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SHORT COMMUNICATION / *COMUNICAÇÃO*

Viability of *Puccinia psidii* urediniospores stored in different environments

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ABSTRACT

The objective of this study was to evaluate the viability and infectivity of urediniospores stored in liquid nitrogen (-196ºC), deep-freezer (-80ºC), modified-refrigerator (5ºC), biochemical oxygen demand (BOD 25ºC) and herbarium specimens (25ºC), for 150 days. The urediniospores were multiplied in Rose Apple plants (*Syzygium jambos*) and stored in the above conditions. Every 30 days, the germination and infectivity were evaluated, the first *in vitro* and the second on *Eucalyptus grandis* plants. The viability and infectivity of urediniospores stored in BOD and herbarium specimens were not preserved. The maximum germination (24.9%) and infectivity (162 pustules/leaf) occurring in modified-refrigerator environment were higher at 17.6 and 30 days, respectively. Urediniospore germination was highest when preserved in deep-freezer (34.3%) and liquid nitrogen (36.3%), at 45 and 40 days, respectively. The highest infectivity occurred at 60 days for the deep-freezer (77 pustules/leaf) and 90 days for the liquid nitrogen (67 pustules/leaf). Urediniospores kept in deep-freezer, liquid nitrogen, and modified-refrigerator maintained their viability and infectivity for 150 days.
Keywords: conservation, rust, liquid nitrogen, freezing.

RESUMO

Viabilidade de urediniósporos de *Puccinia psidii* Winter armazenados em diferentes ambientes

A preservação de urediniósporos de *Puccinia psidii* na ausência do hospedeiro é necessária para vários estudos biológicos, tais como patogenicidade e técnicas moleculares. Neste trabalho, foi avaliada a viabilidade e a infectividade desses urediniósporos em nitrogênio líquido (-196ºC), deep-freezer (-80ºC), geladeira (5ºC), BOD (25ºC) e material herborizado (25ºC). Os urediniósporos foram multiplicados em plantas de jambeiro (*Syzygium jambos*) e armazenados nos ambientes citados por seis meses e, a cada 30 dias, avaliados quanto à germinação *in vitro* e a infectividade em plantas de *Eucalyptus grandis.* Não houve preservação da viabilidade e da infectividade de urediniósporos armazenados em BOD e em material herborizado (25ºC). A germinação de urediniósporos (24,9%) e a infectividade (162 pústulas/folha) em ambiente de geladeira-modificado foram maiores aos 17,6 e aos 30 dias, respectivamente. A maior eficiência na manutenção de germinação dos urediniósporos armazenados em "deep-freezer" (34,3) e nitrogênio líquido (36,3) ocorreu aos 45 e 40 dias respectivamente. Enquanto que, a maior infectividade desses urediniósporos foi de 77 pústulas/folha aos 60 dias para "deep-freezer" e 67 pústulas/folha aos 90 dias para nitrogênio líquido. Os urediniósporos mantidos no "deep-freezer", no nitrogênio líquido e em geladeira-modificado permaneceram viáveis e infectivos até 150 dias.
Palavras chave: preservação, ferrugem, nitrogênio líquido, congelamento.

In vivo and *in vitro* studies with phytopathogenic fungi require preservation of pure cultures, preserving viability, pathogenicity and sporulation ability. In the case of biotrophic fungi, such as rusts, the preservation and multiplication of urediniospores is even more problematical, since it requires the colonization of living hosts for successful preservation. This is especially true when high amounts of inoculum are needed. The preservation of fungi for long periods of time requires methods that are able to keep their viability without morphological, physiological, or genetic modifications during the storage phase.

Rust spores survive in a dormant state in low humidity and regain viability when suitable conditions are restored. Dormancy can also be induced by low temperatures, and reduction of metabolic activity can be achieved by freezing (Smith & Onions, 1994). Rust spores can be successfully preserved at -80°C, although temperatures below -140°C are preferred (Smith & Onions, 1994). These temperatures can be obtained using liquid nitrogen. The preservation of urediniospores in liquid nitrogen requires dry storage (Holden & Smith, 1992; Cunningham, 1973; Prescott & Kerndamp, 1971). Biotrophic fungi unable to develop in growth medium can be stored by the methods cited above (Holden & Smith, 1992).

Loegering & Harmon (1961), using urediniospores of *Puccinia graminis* f.sp. *tritici* Erikss. & Henning were the first to investigate the preservation of rust spores in liquid nitrogen. The authors related a cold-induced dormancy in urediniospores stored at -196ºC, which could be reversed by heat treatments. Since then, the interruption of this cold-induced dormancy has been reported by other authors (Cunningham, 1973; Prescott & Kerndamp, 1971).

The viability and infectivity of *P. gramminis* f.sp. *tritici* urediniospores stored in liquid nitrogen were significantly reduced only ten years after storage (Kilpatrick *et al*., 1971). Studies of this nature still have not been done with *Puccinia psidii* G. Winter urediniospores. This biotrophic pathogen has great importance in the Neotropics, causing rust on plants of the Myrtaceae family, such as *Eucalyptus* spp., *Psidium guajava* and *Syzygium aromaticum* (Coutinho, 1998). Therefore, the present study's main goal was to evaluate different methods of preservation of *P. psidii* urediniospores. The best methods were those which allowed the spores to be kept viable, pathogenic, and with good sporulation capacities.

Urediniospores of *P. psidii* were collected from guava fruits (*Psidium guajava*) proceeding from Carmo do Paraiba, Minas Gerais. These were produced on plants of *Syzygium jambos*, according to Ferreira (1981). *Syzygium jambos* seedlings containing new sprouts were inoculated with a suspension of urediniospores, at a concentration of 1µg/mL. These seedlings were then kept in wet chambers, for 48 hours in darkness at 20±2ºC. After this period the humid chamber was removed and the plants remained in the growth chamber for 15 more days. Meanwhile, large amounts of mature urediniospores were collected from the leaves and stored in microtubes. Part of the infected *S. jambos* leaves were herborized.

Two samples with urediniospores were separated in order to quantify germination and infectivity. These samples were submitted to *in vitro* germination test and infectivity test in seedlings of *Eucalyptus grandis*. After that, remaining urediniospores were placed in screw cap microtubes and stored in five different environments (Table 1). With the exception of the herbarium, each environment received five microtubes filled with urediniospores which were kept this way for 150 days.

The urediniospores stored inside microtubes in the refrigerator were put in a support inside a dessicator containing a solution of 32% sulfuric acid, thereby maintaining the relative humidity of the air at 50 ± 5%. The dessicator was kept in the refrigerator at 5ºC. The urediniospores stored in liquid nitrogen, deep-freezer, and BOD environments did not undergo any previous treatment. The herbarium specimens consisted of twelve leaves with abundant uredia previously placed in wax paper envelopes. The temperature and relative air humidity were monitored by a digital thermohigrometer. Every 30 days, two leaves and a microtube from each environment were used to prepare the urediniospore suspension. This suspension was used in the germination and infectivity tests.

TABLE 1 - *P. psidii* urediniospores storage conditions

Methods	Temperature (ºC)	Humidity (%)
Liquid nitrogen	-196	0
Deep- freezer	-80	0
Modified-refrigerator	5	50
Biochemical oxygen demand (B.O.D)	25	58
Herbarium	25	48

The experiments were conducted in a random outline in which the plots were subdivided 5x6 with three repetitions. The plots were the five different environments, whereas the subplots were the months of storage. Every 30 days, one microtube from each environment was removed and kept at room temperature in order to reach the ideal work temperature. Urediniospore suspensions were prepared at a concentration of 0.9 times $2.5x10^5$/mL of mineral oil and 0.05% of Tween 80 (Tessmann & Dianese, 2002). With the aid of a Drigalsky rod, 100 microliters of this suspension was spread in 5cm Petri dishes containing 2% agar-water medium. Three dishes were inoculated per treatment. The dishes were then incubated in a BOD at 20±2ºC with 48 hours of darkness (Coutinho, 1998; Ferreira, 1981; Furtado *et al.*, 2003; Tessmann & Dianese, 2002). The germination percentage was evaluated using an optical microscope (100x), counting 100 urediniospores per repetition. The urediniospore was considered germinated when the length of the germination tube was larger than the actual spore.

P. psidii urediniospore infectivity was evaluated at 30-day intervals by inoculating *Eucalyptus grandis* seedlings. The experiment was conducted in a random outline in which the plots were subdivided 5x6 with six repetitions and two leaves per repetition. Urediniospore suspensions were prepared for each treatment at a concentration of 1µg/mL of water plus 0.05% of Tween 80. This suspension was used to inoculate the two-month-old *E. grandis* seedlings. The seedlings were then kept in dark humid chambers for a period of 48 hours, at 20±2ºC. After this period, the chambers were removed, and the seedlings remained in growth chambers at 20±2ºC. The infectivity of the urediniospores was evaluated by counting the number of pustules 12 days after inoculation.

In order to test the normality of the distribution of the germination data, the Shapiro-Wilk test and Proc Univariate command of the Statistical Analysis System SAS V8 were used. To adjust the data to the normal distribution curve and to keep the homogeny of the variances between the treatments, the germination data was transformed using the expression $\text{arcsen}\sqrt{\text{germ.}/100}$. This was checked with the Proc Sort command of the SAS program. The variance analyses of the subdivided plots, as well as the interaction between factors, were performed using the Proc GLM and Proc Reg commands of the SAS program. Thus, it was possible to describe the effects of the six-month storage in the germination of the urediniospores. The infectivity was obtained by counting the number of pustules per leaf. Even after the transformation using the expression $\sqrt{No.pustules \quad 0.5}$ the data did not present normal distribution or homogeny of the variance between the treatments, according to the Shapiro-Wilk test and the F_{max} of the SAS V8 program. Thus, the infectivity results were presented by descriptive statistics.

The interaction between environment and storage time was highly significant (Figure 1) only for the germination in liquid nitrogen, deep-freezer, and modified-refrigerator

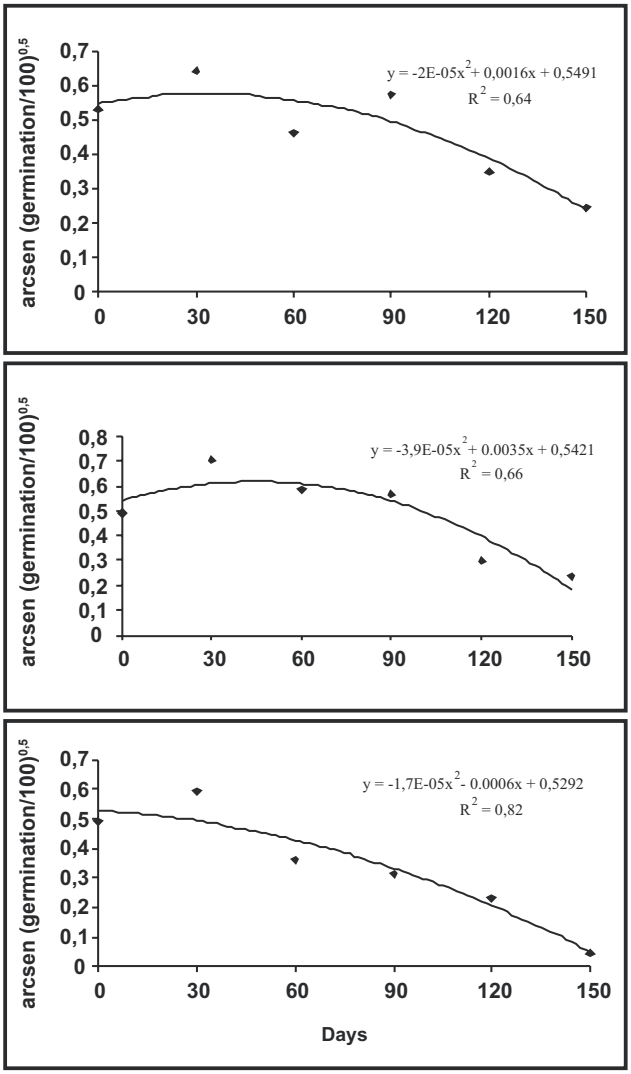

FIG. 1 - *Puccinia psidii* urediniospores germination under three different storage conditions A- deep-freezer, B - liquid nitrogen and C- modified-refrigerator in function of the storage time.

environments. Different environments influenced urediniospore germination in function to the storage period. The highest urediniospore germination value was registered in liquid nitrogen, deep-freezer, and modified-refrigerator environments. The germination rates were 0.6, 0.64 and 0.52% at 45, 40 and 17.6 days, respectively. These results were transformed to $\text{arcsen}\sqrt{\text{germ.}/100}$ and correspond to 34.3, 36.3, and 24.9%, respectively. It can be noted, however, that the shortest time in days to obtain the highest germination value was in the modified-refrigerator environment. This occurred because of the climatic aspects of the environment (50% humidity and 5ºC temperature), which offer enough conditions to keep the biological activity of the urediniospores stimulating germination in the environment. Since the biological activity was not reduced, the decline in urediniospore viability was constant during the storage period, which might explain the rapid loss of viability.

In the liquid nitrogen and deep-freezer environments the freezing temperatures and the absence of humidity induced the dormancy of the urediniospores (Smith & Onions, 1994). With regard to *P. psidii,* the dormancy was only interrupted with the removal of the urediniospores from the freezing environment and with the restoration of ideal germination temperature and humidity. According to Legard & Chandler (2000), the freezing process did not affect the morphological characteristics or the viability of *Colletotrichum fragariae, C. gloeosporioides, C acutatum* and *Phomopsis obscurans,* all pathogens of strawberry plants. These fungi have been maintained in deep-freezer at –95ºC for 21 months. These results confirm those related by Suzuki & Silveira (2003), whereas *P. psidii* urediniospores kept a viability of 3% for 100 days when stored at 4± 2ºC and 40± 5% relative air humidity. *Melampsora medusae-populina* urediniospores were kept viable for 5 years without induction of dormancy when these were freeze-dried and preserved at 1-2ºC (Shain, 1979).

The BOD and herbarium environments did not present significant interaction with the storage period. The results of *P. psidii* germination during the 150 days are presented in Table 2. The elevation of the temperature and the humidity significantly affected the germination of the urediniospores. According to Suzuki & Silveira (2003), the factor that mostly affects *P. psidii* urediniospores' viability during storage is the temperature. *P. psidii* urediniospore germination increased during their storage at 5, -80 and -196ºC. This might be explained by the removal of the auto-inhibition effect on urediniospore germination. According to Tessmann & Dianese (2002), the auto-inhibition effect present on the urediniospores is removed when they are germinated *in vitro* in the presence of mineral oil. Similar results were found by Suzuki & Silveira (2003), when they attributed the increase in germination to the auto-inhibition effect being overcome during the storage period.

The highest infection level (162 pustules/leaf), after the inoculation of the seedlings, was presented by the urediniospores stored in the modified-refrigerator environment, 30 days after storage (Figure 2). The same way that the temperature and humidity influenced the germination, these factors plus the presence of a susceptible host contributed to increase the infectivity in *E. grandis*. The deep-freezer and liquid nitrogen environments showed 77 and 67 pustules/leaf after 90 days of storage, respectively. Seedlings inoculated with urediniospores stored at 5, -80, and -190ºC were more infective, presenting an increase in the number of pustules/leaf during the storage period. The interruption of dormancy and the overcoming of the auto-inhibitor effect, allied to the presence of a susceptible host, may be an explanation for the greater infectivity of these urediniospores. The BOD and herbarium environments presented a significant reduction in the infectivity during the first days of storage, becoming null after 30 days. As in

TABLE 2 - Percentage of germination of urediniospores stored in BOD and herborized leaves at 25ºC

Days	BOD at 25ºC	Herbarium at 25ºC
0	23.16 a	23.16 a
30	1.33 b	2.00 b
60	0.33 b	1.33 b
90	0.00 b	1.00 b
120	0.00 b	0.00 b
150	0.00 b	0.00 b

Means followed by the same letter in the column are equal according to the Tukey test (P>0.05)

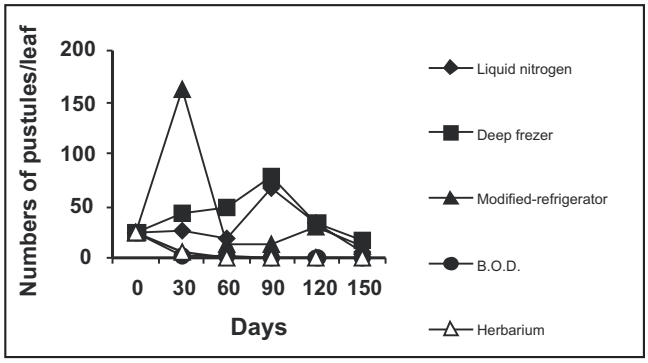

FIG. 2 - Number of pustules /leaf of *E. grandis* seedlings inoculated with urediniospores stored in five different environments during 150 days.

the germination of urediniospores, the number of pustules/ leaf was influenced by high temperatures. Germination and infectivity did not present correlation in any of the studied environments.

Urediniospores kept in microtubes in liquid nitrogen (-190ºC) and deep- freezer (-80ºC) germinated for at least 150 days of storage. The preservation of these spores for shorter periods of time will be satisfactory when kept in microtubes and stored in a dessicator inside a refrigerator, with 50±5% relative humidity and at 5ºC. Under such conditions, the urediniospores remained viable and infective for up to 150 days, even though their highest germination rate occurred after 30 days of storage.

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