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Effect of heat treatment and osmotic storage on the control of fungi associated with short-lived *Inga vera* Willd. Embryos

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ABSTRACT. Seeds sensitive to desiccation remain metabolically active after dispersion with a high-water content, which makes their storage for long periods difficult, due to their deterioration and high proliferation of fungi. One of the ways to maintain or improve the sanitary quality of the seed, preventing the spread and transmission of fungi, is the treatment of these seeds and how they are stored. Thus, *Inga vera* seeds were subjected to thermotherapy in hot water at different temperatures and submersion periods and to storage in an osmotic polyethylene glycol solution (-2.0 MPa) for 25, 50, and 75 days. They were then evaluated for health (incidence and severity), germination and seedling formation. It was found that osmotic storage was more efficient than heat treatment in reducing most fungi found and had a greater germination and normal seedling formation. The association of both types of treatment produces favorable results and the reapplication of heat treatment can increase fungal control throughout storage. The severity assessment made it possible to detect differences between treatments that were not identifiable in the incidence assessment.

Keywords: fungi incidence; osmotherapy in seeds; recalcitrant seeds; thermotherapy in seeds.

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Introduction

The development of research focused on the area of production, viability and conservation of native Brazilian seeds becomes extremely important, since knowledge about seed storage makes it possible to preserve them for future use.

However, seeds that are sensitive to desiccation (known as recalcitrant seeds) present limitations regarding conservation, due to their susceptibility to drying and behavior during storage. These seeds remain metabolically active after dispersal from the mother plant (Colville & Kranner, 2010) with high water content, preventing their storage for long periods. However, reducing water content and the conventional methodology of seed conservation in chambers at sub-zero temperatures have proven to be inefficient or harmful for a variety of seeds sensitive to desiccation, reducing their storage life under these conditions (Faria et al., 2004; Kohama et al., 2006; Delgado & Barbedo, 2007; Bonjovani & Barbedo, 2008; Delgado & Barbedo, 2012; Parisi et al., 2013; Bonjovani & Barbedo, 2014; Parisi et al., 2016; Silva et al., 2018).

Thus, species that present seeds with high lethal desiccation limits must be kept with high water content and temperatures close to or above 0°C. The need to keep these seeds moist favors the proliferation of microorganisms, considerably accelerating the rate of deterioration (Oliveira et al., 2011; Parisi et al., 2013, 2016). Furthermore, the presence of microorganisms can increase the respiratory rates of the seed-microorganism complex, intensifying seed metabolism and affecting the preservation of viability (Parisi et al., 2019).

One way to maintain or improve seed health, preventing the spread of pathogenic microorganisms, especially fungi, is seed treatment (Mendes et al., 2001). Alternative methods to chemical control are currently being studied to mitigate environmental risks. Furthermore, there is no registration of chemical products specifically for the treatment of native forest seeds, making this practice illegal under the law (Brasil, 2020). In this sense, the use of alternative treatments that help control fungal diseases has emerged as promising.

Heat treatment is based on exposing seeds to heat, which acts on the cellular activity of pathogens. Although efficient in controlling seed-associated pathogens, thermotherapy can cause damage to the

physiological quality of seeds (Machado, 2000). In this sense, it is necessary to find an adequate adjustment between temperature and time, since this combination should control pathogens without causing harm to the physiological quality of seeds (Vieira et al., 2011).

Seed storage in osmotic solutions has been used to extend longevity, since by mobilizing water, it is possible to control the intense metabolism of seeds sensitive to desiccation during storage, resulting in reduced deterioration and proliferation of seed-borne fungi (Andréo et al., 2006; Faria et al., 2004).

In this sense, ex situ conservation becomes fundamentally important for biodiversity conservation. *Inga vera* is one of the species most sensitive to desiccation and has the lowest natural longevity (Bilia & Barbedo, 1997) and, therefore, is of great interest in research aimed at increasing the storage capacity of seeds sensitive to desiccation.

The present work aimed to evaluate the effect of heat treatment and osmotic storage on the control of fungi associated with *I. vera* embryos during storage.

Material and methods

Plant material

Ripe fruits of *I. vera* were collected from mother plants located in Parque Villa Lobos (São Paulo, São Paulo State, Brazil). In the laboratory, seeds were manually extracted from the fruits and subsequently, the sarcotesta (integument) was removed, leaving only the embryo.

Germination test

Germination tests were conducted on Germitest paper rolls, previously moistened (Brasil, 2009a) and kept in germination chambers regulated at 25°C and constant light. Evaluations were performed at intervals of three days up to 15 days. The percentage of germination and formation of normal seedlings were recorded. In the case of polyembryonic seeds, only one root and/or seedling per embryo was recorded. For each treatment, four replicates of 15 seeds were used.

Seed health test

The health quality of the embryos was assessed by analyzing the percentage of seeds infected by each fungus (incidence) and the area of the embryo covered by the colony (severity). The seed health test was performed by the Blotter Test as described by Brasil (2009b). The seeds were evenly distributed in Petri dishes (90x15 mm), containing two sheets of filter paper moistened with distilled water and incubated for seven days at 20±2°C and photoperiod of 12 hours of light/dark, using tree replications of 12 embryos. The fungi were identified by examining the fungal colonies developed in the embryos with the aid of a stereoscopic and optical microscope. The calculation of the incidence was expressed as a percentage of the fungi for each embryo (Brasil, 2009b). For the calculation of severity, the development of each fungus on the surface of the seeds was analyzed individually, quantified by a visual scale and expressed as a percentage, as described by Françoso and Barbedo (2016): Zero - uninfected embryo; Traces - growth up to 10% of the embryo, with small and few colonies; Low - growth up to 40% of the embryo; Moderate - growth from 41 to 100% of the embryo, with superficial colonies and slow growth; High - growth from 41 to 100% of the embryo, with dense and well distributed colonies.

Heat treatment of embryos

Heat treatments (in hot water) were used to control pathogens during embryo storage. For this purpose, the embryos were immersed in distilled water at a ratio of 1:5, by weight (embryos: water) in glass beakers and stored in an air circulation oven, at temperatures and periods described below. For each treatment, there was a preheating of 10 min. The beakers were shaken every 10 min. and, at the end of the period, the embryos were cooled with distilled water and placed on filter paper to remove excess surface water. The treatments tested, based on previous studies with recalcitrant seeds (Oliveira et al., 2011; Françoso & Barbedo, 2014, 2016), were: 45°C for 120 min., 55°C for 120, 90, and 30 min., 50°C for 60 min., 60°C for 60 and 30 min. and 65°C for 10 min. At the end of the treatment, the embryos were placed to germinate as previously described and based on these results (Table 1). The treatment of 55°C for 30 min. was chosen as it did not affect germination.

Table 1. Germination of *Inga vera* embryos after heat treatments.

Treatments	Not treated	45°C 120´	50°C 60´	55°C 30´	55°C 90´	55°C 120´	60°C 30´	60°C 60´	65°C 10´
Germination (%)	100	93	73	100	80	87	93	47	67

The embryos to receive heat treatment were divided into three groups. In the first, heat treatment was performed only at the beginning of the experiment (1st day), henceforth referred to as HT1; in the second, the embryos received a first heat treatment on the 1st day and a second after 25 days of storage (HT2); in the third, the embryos received the HT2 treatments, added to a third heat treatment after 50 days of storage (HT3).

Determination of the osmotic potential of incubation

To obtain the hydration/dehydration curves, *I. vera* embryos (three replicates of five embryos each) were weighed and placed on two sheets of Germitest paper previously moistened with distilled water or polyethylene glycol 6000 (PEG) solutions at osmotic potentials of -1.0, -2.0, -3.0, -4.0, and -5.0 MPa, in transparent Petri dishes. The embryos were covered with a third sheet and incubated at 7°C, in the dark, with periodic weighing until they reached a constant weight. Based on the results obtained from the hydration/dehydration curves, the potential of -2.0 MPa was chosen for embryo storage, as it allowed for maintaining a hygroscopic balance equivalent to the initial water content of these embryos.

Storage conditions of embryos

Embryos from the heat treatments described above, as well as those that did not undergo this treatment (NT), were immersed in a single layer in plastic trays containing a PEG solution at -2.0 MPa. To allow gas exchange with the external environment, the embryos were immersed only to half their height. The trays were covered with plastic film to reduce water loss through evaporation and the consequent change in the osmotic potential of the solution and incubated at 7°C throughout the storage period. The solution was replaced every 8-10 days (according to the results of the water potential measurement of the solution) to ensure that it always had the same potential. The osmotic solutions were prepared by adjusting the PEG concentration according to the incubation temperature (Michel & Kauffmann, 1973), with measurement in a WP4 water potential analyzer (Decagon Devices, Inc., Pullman). In addition to storage in PEG, the NT, HT1, HT2, and HT3 embryos were also stored conventionally, in plastic bags (PB), in a BOD chamber, at 7°C.

The embryos were evaluated by the germination, seedling formation and health test, as previously described, before (NT and HT1) and after 25 (NT and HT1), 50 (NT, HT1, and HT2), and 75 (NT, HT1, and HT3) days of storage (Figure 1).

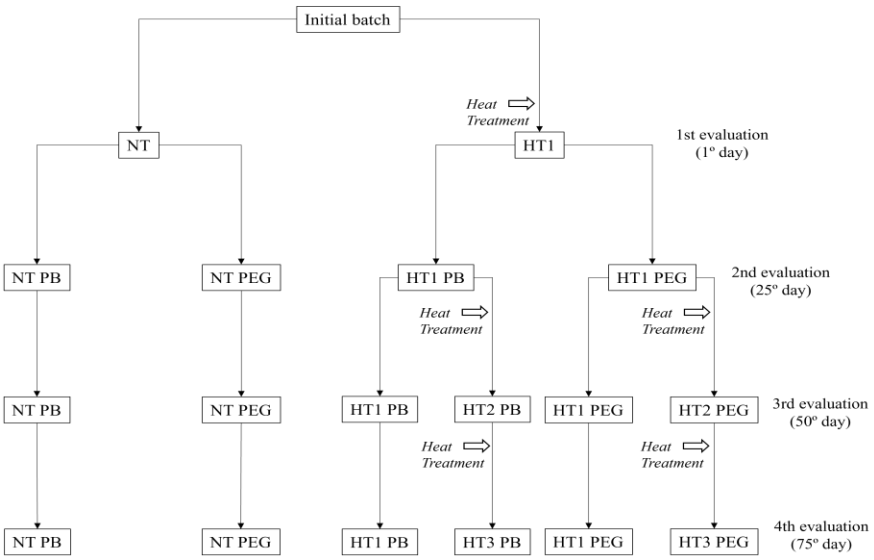

Figure 1. Representative scheme of treatments. Initial batch = freshly processed seeds; NT = non-treated seeds; HT1 = heat treatment performed at the beginning of the experiment; HT2 = heat treatment performed on the 1st day and a second after 25 days of storage; HT3 = HT2 treatments, added to a third heat treatment after 50 days of storage; PB = embryos stored in plastic bags; PEG = embryos stored in polyethylene glycol solution.

Data analysis

The experimental design for all experiments was completely randomized in a 2 x 2 (after 25 days of storage), 3 x 2 (after 50 days), and 4 x 2 (after 75 days) factorial scheme, respectively heat treatment x type of storage. The results obtained were subjected to analysis of variance for each storage period for germination and normal seedling formation by Tukey test ($p < 0.05$) and the means of the health variables were presented with their respective standard deviations, using the SigmaPlot 12.0 program (Systat software, 2011).

Results and discussion

The most frequent fungi in *I. vera* embryos were *Fusarium* sp., *Cladosporium* sp., *Penicillium* sp., and *Ceratocystis* sp. Other genera were detected, but occurred at very low frequency, such as *Phoma* sp., *Phomopsis* sp., and *Alternaria* sp. (Figure 2). *Fusarium* sp. had a higher initial incidence than the sum of the other fungi, a fact that also occurred after thermal and osmotic treatments.

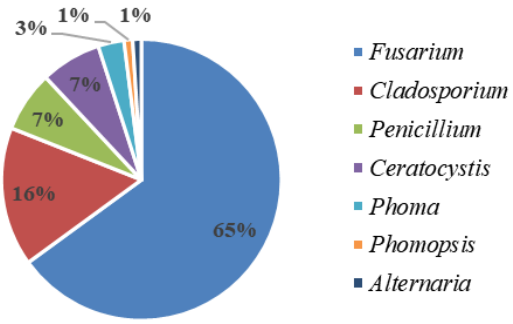

Figure 2. Frequency (%) of fungi initially found in *Inga vera* embryos

Storage in PEG presented the best results in controlling the incidence of *Fusarium* sp. when compared to storage in plastic bags (PB) (Figure 3B-D). In NT treatments, there was a reduction in incidence of 25%, 35%, and 39% and, for heat treatments of 24%, 52%, and 33%, respectively for periods of 25, 50, and 75 days of storage. The reapplication of heat treatment reduced the incidence of *Fusarium* sp. by 10% and 22% for the periods of 50 and 75 days of storage, respectively. Oliveira et al. (2011) also found satisfactory results in reducing the incidence of this fungus after heat treatment > of desiccation-sensitive seeds of *Eugenia pyriformis*. Heat treatment and its reapplication were able to reduce the incidence of *Fusarium* sp. in *I. vera* embryos in both storage conditions (PB and PEG), with the reapplication of the heat treatment being more efficient in controlling the fungus. At 75 days of storage, there was a 36% reduction in the incidence of *Fusarium* sp. for HT3-PB, compared with a 17% reduction for HT1- PB and 18% for HT3-PEG, compared with an 8% reduction for HT1-PEG (Figure 3D). The same occurred for the 50-day storage period. There was a 65% reduction for HT2- PB, compared with a 28% reduction for HT1- PB and 52% for HT2-PEG, compared with a 47% reduction for HT1-PEG (Figure 3C).

Therefore, the reapplication of heat treatment may be a good alternative to maintain the efficiency of the treatment throughout storage, as it does not persist in the seeds like chemicals, as it has no residual effect. After 25 days of storage, heat treatment was able to reduce the incidence of *Fusarium* sp. by approximately 40% for both storage conditions (Figure 3B). In non-stored embryos, there was a low incidence of *Fusarium* sp. and *Ceratocystis* sp., with heat treatment being able to eradicate both (Figure 3A).

Although heat treatments and osmotic storage have been effective in controlling *Fusarium* sp., as the storage period increases the incidence of *Fusarium* sp. also increases (Figure 3). In dry stored seeds, fungi traditionally classified as field fungi, such as *Fusarium* sp., lose their viability during storage (Christensen & Kaufmann, 1965; Marcos Filho, 2015). However, desiccation-sensitive seeds are dispersed with high water content and need to be maintained with values above 20-40% of water (Roberts, 1973; Hong & Ellis, 1996). At this hydration level there is a considerable amount of freezable water (Vertucci & Farrant, 1995) and, therefore, such seeds are also considered sensitive to temperatures below 0°C. Thus, moist storage of seeds at temperatures above freezing levels favors the development and maintenance of the viability of these fungi during storage (Mycock & Berjak, 1990; Calistru et al., 2000). Despite the high incidence of this fungus, in general, seeds were able to germinate and produce normal seedlings above 90%, except for the 75-day storage period for seeds stored in PB, which presented germination below 70% and a maximum of 40% of normal seedling formation (Table 2).

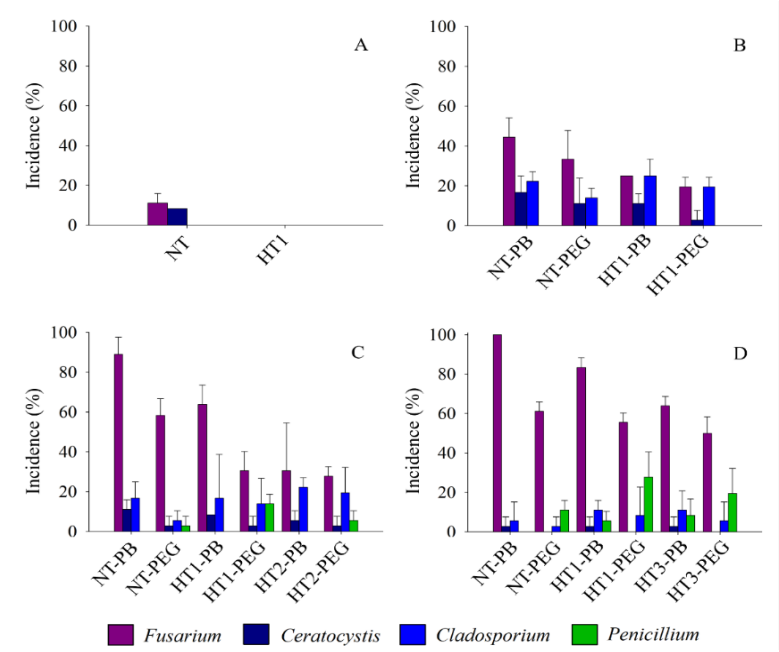

Figure 3. Incidence of *Fusarium* sp., *Ceratocystis* sp., *Cladosporium* sp., and *Penicillium* sp. in *I. vera* embryos subjected to heat treatments (HT1, HT1-PB, HT1-PEG, HT2-PB, HT2-PEG, HT3-PB, HT3-PEG) or not (NT, NT-PB, NT-PEG), before (A) and after storage for 25 (B), 50 (C), and 75 (D) days. NT = non-treated seeds; HT1 = heat treatment performed at the beginning of the experiment; HT2 = heat treatment performed on the 1st day and after 25 days of storage; HT3 = HT2 treatments, added to a third heat treatment after 50 days of storage; PB = embryos stored in plastic bags; PEG = embryos stored in polyethylene glycol solution. Values are average plus standard deviation.

Table 2. Germination and normal seedling formation (%) of *Inga vera* throughout storage.

Treatments	Storage (days)	Germination (%)	Normal seedling (%)
NT	0	100 a	100 a
HT1	0	100 a	100 a
NT PB	25	100 a	100 a
NT PEG	25	100 a	100 a
HT1 PB	25	100 a	100 a
HT1 PEG	25	100 a	100 a
NT PB	50	96 a	93 a
NT PEG	50	96 a	87 a
HT1 PB	50	100 a	97 a
HT1 PEG	50	100 a	95 a
HT2 PB	50	100 a	98 a
HT2 PEG	50	98 a	90 a
NT PB	75	60 c	48 b
NT PEG	75	99 a	88 a
HT1 PB	75	69 b	50 b
HT1 PEG	75	100 a	93 a
HT3 PB	75	57 c	33 b
HT3 PEG	75	99 a	97 a

Means followed by the same letter, within each storage period, do not differ from each other by the Tukey test, at 5% probability.

After 75 days of storage, embryos stored in PB showed a high degree of infection by *Fusarium* sp. on their surface, while those stored in PEG had at most moderate development (Figure 4), making evident the role of PEG in reducing the intensity of fungal infestation, especially in the long term. After 50 days of storage, reapplication of heat treatment was able to reduce the severity of *Fusarium* sp. development, since HT2-PB showed only low and trace-like development, while HT1-PB also showed high and moderate severity. Similarly, HT2-PEG showed low and trace-like development and HT1-PEG also showed moderate severity of the fungus (Figure 4C).

For the genus *Penicillium* sp., heat treatment and osmotic storage were detrimental to the control of the incidence and severity of the fungus (Figures 2C-D and 4). There was no incidence of *Penicillium* sp. in freshly harvested *I. vera* embryos or after 25 days of storage (Figure 3A-B), only after 50 days, and its occurrence and increase were proportional throughout the storage period. Regarding severity, after 50 days of storage, NT-PEG showed only trace development, while for HT1-PEG and HT2-PEG there was an increase in severity in

the form of traces for low development (Figure 5C). Françoso and Barbedo (2016) also reported greater incidence and severity of *Penicillium* sp. in desiccation-sensitive seeds of *E. brasiliensis* subjected to heat and osmotic treatments, mainly after 60 days of storage. According to Garcia et al. (2004), seed deterioration is often not noticeable in the initial phase of storage, becoming more expressive over time. Species belonging to the genus *Penicillium* sp. are more commonly found as contaminants in the post-harvest period, during seed processing, transportation, and packaging, and are therefore classified as storage fungi (Marcos Filho, 2015).

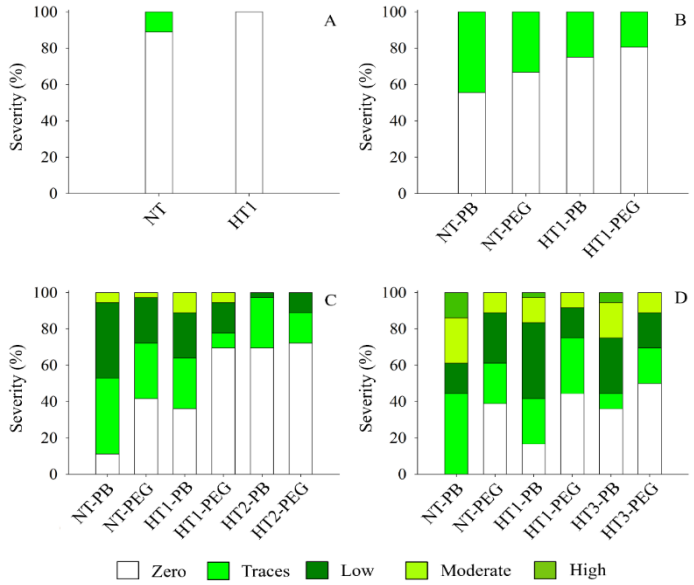

Figure 4. Severity of *Fusarium* sp. in *I. vera* embryos subjected to heat treatments (HT1, HT1-PB, HT1-PEG, HT2-PB, HT2-PEG, HT3-PB, HT3-PEG) or not (NT, NT-PB, NT-PEG), before (A) and after storage for 25 (B), 50 (C), and 75 (D) days. Zero - uninfected embryo. NT = non-treated seeds; HT1 = heat treatment performed at the beginning of the experiment; HT2 = heat treatment performed on the 1st day, and a second after 25 days of storage; HT3 = HT2 treatments, added to a third heat treatment after 50 days of storage; PB = embryos stored in plastic bags; PEG = embryos stored in polyethylene glycol solution. Traces - growth up to 10% of the embryo, with small and few colonies; Low - growth up to 40% of the embryo; Moderate - growth from 41 to 100% of the embryo, with superficial colonies and slow growth; High - growth from 41 to 100% of the embryo, with dense and well distributed colonies.

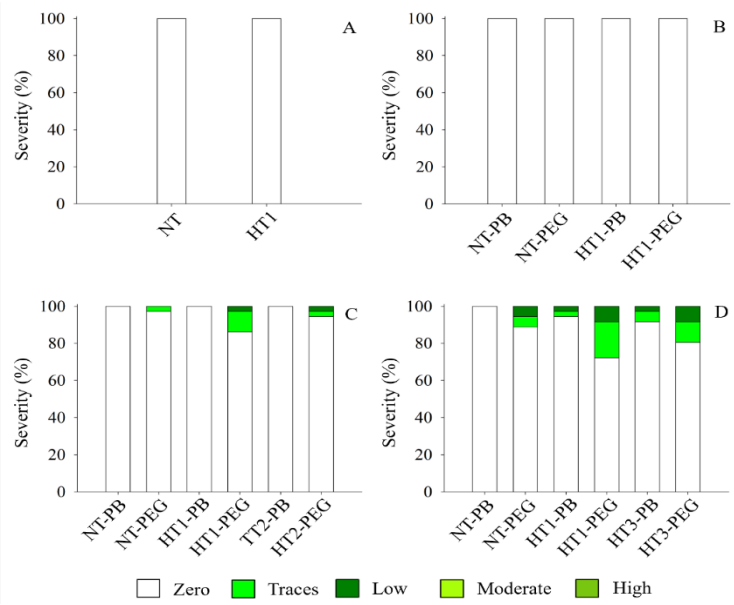

Figure 5. Severity of *Penicillium* sp. in *I. vera* embryos subjected to heat treatments (HT1, HT1-PB, HT1-PEG, HT2-PB, HT2-PEG, HT3-PB, HT3-PEG) or not (NT, NT-PB, NT-PEG), before (A) and after storage for 25 (B), 50 (C) and 75 (D) days. Zero - uninfected embryo. NT = non-treated seeds; HT1 = heat treatment performed at the beginning of the experiment; HT2 = heat treatment performed on the 1st day and a second after 25 days of storage; HT3 = HT2 treatments, added to a third heat treatment after 50 days of storage; PB = embryos stored in plastic bags; PEG = embryos stored in polyethylene glycol solution. Traces - growth up to 10% of the embryo, with small and few colonies; Low - growth up to 40% of the embryo; Moderate - growth from 41 to 100% of the embryo, with superficial colonies and slow growth; High - growth from 41 to 100% of the embryo, with dense and well distributed colonies.

Fungi of the genera *Fusarium* sp. and *Penicillium* sp. have shown some persistence during storage in studies with species producing seeds sensitive to desiccation (Calistru et al., 2000; Mukherjee et al., 2006; Oliveira et al., 2011; Françoso & Barbedo, 2014, 2016; Parisi et al., 2016), even after seed treatment. In contrast, the genus *Ceratocystis* sp. showed a reduction in incidence during storage, as well as in osmotic storage (Figure 2). Thus, embryos stored in PEG for 25 days reduced the incidence of this fungus by 35% (NT) and 73% (HT1) (Figure 3B). At 50 days of storage, there was a reduction of 73% (NT) and 63% (HT1), and a new heat treatment reduced the incidence of *Ceratocystis* sp. by half (Figure 3C). At 75 days, there was no incidence of the fungus in embryos stored in PEG (Figure 3D).

Heat treatment and its reapplication were also effective in reducing the incidence of *Ceratocystis* sp. up to 50 days of storage (Figure 3A-C). In non-stored embryos, heat treatment was able to eradicate the incidence of the fungus (Figure 3A). After 25 days of storage, there was a reduction in the incidence of 35% and 73% for embryos stored in PB and PEG, respectively (Figure 3B). At 50 days, heat treatment and its re-application reduced the incidence of *Ceratocystis* sp. by 27% and 45%, respectively, in embryos stored in PB (Figure 3C). When analyzing only the incidence of the fungus after 75 days of storage in PB, there was no difference between NT and treated embryos (Figure 3D). However, a critical analysis shows a reduction in the development of the fungus from high grade to trace form, for the embryos that underwent thermotherapy (Figure 6), revealing differences that were not identified by assessing incidence alone.

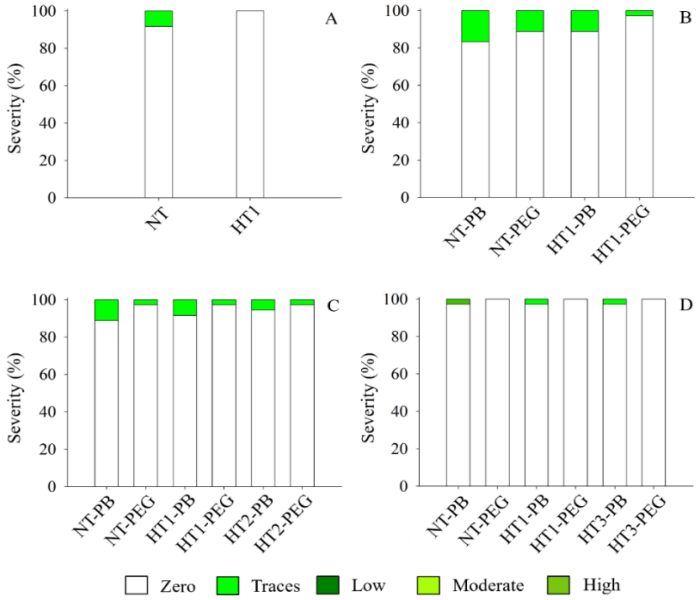

Figure 6. Severity of *Ceratocystis* sp. in *I. vera* embryos subjected to heat treatments (HT1, HT1-PB, HT1-PEG, HT2-PB, HT2-PEG, HT3-PB, HT3-PEG) or not (NT, NT-PB, NT-PEG), before (A) and after storage for 25 (B), 50 (C), and 75 (D) days. Zero - uninfected embryo. NT = non-treated seeds; HT1 = heat treatment performed at the beginning of the experiment; HT2 = heat treatment performed on the 1st day and a second after 25 days of storage; HT3 = HT2 treatments, added to a third heat treatment after 50 days of storage; PB = embryos stored in plastic bags; PEG = embryos stored in polyethylene glycol solution. Traces - growth up to 10% of the embryo, with small and few colonies; Low - growth up to 40% of the embryo; Moderate - growth from 41 to 100% of the embryo, with superficial colonies and slow growth; High - growth from 41 to 100% of the embryo, with dense and well distributed colonies.

As occurred in the genus *Ceratocystis* sp., the incidence of *Cladosporium* sp. is also reduced during storage. Similarly, osmotic storage was also more effective in reducing the incidence of the fungus compared to storage in PB (Figure 3B-D). In NT treatments, there was a reduction in incidence of 41%, 65% and 50% and, for heat treatments, 24, 18, and 27%, respectively for periods of 25, 50, and 75 days of storage. Reapplying the heat treatment reduced the incidence of *Cladosporium* sp. by 14% (HT2) and 45% (HT3), for periods of 50 and 75 days of storage, respectively. These results corroborate those of Oliveira et al. (2011) who, working with desiccation-sensitive seeds of different species of *Eugenia* sp., also reported the efficiency of the osmotic treatment in controlling the incidence of this fungus. However, heat treatment and its replication were ineffective or harmful in controlling the incidence of the fungus, after 50 days of storage (Figure 3C-D).

When analyzing the severity of the embryos, it was found that, for the 50-day period, the treatments NT-PB and HT1-PB, which presented the same incidence, showed an increase in the degree of *Cladosporium* infection in traces form (NT-SP) for a high development of the fungus (HT1-PB); for HT2-PB, there was also

moderate development of the fungus (Figure 7). The same occurs for the 75-day period, where NT-PB and NT-PEG presented only development in the form of traces and HT1-PB and HT3-PEG presented a low development (Figure 7D), showing the negative effect of thermotherapy for this fungus and proving, once again, the sensitivity of the severity analysis. The other way around to what occurs for the genus *Ceratocystis* sp., the application of the heat treatment increased the severity of *Cladosporium* sp., showing that the use of alternative treatments in the seeds can trigger different responses for the different fungal genera found.

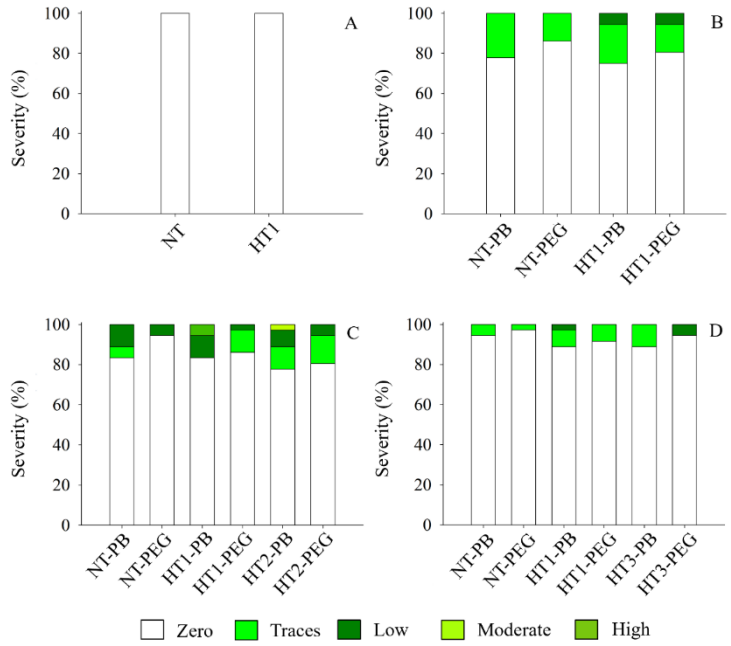

Figure 7. Severity of *Cladosporium* sp. in *I. vera* embryos subjected to heat treatments (HT1, HT1-PB, HT1-PEG, HT2-PB, HT2-PEG, HT3-PB, HT3-PEG) or not (NT, NT-PB, NT-PEG), before (A) and after storage for 25 (B), 50 (C), and 75 (D) days. Zero - uninfected embryo NT = non-treated seeds; HT1 = heat treatment performed at the beginning of the experiment; HT2 = heat treatment performed on the first day and a second after 25 days of storage; HT3 = HT2 treatments, added to a third heat treatment after 50 days of storage; PB = embryos stored in plastic bags; PEG = embryos stored in polyethylene glycol solution. Traces - growth up to 10% of the embryo, with small and few colonies; Low - growth up to 40% of the embryo; Moderate - growth from 41 to 100% of the embryo, with superficial colonies and slow growth; High - growth from 41 to 100% of the embryo, with dense and well distributed colonies

Conclusion

Seeds can harbor a wide range of microorganisms that can cause different sanitary problems. Seed quality and seed longevity may be drastically reduced by fungi that invade seeds before or after harvest. It is known that seed is an efficient way of introducing plant pathogens into a new area. Since many seeds begin to deteriorate during storage, seed storage is an efficient and widely used method for conserving plant genetic resources. In this work, storage in osmotic medium was more efficient than heat treatment in reducing the fungi found, except for *Penicillium* sp. The combination of both types of treatment showed the best results in reducing *Fusarium* sp. and *Ceratocystis* sp. Reapplying heat treatment can increase the control of these fungi throughout storage, in terms of incidence and severity. Storage in PEG showed favorable results in controlling *Cladosporium* sp., while conventional storage was the best option for controlling *Penicillium* sp.

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