

## Respiratory Rates of *Inga Vera Willd.* Subsp. *Affinis* (DC.) T. D. Penn. Seeds

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### ABSTRACT

The deterioration process of recalcitrant seeds is not fully known, mainly regarding the relation between fungi activity and respiration. *Inga vera* seeds are characterized by their high degree of sensitivity to desiccation and for only maintaining viability for a few weeks. The objective of this study was to identify the fungus action on respiratory metabolism of *I. vera* embryos subjected to chemical treatment in order to extend their storability. The results showed that the presence of fungi increases embryo respiration rate, thus affecting their conservation during storage. Treatment with carbendazin + thiram, especially at a dose of 200 ml per each 100 kg of seeds, was effective in controlling fungi, reducing the respiration rate and metabolism of *I. vera* embryos, increasing the maintenance period of their viability in storage.

**Keywords:** fungus, recalcitrant seed, storage.

## 1. INTRODUCTION

The growing demand for seeds of forest species for various purposes of use has a lack of information about quality, especially from the health point of view (Santos et al., 2011; Vechiato & Parisi, 2013). In Brazil, studies on forest seed health began in the 1970s, mainly for species classified as orthodox, but only in recent years research has been intensified on pathogenicity, transmission, damage caused by pathogens and the control of these. However, the absence of information on biochemical and cellular transformations occurring during storage make it difficult to understand the deterioration process resulting from fungus activity, especially in seeds susceptible to dehydration, initially denominated recalcitrant by Roberts (1973).

*Inga vera* Willd. subsp. *Affinis* (DC.) T.D. Pennington is a species from the Atlantic Forest that presents seeds with characteristic recalcitrant behavior, represented by the high degree of sensitivity to desiccation and its conservation of viability for only a few months. As they cannot be subjected to drying, recalcitrant seeds are dispersed with intense metabolic activity, do not demonstrate cellular dedifferentiation, and consequently can only be conserved until germination or rapid deterioration begins (Barbedo et al., 2013; Pammenter & Berjak, 2014).

The best conditions for seed storage to minimize the deterioration process are those that promote reduced metabolic activity, with reflexes on the embryo's respiration levels (Carvalho & Nakagawa, 2000). This process depends on and can be modified by water and thermal variations, as verified by Lamarca & Barbedo (2012) in *Caesalpinia echinata* L. seeds, so that the reduction in temperature and water content of the seeds are the main resources for reducing metabolic activity.

Reducing the temperature to levels that do not freeze tissues reduces metabolism, which decreases the respiratory rates of *I. vera* embryos and increases its storage potential, but this procedure also induces a manifestation of unidentified oxidative reactions (Bonjovani & Barbedo, 2014). Such storage conditions can still lead to embryo deterioration, both due to the metabolism itself and the growth of microorganisms favored by its high degree of humidity. The intense respiratory activity of the embryos, added to the activities of microorganisms, can cause the mass

to warm up, further accelerating the deterioration process (Carneiro & Aguiar, 1993).

The occurrence of storage fungi in recalcitrant stored seeds is one of the main detrimental factors to preserving viability (Berjak, 1995). However, the ones considered as "field fungi" have also been simultaneously detected in *Inga vera* embryos with deterioration progression during storage (Parisi et al., 2013). Fungi can become a major problem, even in cryopreservation of embryos or embryonic axes, which is currently the only way to preserve species with recalcitrant seeds in germplasm banks (Berjak et al., 2014; Walters et al., 2013)

The presence of fungi can increase the respiratory rates of the embryo-fungi complex, intensifying the embryos metabolism, thus affecting viability preservation. Deepening this knowledge will enable developing technologies which will increase the conservation period of forest seeds, resulting in greater availability of high-quality seeds and seedlings. Thus, the objectives of this work were to identify the fungal action on the respiratory metabolism of *I. vera* embryos and to verify the effectiveness of chemical treatment, aiming to maintain the viability time of these embryos during storage.

## 2. MATERIAL AND METHODS

### 2.1. Plant material

Ripe yellow *Inga vera* subsp. *Affinis* (Penn.) fruits were collected from marked branches of 30 matrix trees in parks and around the Piracicaba river in the municipality of Piracicaba, São Paulo (SP) (47°38'00" W, 22°42'30" S, 546 m altitude), in 2010 and 2011. The color of their skins/peels at the time of harvesting was classified as Yellowish (7,8 GY 8/10), according to Munsell Color (1952).

The fruits were manually opened in the laboratory to extract the seeds, not exceeding 24 hours after harvesting, and discarding those that presented damage by insects. The sarcotesta (teguments) were subsequently removed, and the embryos were stored in plastic bags in a BOD (Biological Oxygen Demand) chamber set at a constant temperature of 7 °C, without light until the beginning of the experiments, not exceeding seven days from harvesting.

## 2.2. Seeds physical, physiological and health evaluations

Embryos were evaluated for water content, germination, health and respiratory rates. The water content was determined by a forced air circulation oven at 103 °C for 17 hours (Brasil, 2009), with four replicates of five embryos. The results were expressed as percentage on wet basis.

The germination tests were conducted in germinators with a water curtain on the posterior wall with continuous light at 25 °C, using a paper roll with two sheets for the base and one for the cover. Normal seedlings were evaluated every two days until the 21<sup>st</sup> day after installation and the results expressed as a percentage, as described by Bonjovani & Barbedo (2008). Four replicates of ten embryos were used.

The health tests were performed according to the filter paper method (Parisi & Santos, 2011), and five embryos, equidistant from each other, were plated in 9-cm-diameter Petri dishes containing three sheets of filter paper, moistened with distilled water and maintained for seven days at 20 °C ± 2 °C under a 12-hour photoperiod. The evaluation of fungal structures in the embryos was performed under stereoscopic microscope and under optical microscopy in case of doubts. Each replicate was formed by two Petri dishes using four replicates.

## 2.3. Fungicidal treatment and respiration at different storage temperatures

*I. vera* embryos were divided into two groups in each year of production, one of which was treated with fungicide carbendazin + thiram at a dose of 80 ml for 100 kg of embryos for those harvested in 2010, and 200 ml for 100 kg for those harvested in 2011. The carbendazin-thiram blend has been successfully used to control fungi in Brazilian species of *Eugenia* spp. and *Inga vera* seeds (Oliveira et al., 2011; Parisi et al., 2013).

An analysis of respiratory rates of 100 embryos was performed by incubating them in four 600 ml hermetically sealed glass vials, each vial being a replication, following the methodology described by Lamarca & Barbedo (2012). The water content, germination and health were determined before introducing the embryos into the flasks and at the

end of the experiment, according to the previously described methodologies.

The lids were perforated and the holes were sealed by a rubber septum to collect air sample from the vial, which was measured in an Illinois 6,600 gas analyzer (Illinois Instruments, Inc., Johnsburg, USA). The total volume of air was considered as the volume of the vial subtracted from that of the incubated embryos. Closure of the flasks was considered as the beginning of the incubation period ( $t_0$ ), corresponding to the normal atmosphere (21% oxygen and 0.03% carbon dioxide).

Respiratory rates were analyzed by incubating the embryos at temperatures of 7 °C and 25 °C. Based on previous tests (data not shown), the O<sub>2</sub> consumption and CO<sub>2</sub> release assessments of *Inga Vera* embryos were carried out every five days until completing 15 and 25 days, in 2010 and 2011 respectively for those incubated at 7 °C; and daily until completing four and seven days, in 2010 and 2011, respectively, for those incubated at 25 °C. The difference in the evaluation periods is due to the fact that the respiration of *Inga Vera* embryos at low temperature requires several days for some change in the concentration of gases inside the bottles. The O<sub>2</sub> consumption and the release of CO<sub>2</sub> are so intense at room temperature that only a few days are sufficient for large changes in these concentrations. This was also verified for seeds from other Leguminosae species, such as *Caesalpinia echinata* Lam. (Araujo & Barbedo, 2017; Lamarca & Barbedo, 2012) and *Libidibia ferrea* (Mart. ex Tul.) L.P. Queiroz (Bragante et al., 2018).

O<sub>2</sub> consumption and CO<sub>2</sub> production by the embryos inside the vials were estimated by the difference between the values measured in the period  $n$  and the initial values ( $t_0$ ). The values obtained for O<sub>2</sub> and CO<sub>2</sub> (in percentage volume) were converted to micromoles using the local atmospheric pressure of 0.90 atm and calculations described in Lamarca & Barbedo (2012). These values were divided by the total dry mass of the embryo sample and by the number of days in which they remained incubated, obtaining values in micromoles per gram of dry mass per day ( $\mu\text{mol.gDM}^{-1}.\text{d}^{-1}$ ). The values obtained for O<sub>2</sub> and CO<sub>2</sub> were also presented in  $\mu\text{mol.gDM}^{-1}.\text{d}^{-1}$  for a period evaluated at 25 °C in 2010 (24, 48, 72 and 96 hours) and in 2011 (24, 48, 120, 144 and 168 hours).

The respiratory quotient (RQ) was calculated by the Equation 1 described by Kader & Saltveit (2002):

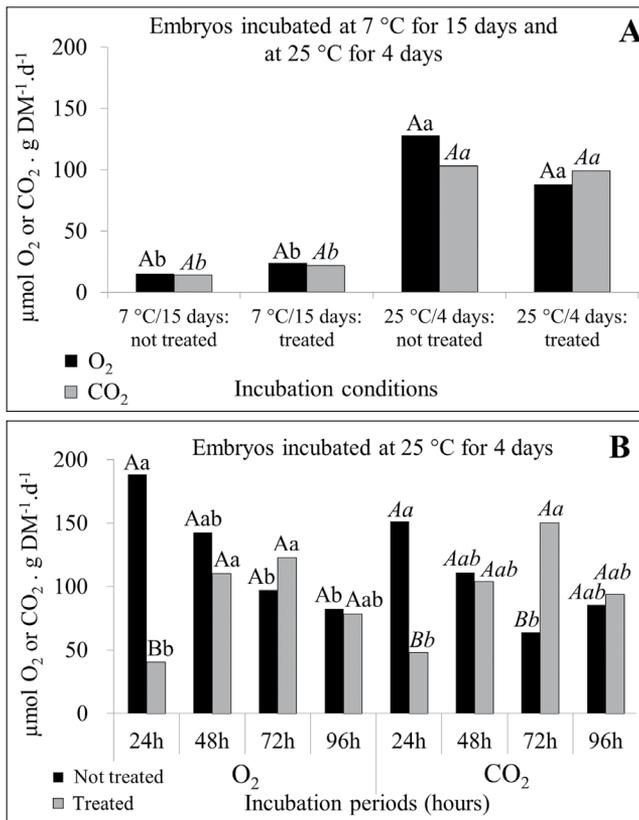
$$RQ = \text{CO}_2 \cdot \text{O}_2^{-1} \quad (1)$$

The results were submitted to analysis of variance at the 5% probability level through the Sisvar program (Ferreira, 2011), and when necessary the data were transformed to  $\log(x)$  and  $(x+0,5)^{0,5}$  to correct normality and heterogeneity (Santana & Ranal, 2004).

### 3. RESULTS AND DISCUSSION

The average water content of the *I. vera* embryos collected in January 2010 was  $60\% \pm 0.2\%$ , while those of 2011 had  $60\% \pm 0.6\%$ . Therefore, there was no major difference in the maturation stage of the embryos of the different years (Barbedo et al., 2013).

The respiratory rates of the embryos in both years, treated or not with the fungicide, were higher at 25 °C than those submitted to the temperature of 7 °C (Figures 1A and B and 3A and B). For example, in one day in 2010, embryos incubated at 7 °C released on average only 1/4 (ca. 25  $\mu\text{mol.gDM}^{-1}$ ) of the  $\text{CO}_2$  released by those incubated at 25 °C (ca. 100  $\mu\text{mol.gDM}^{-1}$ ) over the same period (Figures 1A and B). In 2011 (Figures 3A and B), embryo respiration rates were much higher than in 2010 at both temperatures. However, the difference between temperatures was maintained, and the embryos incubated at 7 °C released on average 1/6 per day (ca. 50  $\mu\text{mol.gDM}^{-1}$ ) of the  $\text{CO}_2$  released by those incubated at 25 °C (ca. 300  $\mu\text{mol.gDM}^{-1}$ ). The decrease in temperature causes lower respiration as a result of the reduced metabolism, thus being more favorable for the embryo conservation of this species (Andréo et al., 2006; Bonjovani & Barbedo, 2008; Faria, 2006).



**Figure 1.** Average daily O<sub>2</sub> consumption and average daily CO<sub>2</sub> production by *Inga vera* embryos harvested in January 2010 in Piracicaba, SP, treated or not with carbendazim + thiram at a dose of 80 ml of the commercial product for 100 kg of seeds, incubated at 7 °C for 15 days and at 25 °C for four days (A), and at each 24-hour period for embryos incubated at 25 °C for four days (B). The means followed by the same letter in normal font for O<sub>2</sub> consumption and in italics for CO<sub>2</sub> production, uppercase comparing chemical treatment, lowercase temperatures (A), uppercase comparing treatment with fungicide and lowercase incubation periods (B), do not differ by the Tukey test at 5% probability.

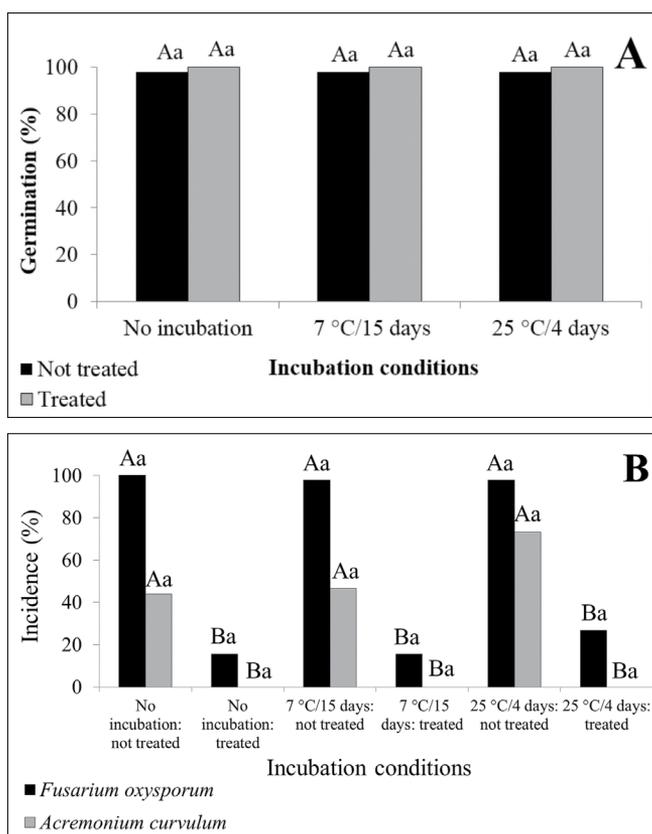
Variations in the environment temperature constitute a factor of great influence in the respiratory rates to determine the speed of the enzymatic reactions that characterize the metabolism of the plants (Marenco & Lopes, 2007).

Another fact that may have affected respiratory rates and germination (Figures 1A and B, 3A and B) was the incidence of fungi in the embryos (Figure 2B and 4B). This group of microorganisms plays a significant role in the post-harvest deterioration of the seeds classified as recalcitrant and of the intermediates (Berjak, 1995; Parisi et al., 2013).

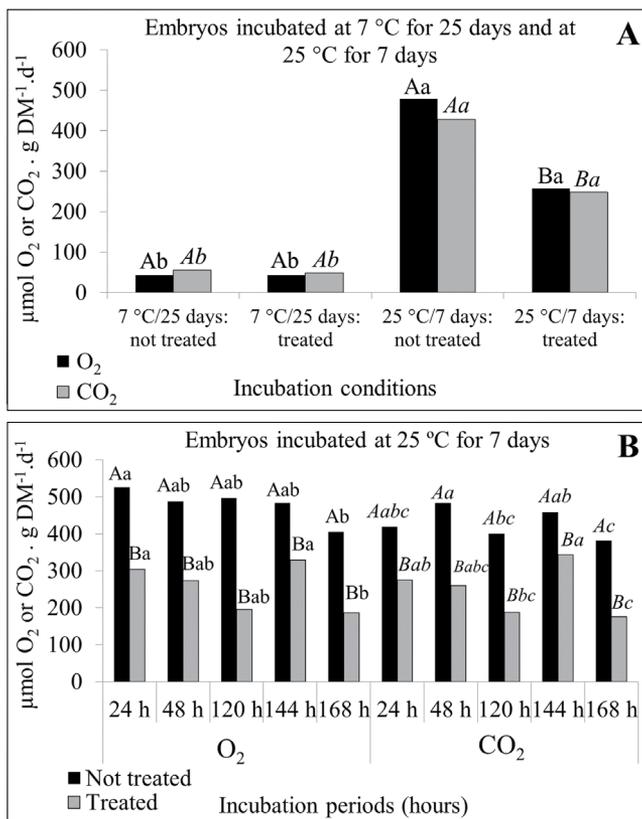
The evident action of the fungicide only on the first respiration reading of the embryos incubated at 25 °C for four days in 2010 may indicate its low efficiency for periods longer than 24 hours (Figure 1B). Therefore, *Fusarium oxysporum* and *Acremonium curvulum* present in the embryos (Figure 2B) may

have developed rapidly in the first hours, since they had favorable environmental conditions (temperature, humidity and substrate).

An important aspect verified for embryos incubated at both temperatures was the proximity of the mean O<sub>2</sub> consumption and CO<sub>2</sub> release values, with RQ near 1.0. These embryos present on average 57.6% of carbohydrates (Mello et al., 2010), which should be the main respiration substrate with complete oxidation occurring. In respiratory metabolism, RQ varies depending on the type of substrate that is oxidized (Marcos Filho, 2005; Marenco & Lopes, 2007), being close to 1.0 for carbohydrates and 0.6 to 0.7 for fatty acids (Tcherkez et al., 2003). However, when the results of respiratory rates are analyzed in the first 72 hours at 25 °C, it is noted that the RQ was very low for the untreated seeds (Figure 1B), indicating that oxidative processes other than respiration were present.



**Figure 2.** Germination (A) and incidence of fungi (B) detected in *Inga vera* embryos harvested in January 2010, Piracicaba, SP, treated or not with carbendazin + thiram at the dose of 80 ml of the commercial product for 100 kg of seeds, before and after incubation at 7 °C for 15 days and at 25 °C for four days. The means of germination and incidence of fungi followed by the same uppercase letters compare treatments with fungicide and lowercase incubation periods, they do not differ by the Tukey test at 5% probability.



**Figure 3.** Average daily O<sub>2</sub> consumption and average daily CO<sub>2</sub> production by *Inga vera* embryos, harvested in January 2011, Piracicaba, SP, treated or not with carbendazin + thiram at a dose of 200 ml of the commercial product for 100 kg of seeds, incubated at 7 °C for 25 days and at 25 °C for seven days (A) and at each 24-hour period for embryos incubated at 25 °C for seven days (B). The means followed by the same letter in normal font for O<sub>2</sub> consumption and in italics for CO<sub>2</sub> production, uppercase comparing chemical treatment, lowercase temperatures (A), uppercase comparing treatment with fungicide, and lowercase incubation periods (B) do not differ by the Tukey test at 5% probability.

In *Caesalpinia echinata* L. seeds, it was also found that the higher the temperature, the lower the RQ values, indicating oxidative processes that were probably related to the rapid deterioration of these seeds (Lamarca & Barbedo, 2012). This fact did not occur when the embryos were treated with fungicide, and therefore the presence of fungi may be associated with an increase of oxidative processes, which may accelerate the deterioration of *I. vera* seeds.

Considering that fungicidal treatment was not necessary to improve germination of the 2010 embryos, which was already high (Figure 2A), but substantially reduced the incidence of *F.oxysporum* and *A.curvulum* (Figure 2B), the respiratory rates of the embryos of that year could suggest that the presence of fungi in the embryos does not alter the respiratory metabolism of these embryos. However, analyzing the

daily incubation results at 25 °C (Figure 1B), it was noted that the respiratory activity of these embryos is not homogeneous in the first days. Thus, it was verified that the fungicide action during the first 24 hours substantially reduced the respiratory rate of the embryos, indicating that the presence of fungi increases O<sub>2</sub> consumption and CO<sub>2</sub> release, which could be by the fungus respiration itself, by the increase in the respiration of the embryos in response to the presence of the fungi, or by both.

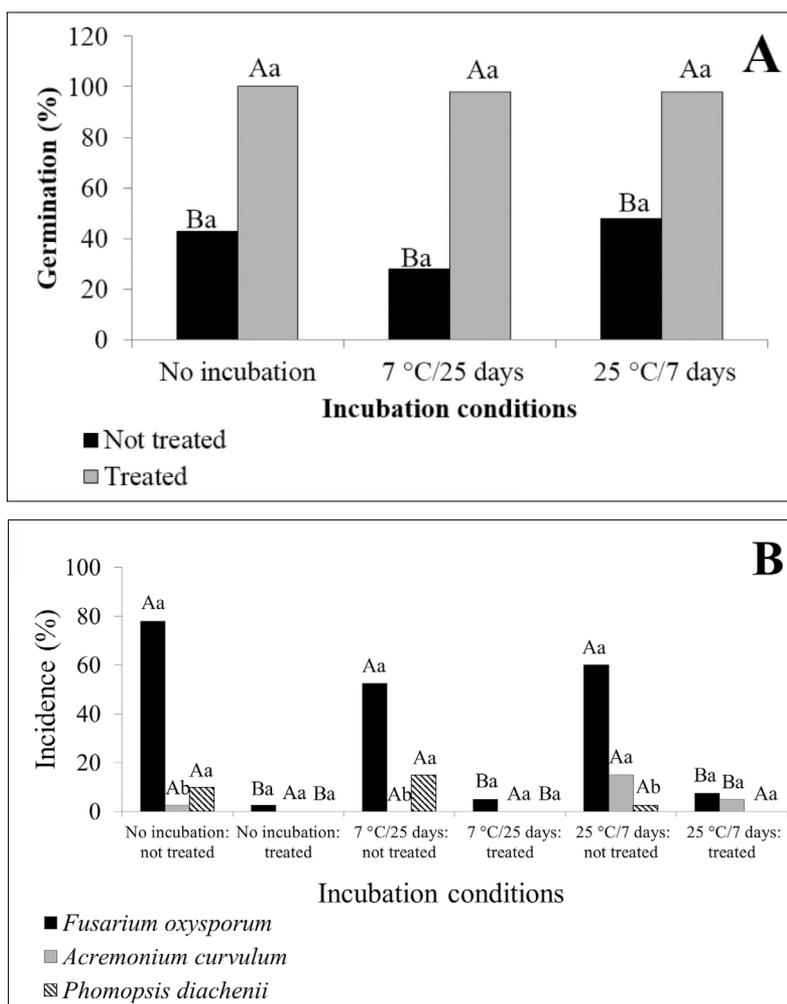
It was evident that the presence of fungi increases the respiratory rates of the embryo-fungus complex, which may represent an increase in the metabolism of the embryos. The fungi present in the untreated embryos incubated at 25 °C for 4 hours must have developed rapidly since they found favorable conditions, thus increasing the respiratory rates of the embryos.

The intense respiratory activity of the seeds, added to the activities of microorganisms, can cause the mass to warm up, further accelerating the deterioration process (Carneiro & Aguiar, 1993).

In the following readings, there was a reduction in the respiratory rates of the untreated fungicide embryos, which could be attributed to the depletion of embryo reserves, to the increase in CO<sub>2</sub> concentration and/or reduction of O<sub>2</sub> in the flask, or both. Meanwhile, there was an increase in respiratory rates up to 72 hours of incubation in the treated embryos, and then reduced. This may have occurred due to the loss in efficiency of the product, since the numerical value

of the incidence of *F. oxysporum* increased with the incubation time (Figure 2B).

There are higher respiratory values for the 2011 embryos in comparing their rates with those of 2010 (Figures 1 and 3). Seed respiration rates are usually lower in the final stages of maturation, as verified in legume seeds such as soybean (Miller et al., 1983), brazilwood (Araujo & Barbedo, 2017), and ironwood (Bragante et al., 2018). Therefore, it is probable that the embryos of 2011 with higher rates of respiration were at a maturation stage prior to 2010. Germination values with or without fungicide treatment reinforce this idea (Figures 2A and 4A).



**Figure 4.** Germination (A) and incidence (B) of detected fungi in *Inga vera* embryos harvested in January 2011, Piracicaba, SP, treated or not with carbendazim + thiram at the dose of 200 ml of the commercial product for 100 kg of seeds, before and after incubation at 7 °C for 25 days and at 25 °C for seven days. Mean of germination and incidence of fungi followed by the same uppercase compare treatments with fungicide, and lowercase incubation periods, they do not differ by the Tukey test at 5% probability.

The treatment with fungicide at the dose adopted in 2010 did not totally eliminate the *F. oxysporum* incidence in *I. vera* embryos, so that in order to control the inoculum in 2011 we opted for treatment with carbendazin + thiram in the dose of 200 ml of the product to 100 kg of seeds. According to Parisi et al. (2016), the same dose used was considered efficient in reducing the incidence of most of the fungi detected in *I. vera* seeds. In 2011, O<sub>2</sub> consumption and CO<sub>2</sub> release rates for *I. vera* embryos maintained at 7 °C (Figure 3A) again demonstrated that there were no differences in respiratory levels between treated and non-fungicidal embryos, even at a higher dose than in 2010, mainly due to the low metabolism of the embryos in this storage condition, independent of the *F. oxysporum* and *Phomopsis diachenii* fungi, which appeared to have a lower incidence in the incubated seeds (Figure 4B). According to Bonjovani & Barbedo (2008), the temperature of 7 °C slightly lower than the basal temperature (that is, the minimum required for the germination process) is the most adequate for storing *I. vera* embryos.

The application of fungicide in the 2011 embryos controlled *P. diachenii*, but even at a higher dose than in 2010 it did not completely eliminate the incidence of *F. oxysporum* or *A. curvulum* (Figure 4B). Also, it was not as efficient in reducing respiratory rates of the embryos incubated at 25 °C, with values close to 500 µmol.gDM<sup>-1</sup>.day<sup>-1</sup> to 400 µmol.gDM<sup>-1</sup>.day<sup>-1</sup>, respectively for O<sub>2</sub> and CO<sub>2</sub>, for values close to 300 µmol.gDM<sup>-1</sup>.day<sup>-1</sup> to 200 µmol.gDM<sup>-1</sup>.day<sup>-1</sup> (Figure 3B), as it was in the embryos of the previous year when they reached values close to 50 µmol.gDM<sup>-1</sup>.day<sup>-1</sup> (Figure 1B).

On the other hand, fungicide use reflected a significant increase in embryo germination (Figure 4A), even with higher respiratory rates (Figure 3A and B) than in the previous year (Figure 1A and B); but it is worth noting that the germination in 2010 was already close to 100% before application of the fungicide (Figure 2A), meaning that the embryos of 2011 were apparently more sensitive to the presence of fungi, again reinforcing the hypothesis of being more immature. It should be remembered that the fruits in both years were harvested at the time of their dispersal. Unlike in the orthodox seeds, the identification of physiological maturity in recalcitrant seeds is quite complex and difficult, because they do not have a characteristic cessation in metabolism

between formation and germination (Barbedo & Marcos Filho, 1998). Particularly in *I. vera* seeds, they can enter the germination phase even before their full development and dispersion, as demonstrated by Caccere et al. (2013). According Barbedo et al. (2013), research should be directed to extending the maturing period of recalcitrant seeds, keeping them bound to the mother plant until the maturation process has been completed, or to a stage as close as possible to the point of physiological maturity. In this way, the damage caused by microorganisms could be more easily proven.

In spite of this difficulty, even though the fungicide action was not 100% efficient in the treated seeds, it enabled us to visualize the increase in respiratory rates of the embryo-fungus complex caused by both the respiration of the fungi present in the untreated seeds and by their action, intensifying their metabolism and thus affecting embryo conservation, not only from the destruction of their tissues, but also by the consumption of their reserves, and without correct targeting for germination. These results showed the importance of defining correct methodologies for measuring respiratory rates for each species, since the dynamics of the gases during the incubation period of embryos or seeds may not be uniform during the evaluation period.

In light of the results, new experiments could be conducted which reduce the time for the first gas reading and also the interval between readings. The use of products and doses which completely eliminate the initial inoculum of fungi in the embryos could also bring important contributions to the knowledge of the real fungi action on the respiratory rates of the embryos and their conservation.

#### 4. CONCLUSIONS

The presence of fungi increases the respiratory rates of the embryo-fungus complex at measurable levels, demonstrably enhancing the metabolism and reducing the conservation of *I. vera* embryos.

The carbendazin + thiram treatment is efficient in reducing fungi associated to *I. vera* embryos and consequently the respiratory rates of the embryos, thus increasing the viability time of these embryos during storage.

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