were exposed in the laboratory with regard to the natural habitat concerned light conditions. Actually, whereas temperature and substratum humidity did not markedly change between the soil in the natural habitat and the germination tests in the laboratory, light conditions were completely different. In the natural habitat, *P. angustifolius* seeds should be in an intensely shaded environment due to the dense canopy cover, whereas in the laboratory, seeds were directly exposed to day light during germination tests. In the literature, there are antecedents of direct exposure to light as a promoting factor of embryo growth and the subsequent break of morphophysiological dormancy (Jacobsen & Pressman 1979, Baskin & Baskin 1990). If this explanation is correct, this may be an efficient adaptive mechanism to detect small gaps in the canopy where seeds can germinate offering real possibilities of survival to young seedlings. Such an interpretation is compatible with field records confirming the low recruitment of *P. angustifolius* under plant cover when it is highly dense (Bonilla pers. observ.).

Other notable results in the study were the highly significant promotion of final cumulative germination and the increase of germination rate when seeds were submitted to a water-immersion pretreatment for 48h, particularly in the case of seeds collected from trees. Fors (1965) found a similar germination response. In some recalcitrant seeds, it has been demonstrated that the embryo requires a high level of hydration to germinate, a level superior to that in embryo tissues at the moment of seed dispersal (Tompsett 1985). In that case, abundant rainfall in the habitat is necessary to increase the germination response (Baskin & Baskin 1998). Similar germination patterns have been registered in other recalcitrant-seeded species (Matias *et al*. 1973). It could be possible that the water-immersion of seeds prior to germination tests, conducted to quickly surpass

the critical level of hydration, accelerated germination once dormancy was lost.

In spite of the water-immersion pretreatment, seeds extracted from soil samples showed a much lower final cumulative germination than that achieved by freshly matured seeds collected from trees (15% versus 70%). Such a difference could be explained by the loss of viability of the former since they had been in the soil for at least one year. The factors involved in this decay of seed viability would be the attack of pathogens (indeed, a proliferation of fungus was detected during germination tests on seeds extracted from the soil and not washed by water immersion) and/or the death of the embryo by aging. However, it was surprising that *P. angustifolius*, even although recalcitrant-seeded, had a non-negligible amount of viable seeds in the soil (c.a. $42/m^2$) in early April, just before the next seed yield. This finding implies that *P. angustifolius* is able to form persistent seed banks, at least over the short-term (i.e. a fraction of seeds remain viable in the soil between two consecutive seed dispersal episodes; *sensu* Thompson & Grime 1979 and Thompson *et al*. 1997). Studies detecting persistent seed banks in recalcitrant-seeded species are scarce in the literature (Ng 1988, Baskin & Baskin 1998). Certainly, two general traits characterize recalcitrant seeds: fast germination and short periods of viability (Ng 1978, Whitmore 1989, Choinski 1990). Accordingly, it is generally assumed that recalcitrant seeds do not live for long periods in the soil, although research under natural conditions on this point is scarce (Baskin & Baskin 1998). The notable observation that *P. angustifolius* has the ability to form persistent seed banks may be explained if their seeds are really semi-recalcitrant or minimally recalcitrant, as demonstrated for seeds of *Podocarpus henkelii* Stapf ex Dallim. & A.B.Jacks. (Farrant *et al*. 1983); semi-recalcitrant conifer seeds which tolerate a certain degree of desiccation. Such a seed trait, along with the abundance of rainfall in the natural habitat and the preservation of soil humidity because of the dense canopy cover and the well-developed

litter layer (Bonilla pers. observ.), may allow some seeds to be hydrated over the critical level of viability for the whole year.

Consequences of the formation of persistent soil seed banks on the plant population dynamics are important, particularly in the case of threatened species, such as *P. angustifolius*. A persistent seed bank can be crucial in ensuring the conservation of a species, since it allows the re-establishment and maintenance of plant populations after disturbances, although no external source of propagules occurs (Baskin & Baskin 1978). In fact, Thompson *et al.* (1997) suggested that short-term persistent soil seed banks play an essential role in the viability of populations, as Martínez-Duro *et al.* (2010) empirically demonstrated for a critically endangered perennial plant species.

In conclusion, *P. angustifolius* seems to have seeds with morphophysiological dormancy, which may be overcome when they are directly exposed to light. This may be interpreted as an adaptive strategy to detect small gaps in the canopy where seedlings would have a higher chance of survival and establishment. The promotion of germination by water immersion of seeds induces us to consider a stimulating effect by embryo hydration. In spite of having recalcitrant seeds, *P. angustifolius* is able to form persistent seed banks in the soil, probably short-termed but essential for population viability of this endangered plant species.

RESUMEN

Podocarpus angustifolius es un árbol endémico de los bosques lluviosos de la región de Pinar del Río y la parte central de Cuba, que se encuentra en peligro de extinción. En este estudio se evaluó la germinación de sus semillas y la naturaleza del banco de semillas del suelo. Específicamente, se analizó la respuesta germinativa de las semillas a dos factores: su procedencia (recolectadas directamente de los árboles *versus* extraídas de muestras de suelo) y el pretratamiento (inmersión en agua durante 48h a temperatura ambiente). La germinación no comenzó hasta las cuatro semanas (≈30 días) en todos los casos. Además, fueron necesarios 9 días más para alcanzar un valor elevado de germinación (en el caso de semillas recolectadas del árbol con pretratamiento), lo que parece indicar la existencia de un letargo de tipo morfofisiológico simple no profundo. El pretratamiento incrementó significativamente la germinación, posiblemente por hidratación de las semillas recalcitrantes. El estudio demostró la existencia de un banco de semillas persistente en el suelo, al menos de corta duración de ≈42 semillas viables por m^2 en los 10 primeros cm del suelo. Dicho hallazgo es destacable, ya que los casos descritos en la literatura sobre bancos persistentes en especies con semillas recalcitrantes son escasos. Además, se discute la importancia que tiene para esta especie amenazada la existencia de un banco de semillas persistente.

Palabras clave: planta en peligro de extinción, letargo morfofisiológico, banco de semillas del suelo persistente, tratamiento pregerminativo, semillas recalcitrantes.

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Rev. Biol. Trop. (Int. J. Trop. Biol. ISSN-0034-7744) Vol. 59 (3): 1061-1069, September 2011

1067

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1069