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Assessment of physiological, ultrastructural and biochemical alterations in naturally and artificially aged *Handroanthus impetiginosus* seeds

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Abstract

Handroanthus, whose species are useful in reclamation of disturbed lands and important for their increasing use for medicinal, is mainly propagated by seeds, which shown alterations in the germination during aged. The objective of the present study was to assess treatments pre-germination and disinfestation in addition to the physiological, ultrastructural and biochemical aspects in naturally and artificially aged *Handroanthus impetiginosus* (Mart. ex DC.) Mattos seeds. The seeds were or not submitted to artificial aging at 42 °C, 100% RH for 24, 48, 72 and 96 h, and assessed for germination, electric conductivity, presence of polyphenol compounds, water potential and cell cycle activity. Were also tested pre-germination treatments (high temperatures (45ºC/seven days) and low (10 °C/15 days), gibberellic acid, potassium nitrate, water imbibitions and seed coat removal) and disinfection of the seeds (sodium hypochlorite and benomyl). Seed germination, depending on the initial quality, present fluctuations during artificial aging and there is not differential response in the disinfestation treatments. Lower germination rates were observed in freshly collected *H. impetiginosus* seeds than in those submitted to all the aging times and treatments pre-germination, except for 48 hours aging with imbibitions treatment followed by coat removal, that stood out from the others, attaining germination of around 80% whereas stored seeds aged for 24, 48 and 96 hours and submitted to imbibitions reached higher germination rates. Thus it can be suggested that *H. impetiginosus* seeds present secondary dormancy that can be broken by artificial aging followed by imbibitions and coat removal. The period of seed exposure to artificial aging that leads to higher germination rates depends on whether the seeds were stored or not. Microtubules were not detected in the *H. impetiginosus* seeds at different times of artificial aging. Regarding DNA content, there were not changes in the 4C value during the artificial aging, indicating the absence of DNA duplication.

Key words: *Tabebuia impetiginosa*, *quality seeds*, *storage*, *forest seeds*

INTRODUCTION

Species of the *Handroanthus* genus are useful in reclamation of disturbed lands and important for medicinal and timber purposes. Their seeds show variations in germination percentage during storage (Figliolia et al., 1988; Cunha et al., 1992; Kageyama et al., 1992; Mello and Eira, 1995; Shibata et al, 2012) and artificial aging (Gemaque, 1999; Shibata et al., 2012).

The variations in response to alterations in germination can be related to the presence of microorganisms, dormancy or by different physiological, biochemical and ultrastructural reactions (Berjak, 1995; Mycock and Berjak, 1995). According to Ferreira (1989), naturally or artificially aged seeds of forest species can be contaminated by several fungus genera and species, that proliferate and maintain metabolic activity in moisture contents higher than 12% -18% (Berjak, 1995). The presence of dormancy is another aspect that can affect germination after storage. Although Carvalho (1994) stated that *H. impetiginosus* seeds do not present dormancy, the variations in germination observed during storage and artificial aging may indicate secondary dormancy, because seeds are submitted to environmental factors that can cause both its induction and release (Desai et al. 1997; Baskin and Baskin, 1998). A factor that has been related to alterations in seed dormancy and germination is the presence of polyphenol compounds, which reduce the availability of oxygen inside the seeds, restricting the germination process (Bewley and Black, 1994). In some seeds, the inhibitory effects on germination from the presence of polyphenol compounds increase with temperature, because the oxygen becomes less soluble and oxidation more intense (Corbineau and Come, 1995). Other factors that can lead to alterations in germination are cell modifications, such as differences in electric conductivity and cell cycle progression, widely reported with the deterioration process (Ganguli and Sen-Mandi, 1990; Basavarajappa et al. 1991).

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The objective of the present study was to assess treatments pre-germination and disinfestation in addition to the physiological, ultrastructural and biochemical aspects in naturally and artificially aged *Handroanthus impetiginosus* (Mart. ex DC.) Mattos seeds.

MATERIAL AND METHODS

This study was carried out in three stages: 1) study of the germination performance of *H. impetiginosus* seeds after storage and artificial aging, submitted or not to disinfestation treatments; 2) study of treatments pre-germination; 3) study of physiological, ultrastructural and biochemical alterations during artificial aging. The seeds were collected at the start of the fruit dehiscence process from various trees in Lavras, MG, Brazil, manually processed and dried in a shed for five days, reducing moisture content from 45 to 9%.

The seeds were stored in polyethylene bags, in acclimatized rooms with controlled temperature and relative humidity (10 -12 °C; 70% RH). Seed moisture content was determined by the oven method at 103 $\pm$ 2 °C for 17 hours (ISTA, 2004), with four replications of 1 g seeds. At all stages, the germination test was performed in a complete randomized block design with five replications of 20 seeds, in sand, at 30 °C and constant light. Assessments were made according to the International Rules for Seed Testing (ISTA, 2004), 14 and 28 days after the start of the test.

The seeds were artificially aged in BOD incubators at 42 °C for 0, 24, 48, 72 and 96 hours. After these periods, the seeds were placed on trays and kept for two days at 15 °C and 70%RH, to homogenize their moisture content, which was around 9% before the tests were performed.

2.1 First stage: Study of the germination performance after storage and artificial aging, submitted or not to disinfestation treatments.

In the first stage, the seeds collected in 2001 were stored and at three-month interval during storage, up to 12 months, the germinability of the seeds submitted or not to artificial aging was assessed. Before the germination test, the seeds were submitted to three disinfestation treatments: 1) sodium hypochlorite (2%; three minutes); 2) benomyl (2%; two minutes); 3) control (seeds without disinfestation).

2.2 Second stage: Study of treatments pre-germination.

In the second stage the seeds were collected in 2000 (one year stored batch) and 2001 (recently collected batch). Before carrying out the germination test with seeds aged or not, the following methods were used, according to the rules for seed analysis (ISTA, 2004): a) high temperature - the seeds were placed in germination boxes containing sand moistened with water and kept at 45 °C for seven days; b) low temperature - the seeds were kept on moistened sand substrate for 15 days at 10 °C; c) GA3 -filter papers were moistened with 0.03% gibberellin solution. The seeds were kept on this substrate for two days; d) KNO3 -filter papers were moistened with solution of 0.2% KNO3. The seeds were kept under these conditions for two days; e) imbibitions in water -the seeds were imbibed for 12 hours at 30 °C between sheets of filter paper moistened with water; f) imbibitions followed by coat removal - the seed coat was removed after imbibitions in water for 12 hours at 30 °C.

2.3 Third stage: Study of physiological, ultrastructural and biochemical alterations during artificial aging.

To carry out the third stage, the *H. impetiginosus* seeds were collected in 2002 and stored for eight months. The seeds were aged and subjected to electric conductivity test, polyphenol assessment, water potential, immunohistochemical detection of microtubules, flow cytometry.

The electric conductivity test was carried out by the mass method. Each replication of 25 seeds was weighed and placed in plastic cups with 75 ml distilled/deionized water at 25 °C for 12 hours. The data obtained were submitted to analysis of variance and the means of the treatments were compared by the Tukey test at 5% probability.

The polyphenols were extracted using the method of Goldstein and Swain (1963), 80% methanol in water as extract and identified according to the Folin Denis method, described by AOAC (1990). The data obtained were submitted to analysis of variance and the means of the treatments compared by the Tukey test at 5% probability.

The water potential was calculated by measuring the water activity, using a water activity meter (HygroLab3, Rotronic), with five replications of five aged and dry seeds (kept for two days in a room at 15 °C and 70%RH) and five replications of five aged and wet seeds (right after aging), according to Decagon (2000).

Immunohistochemical detection of microtubules was carried out according to Xu et al. (1998). After chemical fixation and sectioning with an ultramicrotome, the slides with the sections were incubated with the primary antibody (mouse anti-α-tubulin) diluted in saline phosphate buffer (PBS) containing acetylated bovine albumin (BSAc). The slides were washed in PBS/BSAc and incubated with the secondary antibody (goat anti-mouse IgG conjugated with FITC) diluted in PBS/BSAc and washed again in PBS/BSAc. After applying citifluor, the slides were analyzed using a Confocal Laser Scanning Microscope MRC600, Bio-Rad, and digitized for analysis. *Inga vera* seeds were used as control.

Suspensions of intact nuclei from embryonic axes were prepared to analyze the DNA content. Ten replications of one embryonic axis from each artificial aging treatment were cut with razor blades, in Petri dishes containing 10 mM MgSO4.7H2O, 50 mM KCl, 5 mM Hepes, 1 mg mL^{-1} DTT and 2.5 mg mL^{-1} Triton X-100, 1% (w/v) PVP-40, on ice. The suspension was filtered through a nylon screen (53 µm) and 10 µl propidium iodide (final concentration 1mg/mL) were added (Arumuganthan and Earle, 1991). The analyses were performed in a Backman-Coulter EPICS XL-MCL flow cytometer.

RESULTS

3.1 First stage: Study of the germination performance after storage and artificial aging, submitted or not to disinfestation treatments.

The artificial aging effect on the *H. impetiginosus* seeds was variable in function of the storage time. The germination of the freshly collected *H. impetiginosus* seeds was favored by artificial aging for 96 hours both for seeds without disinfestation treatments and treated with benomyl. After three months of storage, there was a decrease in the germination of seeds aged for 24 hours, an increase in 48 hours aged seeds, and a further decrease in 72 hours aged seeds. With storage for six and nine months, a decline in germination was observed when seeds were aged for 72 hours, followed by an increase after 96 hours of ageing. The greatest oscillation in germination was observed in seeds stored for 12 months, with a fall in germination after 24 and 96 hours of artificial aging (Figure 1).

There was an increase in the germination percentage after storage of up to nine months from 66 to 92%, followed by a decrease. After 12 months, the value observed (76%) was greater than that presented by the freshly collected seeds (66%).

Regarding the disinfestation, the treatments with benomyl and sodium hypochlorite did not affect the variations in germination observed over the aging period, suggesting that the variations are not due the presence of microorganisms in the seeds (Figure 1).

3.2 Second stage: Study of treatments pre-germination

Lower germination rates were observed in freshly collected *H. impetiginosus* seeds than in those submitted to all the aging times and treatments pre-germination, except for 48 hours aging with imbibitions treatment followed by coat removal, that stood out from the others, attaining germination of around 80% (Figure 2) whereas stored seeds aged for 24, 48 and 96 hours and submitted to imbibitions reached higher germination rates.

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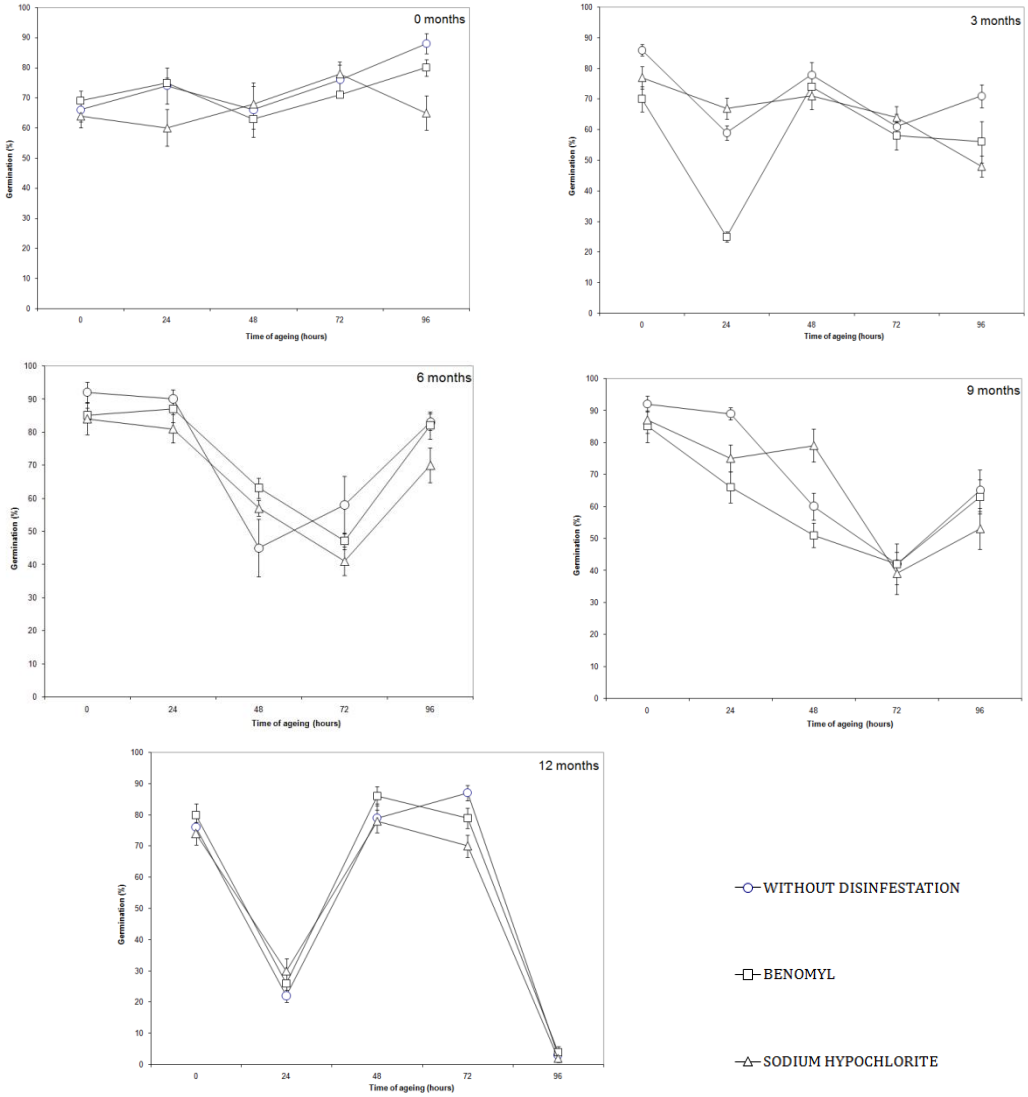

Fig. 1: Germination of *Handroanthus impetiginosus* seeds after storage for different periods (months), followed by artificial ageing. Bars represent SD.

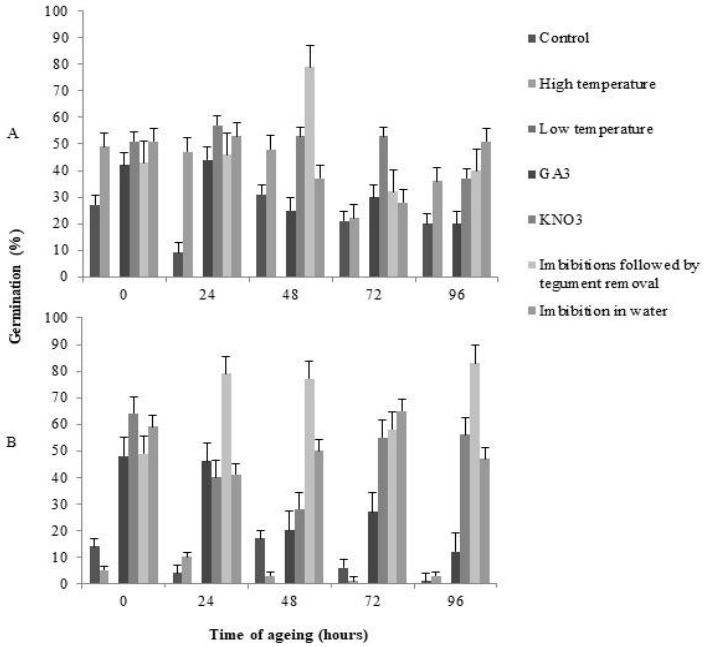

Fig. 2: Germination of freshly collected (A) and stored (B) *Handroanthus impetiginosus* seeds submitted to artificial ageing and treatments pre germination. Bars represent SD.

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Thus it can be suggested that *H. impetiginosus* seeds present secondary dormancy that can be broken by artificial aging followed by imbibitions and coat removal. The period of seed exposure to artificial aging that leads to higher germination rates depends on whether the seeds were stored or not.

3.3 Third stage: Study of physiological, ultrastructural and biochemical alterations during artificial aging

Artificial aging did not cause significant alterations in *H. impetiginosus* seed germination at 14 and 28 days. However, germination at 28 days was favored by artificial aging for 72 hours, although there were no significant differences in the polyphenol values (data not shown) or dry seed water potential (Figure 3).

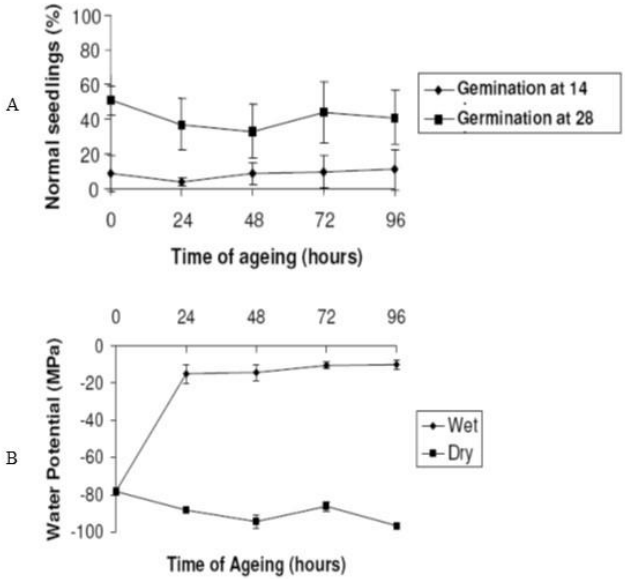

Fig. 3: Normal *Handroanthus impetiginosus* seedlings attained in the germination test of seeds stored for eight months (A) and Water potential of dry and wet *Handroanthus impetiginosus* seeds stored for eight months (B). Bars represent SD.

Microtubules were not detected in the *H. impetiginosus* seeds at different times of artificial aging (Figure 4). Regarding DNA content, there were no changes in the 4C value during the artificial aging, indicating the absence of DNA duplication (Figure 5).

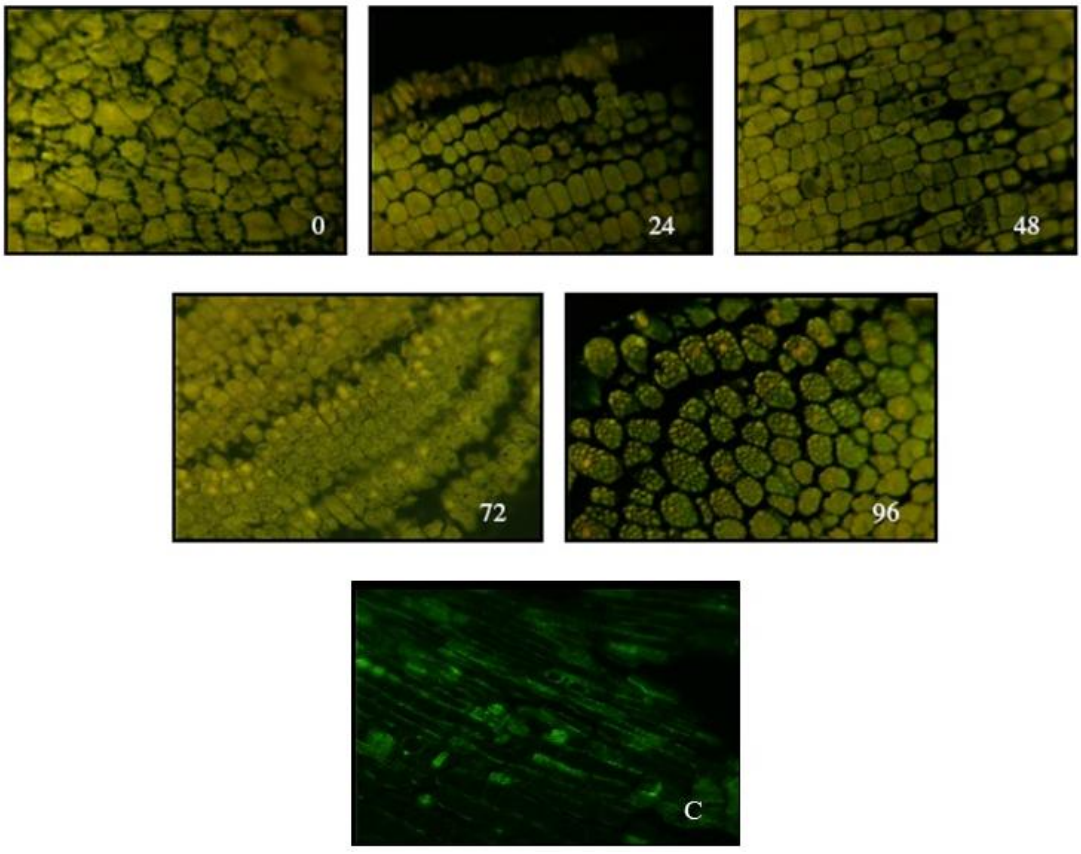

Fig. 4: Immunohistochemical detection of microtubules in *Handroanthus impetiginosus* seeds artificially aged for 0, 24, 48, 72 and 96 hours. (50x magnification). C = *Inga vera* (control)

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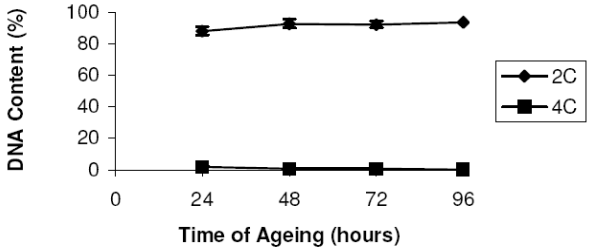

Fig. 5: DNA content of *Handroanthus impetiginosus* seeds stored for eight months and submitted to artificial ageing. Bars represent SD.

DISCUSSION

Handroanthus impetiginosus seed germination presented fluctuations during storage and artificial aging, as observed by Carvalho et al. (1976), Kano et al. (1978), Maeda and Matthes (1984), Figliolia (1988), Figliolia et al. (1988), Cunha et al. (1992), Kageyama et al. (1992), Mello and Eira (1995), Gemaque (1999) and Shibata et al. (2012). Increases in *H. impetiginosus* seed germination during storage were detected by Carvalho et al. (1976) at 63 days of storage, Kano et al. (1978) at 300 days, Figliolia (1988) at 240 days and Mello and Eira (1995) at 540 days. Thus the period and intensity of these fluctuations depend on the characteristics of the batches, such as initial seed quality, degree of maturation at harvest time, presence of microorganisms, dormancy environment and packaging used in storage. Furthermore, the conditions in which the seeds are exposed during development or processing can alter resistance to adverse factors, such as those that are used during the aging method.

A better germinability of artificially aged *H. impetiginosus* seeds was observed by Gemaque (1999), who reported that the aging method, which is performed in a water-saturated environment, promoted membrane restructuring, improving the germination process. Similarly, Schmidt (2000) pointed out that the high moisture content during artificial aging may activate the cell repair mechanism, as observed in *Arachis hypogacea* seeds (Jeng and Sung, 1994). However, alterations were not observed in the membrane system in the present study as measured by the electric conductivity test (data not shown) that could be related to germination increases.

Oscillations in seed germination rates throughout storage can be influenced by the presence of microorganisms and alterations in dormancy. Regarding the presence of fungi, in spite of low pathogen incidence in the *H. impetiginosus* seed batches tested, fungi of the *Alternaria*, *Penicillium* and *Fusarium* genera were detected during the germination test. The presence of these fungi was also observed by Carvalho and Muchovej (1991) and Degan et al. (1997), who reported that *Fusarium* genus is considered a possible pathogen of *H. impetiginosus* seeds and seedlings. Studies report that the artificial aging method may help in pathogen reduction in seeds. Karuna and Aswathaiah (1989) stated that the incidence of seed-borne *Alternaria tenuis* and *Phoma betae* in beetroots and *A. tenuis* in carrots decreased with the increase of the exposure of the seeds to artificial aging. According to Berjak (1995), prolonged heat treatments on wet seeds have been used to treat the seeds, with the advantage that this treatment reaches the inner tissues and any mycelium/inoculum situated more deeply in the seeds.

Cicero (1986) reported the presence of polyphenols, such as floridizin, salicilic acid, cumaric acid, chlorogenic acid and cumarin, in seed coat. According to Bewley and Black (1994), the polyphenols can act as germination inhibitors, because they consume oxygen during the oxidation process.

Although the seed drying process decreases metabolic activities and can inhibit DNA synthesis, accumulation of cells in G1 phase of the cell cycle also occurred in seeds with high water content, such as recalcitrant seeds of *Castanea sativa* (Roberts and Ellis, 1989; Bino et al. 1993) and Inga (Faria et al., 2004). However, Bino et al. (1992) observed that the 4C:2C ratio was constant in tomato seeds that were dried after imbibitions, showing a greater DNA accumulation in the 4C form or G2 phase.

According to Bino et al. (1993), cell accumulation in the G1 phase may have a physiological significance. It has been established that enzymatic and biochemical characteristics of cells in G1 are different from those in G2. Cells in G1 generally remain viable for a longer period of time (Yanishevsky and Stein, 1981), and possibly quiescent seeds whose cells remain in the G1 phase are more resistant to stress conditions (Clowes, 1965 and 1967; Deltour 1985; Bino et al. 1992).

From the results obtained in this study it can be assumed that other factors, like heat shock proteins, regulate *H. impetiginosus* seed germination during storage and artificial aging, and more consistent studies are needed in different phases of seed development and germination of this species. In a study conducted by Shibata et al (2012) was concluded that germination of *H. albus* seeds, when not subjected to accelerated aging, is favored by storage in cold chamber during three to six months, or from nine to 12 months when subjected to accelerated aging process, and storage proteins may be associated to those increases.

CONCLUSIONS

Handroanthus impetiginosus seed germination, depending on the initial quality of the batch, presented fluctuations during artificial aging, and there was no differential response of the disinfestation treatments.

H. impetiginosus seeds have dormancy that can be broken by artificial aging followed by imbibitions and later coat removal.

Ultrastructural and biochemical alterations were not observed for electric conductivity, polyphenol presence, water potential, microtubules and DNA content during the *H. impetiginosus* seed artificial aging process.

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Conflict of interest:

Author declare no conflict of interest.

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