

Effect of seed storage on germination, seedling growth and survival of *Mimosa foliolosa* (Fabaceae): implications for seed banks and restoration ecology

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Abstract: Our goal was to examine the effects of seed storage on seed germination, seedling growth and survival of *Mimosa foliolosa* (Fabaceae). Fresh and stored seeds were set to germinate under optimum conditions after 12, 24 and 36 months of artificial storage. Fresh and 12 month old seeds were sown under greenhouse conditions and seedling growth and survival between the two types of seedlings were compared for a period of seven months. The germinability of stored and scarified seeds was not affected by storage, suggesting high longevity. However, seed storage did reduce germination time indicating alleviation of seed dormancy with time. There were no significant differences in seedling survival or relative growth rates for seedling height and number of leaves. We discuss the implications of seed storage behaviour for seed bank formation under natural conditions and for restoration ecology of rupestrian grasslands using *M. foliolosa* seeds.

Resumen: Nuestro objetivo fue examinar los efectos del almacenamiento de semillas en la germinación de semillas, y en el crecimiento y la supervivencia de plántulas de *Mimosa foliolosa* (Fabaceae). Se pusieron a germinar semillas frescas y almacenadas en condiciones óptimas después de 12, 24 y 36 meses de almacenamiento artificial. Las semillas recién producidas y las que tenían 12 meses de edad fueron sembradas en invernadero, y se compararon el crecimiento y la supervivencia de ambos tipos de plántulas por un período de seis meses. La aptitud para germinar de las semillas almacenadas y escarificadas no se vio afectada por el almacenamiento, lo que sugiere una longevidad larga. Sin embargo, el almacenamiento de semillas redujo el tiempo de germinación, lo que indica una disminución de la dormición de las semillas con el tiempo. No hubo diferencias significativas en la tasa de supervivencia de las plántulas y las tasas de crecimiento relativo en altura de las plántulas y número de hojas. Discutimos las implicaciones del comportamiento del almacenamiento de semillas para la formación de bancos de semillas en condiciones naturales y para la ecología de la restauración de los campos rupestres por medio del uso de semillas de *M. foliolosa*.

Resumo: O nosso objetivo foi examinar os efeitos do armazenamento das sementes na germinação, no crescimento e na sobrevivência das plântulas de *Mimosa foliolosa* (Fabaceae). As sementes frescas e armazenadas foram colocadas para germinar em condições óptimas após 12, 24 e 36 meses de armazenamento artificial. Sementes frescas, e sementes com 12 meses de idade, foram semeadas em estufa e o crescimento e sobrevivência das plântulas entre os dois

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tipos de plântulas foram campos por um período de seis meses. A germinação das sementes armazenadas e escarificadas não foi afetada pelo armazenamento, sugerindo alta longevidade. No entanto, o armazenamento das sementes fez reduzir o tempo de germinação, indicando a redução da dormência das sementes com o tempo. Não houve diferenças significativas na sobrevivência das plântulas ou taxas relativas de crescimento para a altura da planta eo número de folhas. Discutimos as implicações do comportamento das sementes ao armazenamento para o estabelecimento de bancos de sementes sob condições naturais e para a ecologia de restauração de prados rupestres, utilizando sementes de *M. foliolosa*.

Key words: Seed dormancy, seedling survival, seed storage, restoration ecology, rupestrian grasslands.

Introduction

Seed dormancy and germination are controlled by complex interactions between environmental and physiological factors. Viable seeds not experiencing the set of environmental conditions suitable for seedling establishment may deteriorate and lose their ability to germinate (Walters 1998), or may remain in the soil until conditions for establishment become suitable (Thompson *et al.* 2003). The soil seed banks consist of a group of seeds that persist as viable or dormant seeds in the soil or soil surface (Leck *et al.* 1989) playing an important role in natural regeneration, population dynamics and maintaining genetic variability of plant populations (Murdoch & Ellis 2000).

One strategy to assess the potential of soil seed bank formation is to store seeds under controlled conditions and periodically submit seed batches to germination tests. Although controlled laboratory conditions differ from those found under field conditions (Nagel & Börner 2010), artificial seed storage can provide useful information for seed technology and improve our ability to identify suitable species for restoration ecology and germplasm banks. For a number of reasons, the immediate use of fresh seeds in restoration practices may not be feasible; and thus artificial storage of seeds may be necessary. However, seeds of many species lose viability after short periods of storage, eventually dying (Mohamed-Yasseen *et al.* 1994; Walters 1998). Thus, it is of practical interest to determine how storage conditions affect germination and seedling growth in an attempt to maximize sapling production for ecological restoration and germplasm conservation (Berjak & Pammenter 2008; Mohamed-Yasseen *et al.* 1994).

The role of seed banks in regeneration ecology is especially important in the seasonal fire-prone

shrubby altitudinal grasslands such as the threatened *campos rupestres* (rupestrian montane grasslands) in eastern Brazil. The rupestrian grasslands harbor nearly 4000 species, many of which are endemic and threatened by extinction (Echternacht *et al.* 2011; Giuliatti *et al.* 1997). The increasing human pressure on the *campos rupestres* includes mining, intentional fires, environmentally damaging tourism and non-planned soil occupation (Giuliatti *et al.* 1997; Jacobi *et al.* 2007). This ecosystem has a very low resilience (Le Stradic *et al.* 2014), but we lack an ecological framework to ground restoration ecology of this low-productive habitat. Because few studies have investigated the seed bank ecology of the *campos rupestres*, we do not fully understand the natural process of regeneration after disturbances in this ecosystem. Knowledge on seed and seedling biology provides relevant data to improve conservation of endemic species (Fernández Pascual *et al.* 2012) and restoration practices (Negreiros *et al.* 2009; Ranieri *et al.* 2012b) because more reliable seedling establishment could improve outcome of restoration projects and increase cost-effectiveness (Wagner *et al.* 2011). Here, the effects of seed storage on seed germination, seedling growth and survival of *Mimosa foliolosa* Benth. subsp. *pachycarpa* (Benth.) Barneby (hereafter referred to as *M. foliolosa*; Fabaceae) were evaluated. *Mimosa foliolosa* was recently recommended for ecological restoration due to its high relative growth rate and soil cover in unfertile soils (Negreiros *et al.* 2009).

Materials and methods

Study site and species

Mimosa foliolosa is a shrub commonly found in the sandy soils of rupestrian grasslands and on the

edges of sandstone outcrops of Serra do Cipó between 1100 and 1300 m a.s.l. (Barneby 1991). The presence of a lignotuber facilitates seedling establishment and survival and functions as a reservoir organ allowing resprouting after fire events (Rizzini 1963). Seeds of *M. foliolosa* are dispersed during the dry season and are physically dormant (Silveira & Fernandes 2006). Seeds were collected from Serra do Cipó (19° 17' S and 43° 33' W), in the southern portion of the Espinhaço Range, southeastern Brazil. The main vegetation above 900 m in the Espinhaço range is rupestrian grassland established on quartzitic-derived, shallow, acidic, nutrient-poor, and excessively drained soils. The climate is characterized by dry winters and rainy summers with an average annual rainfall of 1350 mm (Madeira & Fernandes 1999).

Seed storage and germination

Seeds were collected from 40 individuals in the same population. Fresh apparently viable seeds (collected in April 2000) were sterilized with a 1 % sodium hypochlorite solution for 5 minutes and then washed with running water for 10 minutes. Non-scarified seeds (control group) were placed in Petri dishes (4 replicates of 25 seeds each) layered with double sheet of filter paper and moistened with nistatin solution whenever necessary to avoid fungal growth. The Petri dishes were incubated at constant temperature of 25 °C and were exposed to a 12-hour photoperiod (Silveira & Fernandes 2006). Seeds were considered as germinated when the radicle protrusion was observed.

Fresh apparently viable seeds were stored in hermetically closed glass pots at room temperature ranging between 18 and 25 °C. Seeds were stored for 12, 24 and 36 months and at each interval, seed batches were set to germinate under optimum conditions (Silveira & Fernandes 2006). As seeds are physically dormant, they were mechanically scarified through the friction of the external tegument against sandpaper (Silveira & Fernandes 2006). For all treatments, mean germination time (MGT) was calculated following Ranal & Santana (2006):

$$MGT = \frac{\sum_{i=1}^k n_i t_i}{\sum_{i=1}^k n_i}$$

where, n_i is the number of seeds germinated at the time i , t_i is the time from the start of the experiment to the i^{th} observation, and k is the time of last germination.

The seed germination experimental design was

completely randomized. The values in percentage were transformed into arcsin to obtain data normalization. ANOVA was used to compare the means of seed germination among treatments followed by the Tukey's test ($\alpha = 0.05$) (Zar 1996).

Seedling growth and survival

To evaluate the effect of seed storage on seedling growth and survival, scarified fresh and stored seeds of *M. foliolosa* were directly sown in 3 l dark polyethylene containers under greenhouse conditions in Serra do Cipó. In total, 63 fresh seeds and 63 1-year stored seeds were sown directly on a 1:1:1 horticultural substrate composed of soil, charcoal powder and sphagnum moss mixture (Ranieri *et al.* 2004) in July 2001. All seeds were sown at the same depth (1 cm) and at the same spatial orientation (radicles oriented downwards). The containers were arranged in a completely randomized design and were irrigated through 10-min aspersion three times a day.

To evaluate plant growth rate, non-destructive measures were made every month after sowing to calculate the monthly relative growth rate (RGR). Primary shoot length and leaf number of each individual were used to calculate RGR following Hunt (1982):

$$\text{RGR for shoot length} = (\ln L_{i+1} - \ln L_i) / (t_{i+1} - t_i)$$

$$\text{RGR for number of leaves} = (\ln N_{i+1} - \ln N_i) / (t_{i+1} - t_i)$$

where, L is shoot length, N is total number of leaves, and t is time. RGR data did not fit a normal distribution even after transformations. In addition, variations of the residuals were not homogeneous for the groups studied (species and treatments). Hence, the data were analyzed by the non-parametric test of Mann-Whitney to compare RGR between treatments in each interval.

The surviving seedlings were counted, and primary shoot length (until last node), the number of leaves, lateral shoots, inflorescences, and remaining cotyledons were recorded monthly throughout a 7-month study interval under greenhouse conditions (see details in Negreiros *et al.* 2009). This interval corresponds to the establishment season at the rupestrian grasslands (Silveira *et al.* 2012).

To test whether storage potentially affect seedling survival, we used survival analysis to compare total survival and mortality rates between the two storage treatments (proportional hazards, JMP 10.0, SAS Institute Inc 2012). Survival analysis is used to estimate a non-parametric model (proportional hazards) by maximum likelihood of exact

failure times of the censored survival data (Ranieri *et al.* 2012a). The censored seedlings offer the information of survival rate because they all have the same value of time (the maximum possible at the end of the experiment). This analysis can examine how survival is influenced by categorical covariates (in this case seed storage treatment; Lawless 1982). The proportional hazards method computes a coefficient for each predictor that indicates the direction and degree of flexing that it has on a baseline survival curve (Lawless 1982). Such an analysis is analogous to a regression model, but allows the use of seedlings that have never died (which would violate the assumptions of typical regression models) to be included in the analysis.

Results

Seed storage and germination

We found a significant effect of storage and scarification on both germinability and MGT of *M. foliolosa* seeds (Table 1). The germinability of stored and scarified seeds was not affected by storage, but storage did reduce MGT (Fig. 1B). Storage increased germination percentage by nearly 8-fold and reduced germination time of non-scarified seeds by half (Fig. 1B). Despite significant scarification \times storage interactions (Table 1), storage favored seed germination.

Seedling growth and survival

All seedlings, both from fresh and stored seeds, kept their cotyledons after 1-month period. However, after 2 months, seedlings from fresh seeds lost their cotyledons more than seedlings from stored seeds ($U = 911$, $P < 0.001$). After three months, the presence of cotyledons was not observed in any seedling. Lateral shoots were observed only in two individuals after 6 months. After seven months, a single inflorescence was observed.

Overall, there were no significant differences in RGR for seedling height and leaf number (Fig. 2). In both treatments, seedlings showed a fast growth in the first month and a strong reduction in RGR in the following month. After high RGR values in the third month, height RGR decreased and remained constant for both treatments (Fig. 2A). For RGR in number of leaves, seedlings from fresh seeds initially produced slightly more leaves compared to seedlings from stored seeds. However, differences in RGR for number of leaves between

treatments disappeared in the following intervals (Fig. 2B). At the end of the experiment, seedlings from stored and fresh seeds measured 16.13 ± 0.7 (SE) and 15.72 ± 0.69 cm, and produced on average 12.7 and 13.6 leaves, respectively (Fig. 3).

Seedling survival was higher than 80 % for seedlings emerging from both fresh and stored seeds. For both treatments, seedling mortality was higher at the first month and remained relatively constant through time. Total seedling survival and mortality rates did not differ between treatments (proportional hazards, lowest log likelihood $c2 = 1551$, d.f.= 1, $P = 0.213$; Fig. 4). At the end of the experiment, 56 and 51 individuals from fresh and stored seeds survived, respectively.

Discussion

This study provides strong evidence that seed storage for short periods does not reduce seed germination, seedling growth and survival of *M. foliolosa*. Seed storage period had minor effects on seed germination and in seed reserve mobilization (indicated by different cotyledon abscission times). However, these changes did not translate into subsequent life-stage differences, i.e. the early seedling growth phase. Overall, seedlings from stored seeds showed similar growth parameters during the first growing season as fresh seeds, with seed storage period having minor effects on seedling survival.

The seeds of *M. foliolosa* were able to maintain viability for at least three years under artificial conditions. The seed longevity of this species may be attributed to the hard, water-impermeable seed coat. The seed coat is the seed's primary defense against adverse environmental conditions providing physical and chemical barriers to unfavourable conditions and protecting the embryo against infection and deterioration caused by microorganisms (Baskin & Baskin 2000; Mohamed Yasseen *et al.* 1994). Increasing storage periods of *M. foliolosa* seeds resulted in increased germination percentages and decreased germination time for non-scarified seeds. These data suggest alleviation of seed dormancy with storage time, which is likely to result from seed coat softening under artificial conditions. However, one must be careful to extrapolate this hypothesis under field conditions to make inference on seed bank dynamics (see Baskin & Baskin 2000).

There is growing evidence that many small-seeded species from the *campos rupestres* are able to form seed banks through photoblatism and/or

Table 1. Results of the two-way ANOVA for the effects of storage time and scarification on germinability and mean germination time (MGT) of *Mimosa foliolosa* seeds from the rupestrian grasslands.

Source of Variation	DF	SS	MS	F
Germinability (%)				
Storage	3	1094.6	364.9	14.70
Scarification	1	22016.7	22016.7	887.1
Storage × Scarification	3	2006.0	668.7	26.9
Residual	24	595.7	24.8	
MGT (days)				
Storage	3	435.9	145.3	63.88
Scarification	1	642.3	642.3	282.34
Storage × Scarification	3	254.9	84.9	37.35
Residual	22	50.1	2.3	

All F values are significant at $P < 0.001$.

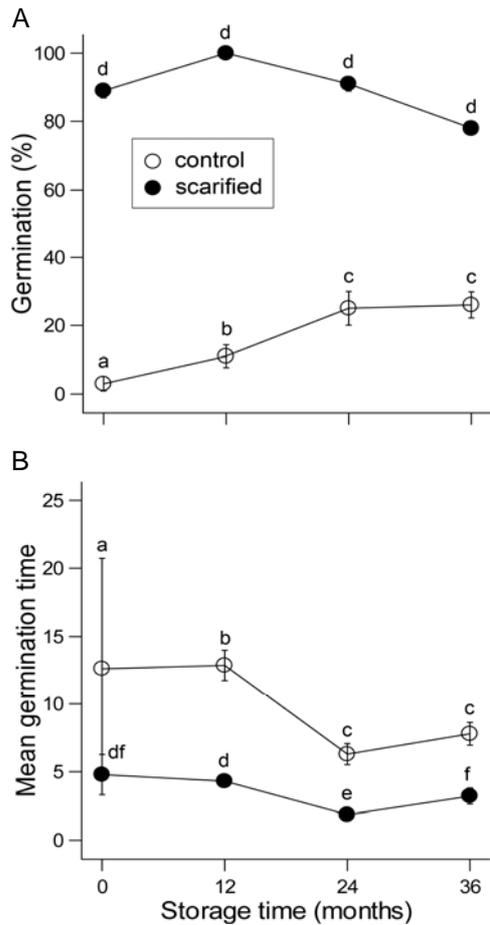


Fig. 1. Germinability (A) and mean germination time (B) of fresh and stored seeds of *Mimosa foliolosa*. Bars indicate SE.

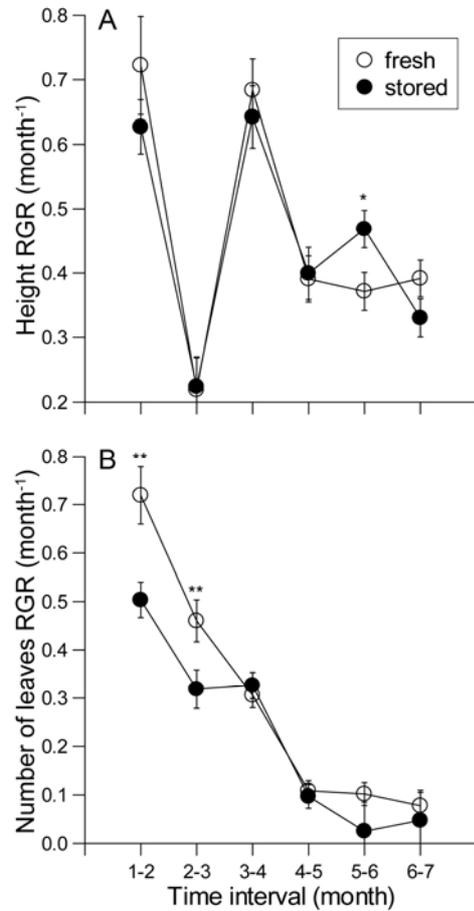


Fig. 2. Monthly relative growth rate (RGR) for primary shoot length (A) and number of leaves (B) in *Mimosa foliolosa* seedlings emerging from fresh and stored seeds. *indicates significant differences between treatments ($\alpha = 0.05$), ** indicates significant differences between treatments ($\alpha = 0.01$) and error bars indicate SE.

physiological dormancy (Cheib & Garcia 2012; Garcia *et al.* 2012; Medina & Fernandes 2007; Munné-Bosch *et al.* 2011; Silveira *et al.* 2012). Our study suggests that physically dormant seeds of *M. foliolosa* are also able to form persistent seed banks. Although seed dormancy is not strictly necessary for incorporation into soil seed banks (Thompson *et al.* 2003), physical dormancy plays a major role in controlling seed bank dynamics in Fabaceae. Daily fluctuating temperatures and fires account for breaking physical dormancy in tropical legumes (Moreno-Casasola *et al.* 1994). The dormant seeds of *M. foliolosa* are dispersed in the middle of the dry season, when conditions for establishment are unsuitable and fires are common (Silveira & Fernandes 2006). *M. foliolosa* seeds may remain for 2-3 months in the soil during

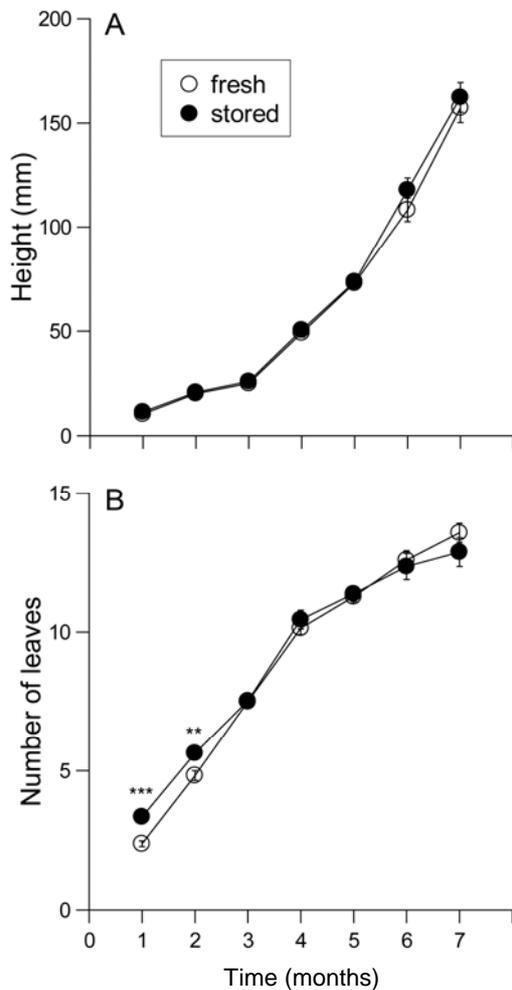


Fig. 3. Total seedling height and number of leaves of *Mimosa foliolosa* seedlings emerging from fresh and stored seeds. **indicates significant differences between treatments ($\alpha=0.01$), ***indicates significant differences between treatments ($\alpha=0.001$) and error bars indicate SE.

the rainy season, so fire and high fluctuating temperatures at the end of the dry season are expected to act as environmental signals (Baskin & Baskin 2000; Moreno-Casasola *et al.* 1994). We therefore, argue that germination synchronized at the onset of the rainy season, coinciding with the optimum conditions for establishment at the seasonal rupestrian grasslands (Silveira *et al.* 2012), positively affect plant fitness.

Because our experiment was conducted under controlled conditions of temperature rather, than under the natural environment, dormancy alleviation with seed storage period seems to be underestimated for *M. foliolosa* seeds. Regardless of that, seeds stored in the soil for different periods, have different germination probabilities.

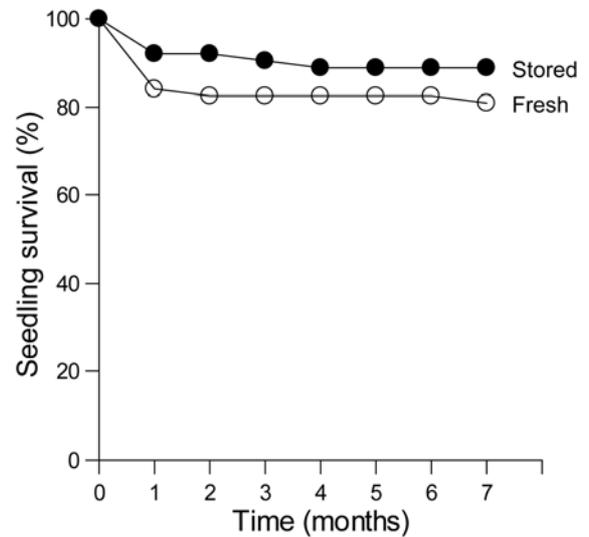


Fig. 4. Relative survival of *Mimosa foliolosa* seedlings emerging from fresh and stored seeds.

How population dynamics is to be affected by increasing recruitment from seed as dormancy is alleviated is still to be investigated. Following germination from seed banks, the seedlings would have increased likelihood of establishment as they tolerate poor nutritional conditions of the natural soils of the *campos rupestres* (Negreiros *et al.* 2009).

Our study has important implications for ecological restoration of the low-resilient *campos rupestres*. Under the scenario of limited resources for biodiversity conservation, ecological restoration relies on prioritizing target species for reintroduction. Producing seedlings under controlled conditions may be a viable strategy for preventing early-stage mortality (Negreiros *et al.* 2009; Ranieri *et al.* 2004) and decrease the costs of restoration of the *campos rupestres* (Wagner *et al.* 2011). The high seedling survival of *M. foliolosa* under controlled conditions suggests planting seedlings in degraded areas is a feasible strategy. However, experiments examining seedling survival after transplantation are needed to determine the balance between losses during transplantation and improved survival under greenhouse conditions. Seedlings of *M. foliolosa* are able to cope with a great variation of soil conditions (Negreiros *et al.* 2009), often enriching the degraded *campos rupestres* soils (Le Stradic *et al.* 2014). Moreover, *M. foliolosa* stands out as a species showing higher crown cover, seedling survival, plant height and flowering individuals among 17 planted species (Le Stradic *et al.* 2014).

Therefore, we predict that reintroducing *M. foliolosa* seedlings into degraded *campos rupestres* will aid ecosystem recovery after disturbance.

In conclusion, the physically dormant seeds of *M. foliolosa* are able to maintain viability and vigor, germinating with high percentages and producing fast-growing seedlings, even when stored. Thus, artificial seed storage of *M. foliolosa* is not a constraint to seed-based restoration techniques. Further studies addressing seed longevity in other endemic and threatened species from the *campos rupestres* are encouraged to increase our knowledge on *ex situ* conservation strategies and the success of ecological restoration.

Acknowledgments

We thank E. Christie and L. G. Lima for help in field work. GWF thanks the support of CNPq (3304/2009-3, 303352/2010-8), Fapemig and Planta Tecnologia Ambiental. The comments by two reviewers significantly increased manuscript quality.

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(Received on 27.11.2012 and accepted after revisions, on 10.06.2013)