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ORIGINAL ARTICLE

Fungicide and drying effects on the viability of recalcitrant seeds of *Inga vera* subsp. *affinis*

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Abstract Studies on the health and quality of seeds from native tree species are scarce in the tropics, which is the case of *Inga vera*, a recalcitrant-seeded Brazilian species used in reforestation. In this study a series of experiments were conducted to analyze the effects of fungicides and hydration levels on the suppression of seed borne fungi and seed physiology. Firstly, three commercially available fungicide formulations (thiram, carbendazim + thiram and carboxin + thiram) for seed treatment were evaluated. Secondly, embryos were subjected to a combination of four drying levels (62, 52, 44 e 34 % water content) with or without fungicide (carbendazim + thiram). All three preliminary screened fungicide formulations were not phytotoxic, with carbendazim + thiram performing best. Seed deterioration was associated with a high incidence of fungi at any level of drying. Fungicide treatment reduced the incidence of the most common fungi (*Acremonium curvulum* and *Phomopsis diachenii*) and extended the lifespan of non-dried embryos from 90 to 120 days.

Keywords Recalcitrant seed · Seed storage · Seed treatment

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Introduction

The use of native forest species for recovering degraded areas and rebuilding landscapes and riparian woodlands is challenging for species with short seed longevity (Barberdo et al. 2013). These seeds should be sown immediately after harvesting, which may limit their supply throughout the year. Differential seed longevity among plant species may result from the selection pressure under distinct climatic conditions. While some exhibit a high degree of desiccation tolerance, and may be stored for centuries, the so-called orthodox seeds, the desiccation-sensitive, also known as recalcitrant seeds, can be preserved only for short periods, generally a few weeks or months (Roberts 1973; Berjak and Pammenter 1994; Barberdo et al. 2013). *Inga vera* Willd. subsp. *affinis* (DC.) T.D. Penn., popularly known in Brazil as ingá, typically produces recalcitrant seeds that maintain their viability only for a few days under natural conditions and 2 to 3 months when stored in cold conditions (Bilia et al. 2003). This Brazilian-native tropical tree species is found by lakes or rivers and plays a key role in the preservation of water resources (Bilia et al. 2003).

The viability of inga seeds during storage can be extended by the use of partial dehydration, treatment with abscisic acid or polyethylene glycol, and cold storage (Andréo et al. 2006; Faria et al. 2006; Bonjovani and Barbedo 2008). However, the efficacy of those treatments is limited by the accelerated seed metabolism and microorganism development under high seed moisture. Several fungal species have been reported in association with recalcitrant seeds (Mittal 2003; Parisi et al. 2013; Oliveira et al. 2011) but their effect on seed physiology is barely known, especially whether seed deterioration is associated with fungal colonization. Non-infected or disinfected recalcitrant seeds have shown extended viability compared to infected seeds (Berjak 1995).

In our previous study we found that mature embryos of *I. vera* had a greater storage potential than immature ones. We also found that seed borne fungi, such as *Fusarium oxysporum*, *Colletotrichum gloesporioides* and *Phomopsis diachenii*, were commonly infecting seeds associated with seed deterioration, differing from common patterns found for orthodox seeds (Parisi et al. 2013). Therefore, this study aimed to analyze the effects of fungicide treatment combined with drying levels on the long-term preservation and viability of inga embryos.

Material and methods

Study area, seed origin and preparation

Three annual collections (Dec 2009, Jan 2010 and Jan 2011) of *I. vera* fruit were made from 30 trees of natural vegetation located in parks and the surrounding area of the Piracicaba river, in the municipality of Piracicaba, state of São Paulo. Fruit were classified at harvest as yellow (FA) or yellowish (7.8 GY 8/10), according to the color charts for plant tissues patterns (Munsell 1952). These fruit were transported to the laboratory within 24 h after harvesting, where the embryos were manually extracted from the fruit. Fruit exhibiting damage by birds were discharged. The sarcotesta (fleshy seed coat) was removed and the non-disinfected embryos were placed in plastic bags and stored under continuous dark in a growth chamber set at 7 °C. The whole process was made within 3 days after harvesting. Embryos were assessed for water content, germination and fungal infection as described below.

Water content and germination assays

Water content (WC) was determined in a forced air oven at 103 °C for 17 h (Brasil 2009) in four replications of 10 embryos. Results were expressed as percentage of the initial fresh weight. Germination tests were carried out on germinators with a water curtain under continuous light at 25 °C (Bonjovani and Barbedo 2008) using the paper roll method (Brasil 2009). Evaluations of normal seedlings were performed at 2-day intervals up to 20 days as described (Bonjovani and Barbedo 2008). The germination percentage was assessed in four replications of 15 embryos for seeds collected in 2010 or 25 embryos for seeds collected in 2009 and 2011.

Seed health test and fungi identification

The blotter test (Neergaard 1979) was used to assess fungal incidence as described (Parisi and Santos 2011). Petri dishes (9-cm diameter) containing embryos were incubated for 7 days at 20 ± 2 °C, under a 12 h light/dark cycle. Stereoscopic microscope aided the identification of fungal colonies and, whenever needed, slides were prepared and fungal structures examined using light microscopy. The fungi detected on the embryos were recovered and cultured on BDA medium for further species identification by the mycology laboratory of the Universidade Federal de Pernambuco (Recife, PE, Brazil).

Fungi were grown on potato dextrose agar (PDA) culture medium, at 30 ± 2 °C, under a 12-h light (40-W "daylight" fluorescent lamps)/dark regime and analyzed after 7 days of incubation. Macroscopic (color, appearance and diameter of colonies) and microscopic characteristics (somatic and reproductive microstructures) were observed by light microscopy. The identification of fungi was based on specialized literature (Ellis 1971, 1976; Barnett and Hunter 2003; Leslie and Summerell 2006; Domsch et al. 2007; Seifert and Gams 2011). The same number of replicates and embryos per replication used in the germination assay was used to assess fungal incidence.

Screening of fungicides—seeds collected in 2009

Three fungicide formulations, at different dosages, were screened for their efficacy to reduce fungal incidence in non-dried and non-disinfected embryos from seeds collected in 2009. These formulations were selected among those commonly used in the market, and which are recommended for seed treatment of a range of crops. The treatments, including a disinfestation and non-treated check, consisted of: *i*) thiram (1.44 g of active ingredient (a.i.)/ kg of embryos); *ii*) carbendazim plus thiram (0.6 + 0.6 and 0.9 + 0.9 g a.i./kg of embryos); *iii*) carboxin plus thiram (0.12 + 0.28 and 0.3 + 0.7 g a.i./kg of embryos); *iv*) 1 % sodium hypochlorite for 3 min; and *v*) control (no treatment).

Distilled water was added at 0.5 % of the weight of the embryos for both treated and non-treated samples. Treatments were applied using 5 L plastic bags, under stirring for 5 min. Immediately after the fungicide treatment, embryos were assessed for water content, germination and fungi incidence as described. The experimental design used was completely randomized with seven treatments.

Effect of drying and carbendazim plus thiram—seeds collected in 2010

For the seeds collected in 2010, non-dried embryos with 62 % WC were dried in a forced air oven at 30 °C for reducing WC to 52, 44 and 34 %. Treatments were comprised of the combination of the four WC levels, with or without fungicide, which was the mixture of carbendazim (0.12 g a.i./kg) plus thiram (0.28 g a.i./kg) selected previously. Embryos were placed on transparent polyethylene bags (30 × 40 cm in width and length, 2 mm thick, 6.5 L volume, resistant to gas exchanges), filled up to one quarter of their volume, manually wrapped and stored at 7 °C. Temperature was reduced to slightly below basal level (9 °C), following Bonjovani and

Barbedo (2008), to avoid the germination of embryos during storage. Physical, physiological, and sanitary evaluations were made as described, at time zero and after 30, 60, 75, 90, and 120 days of storage (dos). The experiment was in a complete randomized design with four seed drying levels, two fungicide levels and six storage times.

Effect of carbendazim plus thiram mixture—seeds collected in 2011

For seeds collected in 2011, the effect of carbendazim plus thiram (CS, 0.3 and 0.7 g a.i., respectively, per kg of non-dried embryos) was evaluated. The same evaluations of the previous experiment were made. Fungicide-treated and untreated embryos were stored at 7 °C and evaluated after 30, 60, 90,120 and 150 dos. The experimental design was a 2 × 6 factorial.

Data analysis

Analysis of variance was conducted with data of each experiment and the logarithm or root-square transformation ($\sqrt{x}+0.5$) was applied to the germination data (Santana and Ranal 2004). Single factors and their interactions were tested at 5 % probability, and means were compared by Tukey's test when significant. The analyses were conducted in SISVAR (Ferreira 2003).

Results

Fungicide screening

None of the fungicides were phytotoxic, as 100 % seedling emergence was recorded after 10 dos. Five genera/species were detected, with the *Fusarium oxysporum* species complex being the most predominant at incidence levels reaching >95 % in the non-treated check and some fungicide treatments (Table 1). Other genera with incidence ranging from 0 to 4 % included *Aspergillus*, *Pestalotiopsis* and *Phoma*. *Cladosporium* was found at low incidence levels, but was exceptionally higher in the sodium hypochlorite treatment (14 %). The best control was provided by the two mixtures of carbendazim plus thiram (Table 1).

Table 1 Incidence (%) of *Fusarium oxysporum* detected on *Inga vera* embryos treated with different fungicides. Embryos harvested in December 2009

Treatments	Dosage[a]	Incidence[b]
Carbendazim plus thiram	(0.12 + 0.28)	1 e
Carbendazim plus thiram	(0.30 + 0.70)	2 e
Carboximplus thiram	(0.6 + 0.6)	90 b
Carboxinplus thiram	(0.9 + 0.9)	69 d
Thiram	(1.44)	62 c
Sodium hypochlorite	(1 % for 3 min)	92 b
Non-treated check	–	98 a

[a] g of active ingredient/kg of embryos, except as indicated for sodium hypochlorite

[b] Incidence levels followed by the same letter do not differ according to Tukey's test at 5 % probability

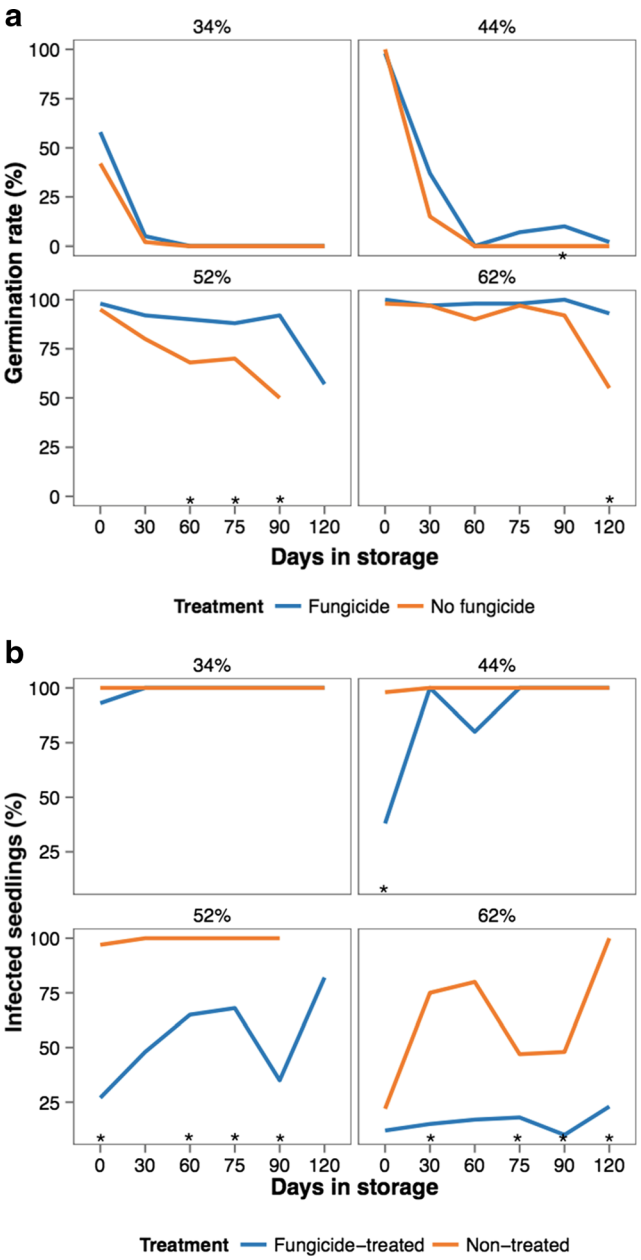

Fig. 1 **a** Means of germination (% of normal seedlings) and **b** of infected seedlings (%) obtained from germination of mature embryos of *Inga vera* harvested in 2010. Seeds showed 62 % of water content and were subjected to drying levels (34, 44, 52 %) and treated or not with fungicide (carbendazim + thiram, 0,12 g + 0,28 g a.i./kg of embryos), and stored for 120 days. *Asterisks* at storage times indicate significant difference between fungicide-treated and non-treated seeds

Effect of drying levels and carbendazim + thiram mixture

The 2010 embryos with 62 % WC were dried to 52, 44, and 34 %, which were close to the target values. Anova showed significance for the three-way interaction ($P<0.05$). Higher germination rate and extended viability of the embryos were found for non-dried (62 %) and fungicide-treated embryos, which kept their viability for up to 120 dos (Fig. 1a). In general, embryo germination was reduced with the decrease of water content and the increase of storage time. Germination was reduced by more than 50 % just after drying for embryos with 34 % WC, and after 30 dos for embryos with 44 % WC (Fig. 1a). The best results were for embryos with 52 % WC treated with fungicide, which kept their viability for up to 90 dos. Non-treated embryos started to lose viability when stored for 30 days.

The incidence of infected seedlings tended to increase with the decrease of drying levels and no fungicide. Non-dried embryos (62 % WC) showed 22 % infected seedlings at the beginning of storage, while drying the embryos to any level led to 100 % seedling infection at this time. The fungicide treatment benefited embryos with 52 and 62 % WC during the storage period (Fig. 1b).

Four fungal species were found associated with the embryos, notably *Fusarium oxysporum, Colletotrichum gloeosporioides, Phomopsis diachenii,* and *Acremonium curvulum* (Fig. 2). Those of the *Fusarium* and *Acremonium* genera were found at high incidence since the beginning of the storage period, regardless of the water content (Fig. 2). *Phomopsis diachenii* was detected after 30 dos in non-treated embryos at all drying levels. The incidence of *Fusarium, Acremonium and Penicillium* spp. increased on fungicide-treated embryos during the entire storage period, regardless of water content (Fig. 2).

Effect of carbendazim plus thiram mixture

The incidence of *F. oxysporum* and *Penicillium* spp. tended to increase during the storage of seeds harvested in both 2010 and 2011 (Figs. 2 and 3). However, fungicide

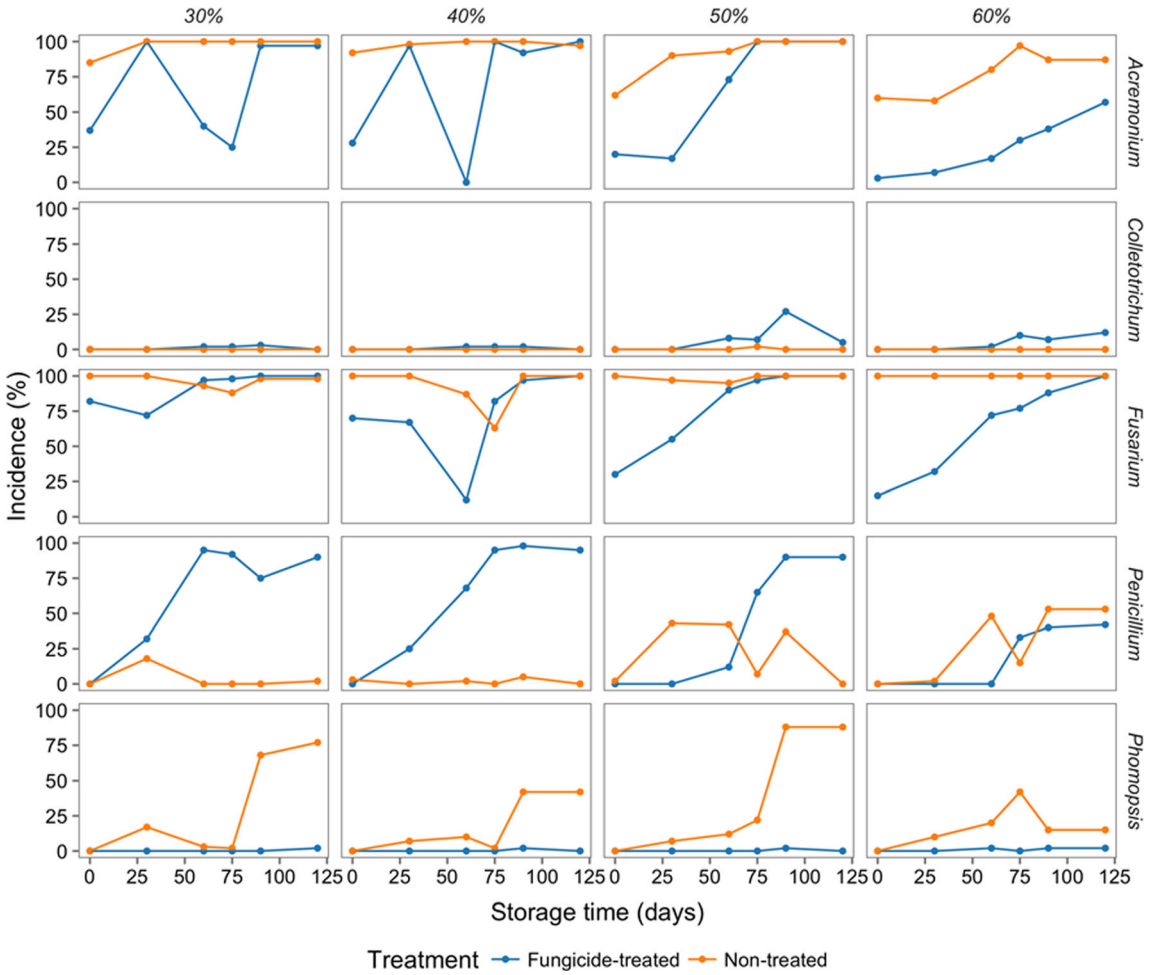

Fig. 2 Incidence (%) of fungi detected in mature embryos of *Inga vera* with different water contents (30 to 60 %) with or without fungicide treatment (carbendazim + thiram, 0,12 g + 0,28 g a.i./kg of embryos), and submitted to different storage time in days. Embryos harvested in 2010

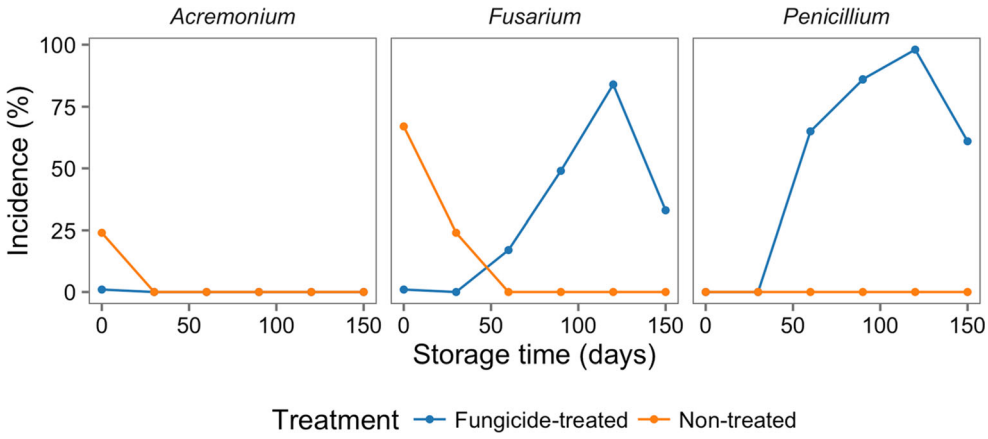

Fig. 3 Incidence (%) of fungi on mature embryos of *Inga vera* with a water content of 61 %, with or without fungicide treatment (carbendazim + thiram, 0,3 g + 0,7 g a.i./kg of embryos), and submitted to different storage times in days. Embryos harvested in 2011

treatment for longer time suppressed other fungi, allowing the development of *Colletotrichum gloeosporioides*, which was detected after 60 days only on fungicide-treated embryos harvested in 2010, and after 120 days on the ones harvested in 2011. These embryos lost their viability after the first 30 dos. Fungicide-treated seeds showed 90 % germination for up to 60 days. However, the germination was reduced to 20 % after 120 dos (Fig. 4). Compared to the viability of untreated embryos in 2010 and 2011 (Figs. 1a and 2), we observed that the ones harvested in 2010 showed a greater physiological potential suggested by the longer period of time until deterioration.

Discussion

The use of carbendazim + thiram was the most effective treatment, reducing fungal incidence on *I. vera* embryos to very low levels. This is in agreement with results reported for

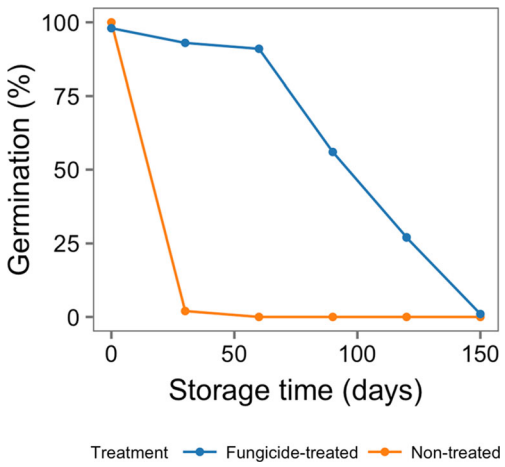

Fig. 4 Germination (% of normal seedlings) of mature embryos of *Inga vera* non-treated or treated with fungicide (carbendazim + thiram, 0.3 g + 0.7 g a.i./kg of embryos) and stored for 150 days. Embryos harvested in 2011

Eugenia dysenterica (Mittal et al. 1999) and *E. pyriformis*, *E. brasiliensis* and *E. uniflora* (Oliveira et al. 2011). However, it contradicts a study using *E. brasiliensis* and *E. uniflora* seeds (Françoso and Barbedo 2014). These seeds are all of the recalcitrant type, and inconsistencies in the results for the same species suggest that other uncontrolled factors may influence the efficacy of fungicides.

Because of their high water content, typical of recalcitrant seeds, inga embryos exhibit very high metabolic activity even after shedding (Barbedo and Marcos Filho 1998; Caccere et al. 2013), from which derives their short longevity, as corroborated by the results found on the germination test of the present study. The dehydration of embryos collected in 2010 probably led them to an uncontrolled metabolism (Berjak and Pammenter 2013), accelerating the deterioration and the development of latent fungi.

The physiological potential differences found between embryos collected in 2010 and 2011 might have resulted from different environmental conditions during the development of embryos in these 2 years. However, regardless of the reasons for the different maintenance of embryo viability, we observed that for the most vigorous ones harvested in 2010 and not dried, the benefits of chemical treatment were seen only after 120 days. For embryos harvested in 2011, due to their reduced vigor, the treatment was necessary since the first storage month. This suggests that fungi remain latent on embryos, not causing any symptoms, until stress conditions trigger the deterioration process.

The higher germination percentage obtained for fungicide-treated embryos during storage may be related to the reduced incidence of fungi. According to Berjak (1995), the lifespan of recalcitrant seeds is prolonged when the primary source of inoculum is removed. Our results confirm that fungal activity plays a key role in the deterioration of *I. vera* embryos. In a previous study we showed that fungi such as *Fusarium oxysporum*, *Colletotrichum gloesporioides* and *Phomopsis diachenii*, considered important pathogens of crop species, were found in association with *I. vera* embryos during storage,

which could be associated with their deterioration (Parisi et al. 2013). According to Bacchi (1961) and Berjak and Pammenter (2013), the high water content, which is necessary to preserve the viability of recalcitrant seeds, is also favorable for the development of fungi during seed storage.

Our data shows that drying, storage and low initial vigor (as observed for embryos from seeds collected in 2011) contribute to a more favorable environment for the development of fungi on inga seeds, which accelerates their deterioration. It was also shown that fungicide treatment with the mixture of carbendazim plus thiram can extend the viability period of inga embryos during storage at 7 °C, as long as they are non-dried, by suppressing fungal development. Attention should be given to the control of seed borne fungi to increase the period of maintenance of viability of *I. vera* embryos during storage and to improve the supply of seedlings throughout the year, especially for seeds with low embryo vigor.

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References

Andréo Y, Nakagawa J, Barbedo CJ (2006) Mobilização de água e conservação da viabilidade de embriões de sementes recalcitrantes de inga (*Inga vera* Willd. subsp. *affinis* (DC.) T.D. Pennington). Rev Bras Bot 29:309–318

Bacchi O (1961) Estudos sobre a conservação de sementes. IX. Inga. Bragantia 20:805–814

Barbedo CJ, Marcos Filho J (1998) Tolerância à dessecação em sementes. Acta Bot Bras 12:145–164

Barberdo CJ, Centeno DC, Ribeiro RCLF (2013) Do recalcitrant seeds really exist? Hoehnea 40:583–593

Barnett HL, Hunter BB (2003) Illustrated genera of imperfect fungi, 4th edn. APS Press, Saint Paul

Berjak P (1995) The role of microorganisms in deterioration during storage of recalcitrant and intermediate seeds. In: Ouédraogo AS, Poulsen K, Stubsgaard F (eds) Intermediate/recalcitrant tropical forest tree seeds: proceedings of a working on improved methods for handling and storage of intermediate/recalcitrant tropical forest tree seeds. IPGRI, Rome, pp 121–126

Berjak P, Pammenter NW (1994) Recalcitrance is not an all-or-nothing situation. Seed Sci Res 4:263–264

Berjak P, Pammenter NW (2013) Implications of the lack of desiccation tolerance in recalcitrant seeds. Front Plant Sci 4:1–9

Bilia DAC, Barbedo CJ, Cicero SM, Marcos Filho J (2003) Inga: uma espécie importante para recomposição vegetal em florestas ripárias, com sementes interessantes para a ciência. Informativo Abrates 13: 26–30

Bonjovani MR, Barbedo CJ (2008) Embriões recalcitrantes de *Inga vera* Willd. subsp. *affinis* (DC.) T.D. Penn. toleram temperatura subzero. Rev Bras Bot 31:345–356

Brasil (2009) Ministério da Agricultura e Reforma Agrária, Regras para Análise de Sementes. SNDA/DNDU/CLAV, Brasília

Caccere R, Teixeira SP, Centeno DC, Figueiredo-Ribeiro RCL, Braga MR (2013) Metabolic and structural changes during early maturation of *Inga vera* seeds are consistent with the lack of a desiccation phase. J Plant Physiol 170:791–800

Domsch KH, Gams W, Anderson T (2007) Compendium of soil fungi. IHW–Verlag, San Francisco

Ellis MB (1971) Dematiaceous Hyphomycetes. Commonwealth Mycological Institute, Kew

Ellis MB (1976) More Dematiaceous Hyphomycetes. Commonwealth Mycological Institute, Kew

Faria JMR, Davide LC, Silva EAA, Davide AC, Pereira RC, Van Lammeren AAM, Hilhorst HWM (2006) Physiological and cytological aspects of *Inga vera* subsp. *affinis* embryos during storage. Braz J Plant Physiol 18:503–513

Ferreira DF (2003) Sisvar: versão 4.2. UFLA, Lavras

Françoso CF, Barbedo CJ (2014) Tratamentos osmóticos e térmicos para controle de fungos em sementes de grumixama e pitanga. Hoehnea 40:541–552

Leslie JF, Summerell BA (2006) The *Fusarium* laboratory manual. Blackwell Publishing, Ames

Mittal RK (2003) Mycoflora of recalcitrant tree seeds and its effects on germination and storability. J Mycopathol Res 41:127–134

Mittal RK, Hansen HJ, Thomsen K (1999) Seed mycoflora of *Eugenia dysenterica* and its effect on germination and storability. IUFRO Seed Symposium 1998 "Recalcitrant seeds": Proceedings. IUFRO, Kuala Lumpur, pp 87–95

Munsell CC (1952) Munsell color charts for plant tissues. Munsell Collor Company, Baltimore

Neergaard P (1979) Seed pathology, vol 1. McMillan Press, London

Oliveira CF, Oliveira DC, Parisi JJD, Barbedo CJ (2011) Deterioração de sementes de espécies brasileiras de *Eugenia* em função da incidência e do controle de fungos. Rev Bras Sementes 33:520–532

Parisi JJD, Santos AF (2011) Métodos convencionais de detecção de fungos em sementes. In: Santos AF, Parisi JJD, Menten JO (eds) Patologia de Sementes Florestais. Embrapa Florestas, Colombo

Parisi JJD, Biagi DJ, Barbedo CJ, Medina PF (2013) Viability of *Inga vera* Willd. subsp. *affinis* (DC.) TD Penn. embryos according to the maturation stage, fungal incidence, chemical treatment and storage. J Seed Sci 35:70–76

Roberts EH (1973) Predicting the storage life of seeds. Seed Sci Technol 1:499–514

Santana DG, Ranal MA (2004) Análise da Germinação: Um Enfoque Estatístico. Universidade de Brasília, Brasília

Seifert KA, Gams W (2011) The genera of Hyphomycetes—2011 update. Persoonia 27:119–129