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Re-Isolation Methodologies for Recovering Sporulation of Eucalyptus *Pestalotiopsis grandis-urophylla* Isolates after 14 Months Storage

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Authors' contributions

This work was carried out in collaboration among all authors. Authors LRS and DDCC contributed in the concept, data collection and data analysis. Authors MGM and PHPCM contributed in the data collection, data analysis, interpretation, in the manuscript preparation and critical revision, added the intellectual content. Authors TASO, EAAD, FR and SXS contributed in the data analysis and interpretation and in the critical revision, added intellectual content. Authors LRS and DDCC designed the study and interpretation and participated in the manuscript preparation and in the critical revision added the intellectual content. All authors read and approved the final manuscript.

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ABSTRACT

After long periods of storage, plant pathogen isolates lose their sporulation capacity. The objective of this study was to evaluate re-isolation methodologies for recovering sporulation of *Pestalotiopsis grandis-urophylla* isolates after subjection to a long period of storage. Isolates of *P. grandis-*

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urophylla were kept for 14 months on Petri dishes with PDA medium at 10°C. After this period, the isolate colonies showed reduced mycelial growth and no sporulation. The isolates were inoculated on healthy *Eucalyptus grandis-urophylla* leaves, and after ten days they were subjected to three re-isolation methods: scraping of the lesions (S) removing of injured plant tissue fragments, followed by disinfestation (D) and without disinfestation (WD). Then, the purified isolates were evaluated for the recovery of its sporulation ability. The different methods for re-isolation resulted in the occurrence of differences among the isolates, showing that sporulation is an isolate-dependent feature. The three methods (S, WD and D) allowed the sporulation recovery of *P. grandis-urophylla*, even after these isolates have been subjected to 14 months.

Keywords: Pathogenicity; mycelial growth; fungi storage.

1. INTRODUCTION

The *Eucalyptus* species is among the most important forest crops in the world [1]. Its spread to diverse production areas after the discovery of its economic value, showing fast diffusion and the ability to adapt to the most diverse climate conditions, from hot and dry, to humid and cold weather [2]. It presents a wide range of uses, including cellulose production, recovery of degraded areas, forests restoration, oils for pharmaceutical industries, ornamentation, windbreaks, firewood and charcoal production [3,4]. However, among the limiting factors to the crop, phytopathogenic agents, mainly fungi, can be present in different cycle stages, occurring in many places, species and seasons of the year, which can cause considerable reduction in the development and yield of eucalyptus [5].

Thus, leaf spots caused by fungal pathogens are considered the main agents responsible for losses in the field, especially in adult crops [6,7]. The fungus *Pestalotiopsis* sp. (Steyaert 1949) has been reported as a causal agent of lesions in eucalyptus, capable of penetrating even healthy leaf tissues, giving rise to necrotic areas with high development indexes, besides being related as a secondary pathogen in woody tissue [8]. The pathogen is also reported in a great number of alternate hosts, causing damages linked with different symptoms in native, ornamental, coniferous and fruit forest species [8].

Pestalotiopsis sp. is considered an anamorphic fungus and their spores (conidia) are easily spread, whose penetration occurs in vegetal tissues by natural wounds or openings [9]. In this context, studies involving virulence level of different isolates and other characteristics of pathogenicity are needed [10].

Therefore, the isolation procedures, conservation and use of microorganisms, although established in laboratory routine, are important for the research development and obtaining products of economic interest [11]. In such a way, the method of maintenance of microorganisms requires that these should be conserved under low biological activity, focusing on the preservation of sporulation and pathogenicity characteristics [12]. However, after successive replications, fungal isolates may lose sporulation capacity or even virulence [13,14].

Recently, phylogeny based on combined ITS, TUB and EF1-α DNA sequences evidenced *P. grandis-urophylla* as a new species [15]. In this way, the objective of any recovery method is to preserve the microorganism viability as long as possible, avoiding excessive occurrence of mutations that change its characteristics and, in addition, adapting the recovery to the most appropriate method [16]. Accordingly, it will also imply that it is not needed to work with host plants to recover fungi viability [17]. The objective of this work was to evaluate re-isolation methodologies developed for recovering sporulation of *Pestalotiopsis grandis-urophylla* isolates that lost this capacity after being submitted to a 14 months storage period.

2. MATERIALS AND METHODS

2.1 Achievement and Preservation of Fungi Isolates

In this study, *P. grandis-urophylla* isolates E-72-02, E-72-03, E-72-04 and E-72-06 of Plant Pathology Laboratory of State University of Goiás (Ipameri *Campus*) were stored for 14 months on Petri dishes with 20 ml of potato dextrose agar (PDA) medium at 10°C. After this period, the colonies of the isolates, which previously had good rates of mycelial growth and sporulation, presented a reduced growth capacity and nonexistent sporulation.

2.2 Evaluation of Pathogenic Potential after a Long Storage Period

Thus, the isolates were inoculated on healthy leaves of *E. urograndis* 'GG 100' with ages ranging from 18 to 24 months. For this step, the collected leaves were washed in tap water and allowed to dry in a laminar flow chamber for 10 min. Five holes were placed in the center of the leaf blade with the help of a platinum needle and an agar disc (5 mm Ø) containing mycelium deposited from each isolate. The inoculated leaves were submitted to controlled wet conditions in transparent acrylic boxes of the gerbox type (11x11x3.5 cm) containing a sheet of germination paper with constant moisture maintenance only on paper (six leaves of *E. urograndis* per isolate; a leaf of *E. urograndis* per gerbox). Then, measurements of the lesions on the abaxial face of the leaves were performed at 4, 6, 8 and 10 days after inoculation (DAI), with the aid of a digital caliper, obtaining the lesion size in mm^2.

2.3 Re-Isolation

After development of leaf lesions, the pathogen was isolated by three methodologies: (1) scraping of fungal structures contained on the injured plant tissue with a platinum needle, and subsequent transfer to Petri dishes containing potato dextrose agar (PDA) medium (S); (2) removal of fragments of injured plant tissue, after subjecting the fragments to a series of disinfestation steps: (a) immersion of the fragments in 70% ethanol (60 s), (b) subsequent immersion in 2% sodium hypochlorite (60 s) and (c) washing in sterile distilled water (SDW) for three times, 60 seconds each (D); (3) removal of fragments of injured plant tissue without disinfestation (WD).

The fragments obtained according to the WD and D (disinfestation) methodologies were transferred to Petri dishes containing PDA medium (1 fragment of 5 mm^2 $plate^{-1}$). Six Petri dishes were used for each *P. grandis-urophylla* isolate per each isolation method (S, WD and D).

The Petri dishes were then incubated in a BOD at 25°C with no light for evaluation of mycelial growth (based on the mean of two diametrically opposed measurements) and elimination of contaminated samples.

2.4 Purification and Evaluation of Isolate Sporulation

After the new isolation, the isolates obtained from each of the three employed methodologies (S, WD and D) were subjected to three replications until pure colonies were obtained. Then, the isolates were evaluated for sporulation, in order to verify if this capacity was recovered, using six Petri dishes per isolate. After 10 days of cultivation at 25°C and 12 h photoperiod, a total of 10 ml of sterilized distilled water (SDW) were added to the petri dish, followed by release of the spores with a Drigalsky's handle. The spores were then collected in a Becker containing 10 ml of SDW and filtered through sterilized gauze. The concentrations of the suspensions obtained were measured in a Neubauer chamber, counting spores in five quadrants of the chamber for each plate [18]. The experimental design used in all the experiments was completely randomized.

2.5 Statistical Analysis

To evaluate the pathogenic potential and re-isolation, data on lesions caused by *P. grandis-urophylla* and mycelial growth from 4 to 10 DAI, respectively, were submitted to regression analysis to obtain significant models. The severity and mycelial growth were integrated as an area below the disease progress curve (ABDPC) and area under the mycelial growth curve (AUMGC), respectively, using the formula ABDPC / AUMGC = Σ $[(y_1 + y_2) / 2) * (t_2-t_1)]$, where y_1 and y_2 are two consecutive evaluations performed at times t1 and t2, respectively. The sporulation evaluation of the isolates after re-isolation were submitted to variance analysis and Scott-Knott test ($P<0.05$). In all analysis, the statistical program used was Sisvar software 5.3 [19].

3. RESULTS AND DISCUSSION

After inoculation, typical symptoms of *Pestalotiopsis* infection were reproduced, characterized by dark brown necrotic lesions at 10 DAI (Fig. 1). There was no statistically significant difference between the isolates for *P. grandis-urophylla* lesion leaf area (LLA), according to Table 1. After regression analysis for the increase of leaf lesions over time, isolates E-72-02 and E-72-06 were fitted by a linear model, since isolates E-72-03 and E-72-04 were fitted by a polynomial of second degree. All the models were significant, even though with a great variation of the determination coefficient

(r^2). The isolate E-72-02 was superior to the others, presenting an area below the disease progress curve (ABDPC) with a mean of 188.4. The coefficients of variation were low, with a value of 16.07% for LLA and 19.65% for ABDPC.

According to Fig. 2, all isolates showed lesion growth from the first evaluation (4th day of inoculation). However, the isolates behavior was different, being that E-72-02 and E-72-06 presented continuous and linear growth of the lesions in all the evaluations, which was not observed for the E-72-03 and E-72-04, whose peak growth was recorded at 8 DAI.

In this evaluation, comparing the general mean of the methodologies used, it was verified that the re-isolation of *P. grandis-urophylla* by obtaining fragments of plant tissue without disinfestation (WD) was superior to the other methodologies, since the mean AUMGC for WD was 142.0 (Table 2). Afterwards, the scraping methodology was performed (S), with a mean AUMGC of 120.8, followed by obtaining colonies from fragments of disinfested lesions (D), with only 99.9 of AUMGC. The isolates E-72-02 and E-72-03 (Table 2) were superior to the others (146.0 and 142.7, of AUMGC, respectively) through comparison by means of the general AUMGC followed by E-72-04 (117.6 of AUMGC) and finally the isolate E-72-06, which presented the lowest mean among the four isolates evaluated (77.3 of AUMGC only).

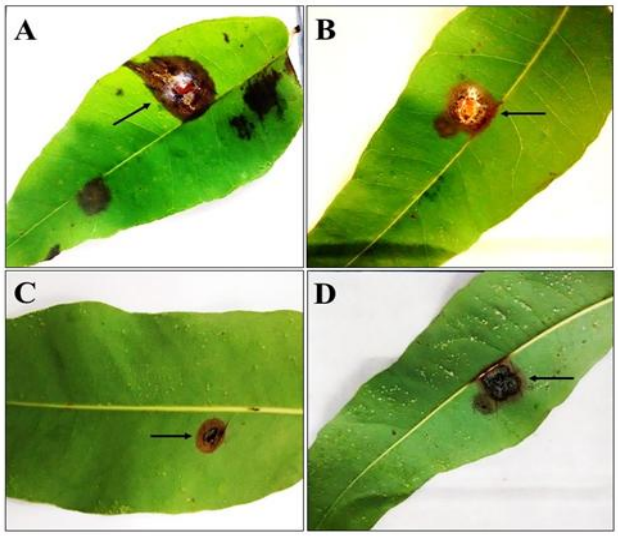

Fig. 1. *Eucalyptus urograndis* 'GG100' leaves inoculated with *P. grandis-urophylla*, at 10 days after inoculation (DAI), exhibiting symptoms and necrotic dark brown lesions. (A) isolate E-72-02: arrow shows typical *Pestalotiopsis* lesions, containing fungal mycelia developed on necrotic leaf tissue; (B) isolate E-72-03; (C) isolate E-72-04; (D) isolate E-72-06: lesion containing dark aggregations corresponding to acervuli development

Table 1. Lesion leaf area of *Eucalyptus urograndis* "GG100" (LLA) by *P. grandis-urophylla* 10 days after inoculation (DAI), regression models of development of leaf lesions and area under the disease progress curve (AUDPC) from 4 to 10 DAI

Isolate	LLA at 10 DAI (mm^2)[(†)]	Regression model	r^2 (%)	(P≤X)	AUDPC[(†)]
E-72-02	31.8 a	Y = 0.3535x + 28.7263	60%	0.05	188.4 a
E-72-03	27.8 a	Y = -1.6483x^2 + 27.2446x – 81.2160	93%	0.01	136.1 b
E-72-04	29.4 a	Y = -0.6328x^2 + 12.8039x – 34.9112	99%	0.05	128.3 b
E-72-06	28.2 a	Y = 2.3900x + 6.7556	83%	0.05	145.7 b
CV	16.07 %	-	-	-	19.65 %

[(†)] Values followed by the same letters in columns do not differ statistically among themselves, according to Scott-Knott test (P≤0.05)

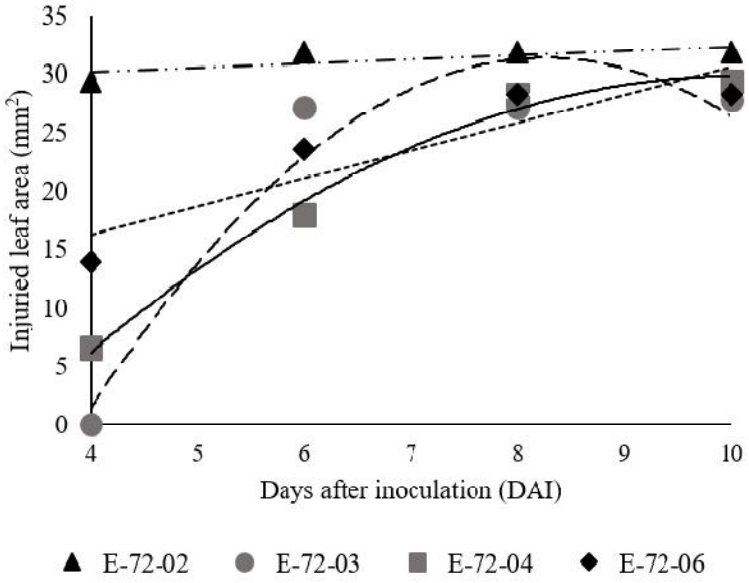

Fig. 2. Growth of *P. grandis-urophylla* lesions (mm^2) from 4 to 10 days after inoculation (DAI) on leaves of *Eucalyptus urograndis* "GG100"

Table 2. Area under the mycelial growth curve (AUMGC) of *P. grandis-urophylla* after re-isolation on PDA medium by using different methodologies for pathogenicity recovery

Isolate	AUMGC(‡)			
	S	D	WD	Average
E-72-02	166.2 aA	98.8 bB	173.0 aA	146.0 a
E-72-03	121.7 bB	128.3 aB	178.2 aA	142.7 a
E-72-04	116.3 bB	101.3 bB	135.3 bA	117.6 b
E-72-06	79.3 cA	71.1 cA	81.4 cA	77.3 c
Average	120.8 B	99.9 C	142.0 A	-
CV	9.27 %	23.28 %	13.92 %	15.42 %

(†) Means followed by the same lowercase letters in columns and capital letters in lines do not differ statistically among themselves, according to Scott-Knott test (P≤0.05)

(‡) S: Scraping injuries using platinum needle; D: Disinfection of infected vegetal tissue, with subsequent transplanting to PDA medium; WD: infected vegetal tissue transplanting directly to PDA medium (without disinfection)

In relation to the evaluation of sporulation of the isolates using the scraping (S) methodology for recovery, it was verified that the isolate E-72-06 was superior to the others regarding sporulation, presenting on average of 12.8 x 10^5 spores ml^{-1} (Table 3). In this methodology it was not possible to visualize any conidia of the fungus for isolates E-72-02 and E-72-04. For the disinfestation methodology (D), the isolate E-72-03 was superior to the others, presenting a mean of 13.7 x 10^5 spores ml^{-1}, followed by the isolate E-72-04 (11.2 x 10^5 spores ml^{-1}), and finally with a lower sporulation index, but not differentiating between them, isolates E-72-02 and E-72-06. Following the same results obtained in methodology (D), the isolate E-72-03 was superior to the others using the WD methodology (16.6 x 10^5 spores ml^{-1}).

No significant difference regarding the overall mean of sporulation recovery methodologies was observed, obtaining a mean value (average) ranging from 5.5 x 10^5 to 6.8 x 10^5 spores ml^{-1} (Table 3). When the average sporulation of each isolate was evaluated, it was verified that the isolate E-72-03 was superior to the others (13.2 x 10^5 spores ml^{-1}), followed by the isolate E-72-06 (7.4 x 10^5 spores ml^{-1}), isolate E-72-04 isolate (3.7 x 10^5 spores ml^{-1}), and isolate E-72-02, with only 0.4 x 10^5 ml^{-1}spores (Table 3).

In the present work, the pathogenicity of four *P. grandis-urophylla* isolates on eucalyptus leaves was investigated. However, other species of *Pestalotiopsis* have also been evaluated in such a way by other authors in eucalyptus crops [20]. According to [21], the species *Pestalotiopsis psidii*, *P. microspora*, *P. neglecta and P. clavispora*, caused lesions on leaves of guava (*Psidium guajava* L.), a tropical fruit plant, with similar characteristics in comparison to those presented on the present study, mainly regarding the size of the lesions, which varied between 18.2 and 36.4 mm^2. In a similar study, [22] identified *P. microspora* occurring in the ornamental plant *Hypericum patulum*, causing severe lesions in the leaf area, but with a lesion area of greater extent than in the present study, from 20 mm^2 reaching 113 mm^2 in size. Such behavior suggests that the size of lesions caused by *P. grandis-urophylla* may vary not only in function of the pathogen population, but also in function of the host type. In addition, the fact that there was no difference between *P. grandis-urophylla* isolates from this study (E-72-02; E-72-03; E-72-04; E-72-06), as to the size of the lesion leaf area, can be related to the fact that they belong to the same population and, therefore, descendants of a common ancestor [23].

Although no statistical difference was detected in lesion size, after regression analysis for the increase on leaf lesions over time, isolates E-72-02 and E-72-06 behaved differently from isolates E-72-03 and E-72-04. This event evidences different behaviors regarding the growth and evolution of lesions among isolates of the same *Pestalotiopsis* species [24]. According to [25], within the same group, there may exist organisms with special or different characteristics and this is due to the environment or mutations occurring within the isolates population.

Symptoms evidencing the appearance of lesions from the 4th day of inoculation. These symptoms consisted of necrotic lesions of dark brown color, with later agglutination of spores of black coloration, ranging in size from 27 to 31 mm^2 at 10 DAI. The rapid onset of symptoms at 4 DAI, observed for the E-72-02 and E-72-06 isolates, can be identified as an unusual result. [26], inoculating ten isolates of *Pestalotiopsis* spp. by spore suspension on leaves of Pecan (*Carya illinoinensis* [Wangenh.] K. Koch), observed the first symptoms only after 13 DAI. The initial performance observed for E-72-02 and E-72-06 can be explained by the fact that, although species of fungi of the genus *Pestalotiopsis* are considered weak pathogens, when areas containing previously injured tissues are present, the rapid growth of the pathogen is favored [27], suggesting that this fungus is an opportunistic pathogen.

A low value of the coefficient of determination (r^2) was observed for E-72-02 and E-72-06 in relation to the others. This may occur due to the diversity of the isolates, varying from more to less in terms of aggressiveness [28]. Incubation conditions were uniform for all, which supposedly would result in equal behavior. However, as of the ninth day, the E-72-02 and E-72-06 isolates generated a continuous lesion growth, which did not occur with E-72-03 and E-72-04. This indicates a certain level of complexity among the studied isolates. Variation in pathogenicity of fungal isolates have been frequently observed and is widely discussed by several authors due to isolate virulence, host specificity or tolerance and the consequences linked with genetic variability [29,30]. This indicates that there is enough scope for the inclusion of new studies related with the pathogenicity of *P. grandis-urophylla*.

In relation with the disease severity data, an area under disease progress curve (AUDPC) was calculated. The isolate E-72-02 AUDPC was higher in relation to the others. An explanation for such an event lies in the rapid early colonization of the leaf parenchyma affected by this isolate, since a mean lesion area of approximately 30 mm^2 was measured at only 4 DAI.

After evaluating the three methodologies for the re-isolation of *P. grandis-urophylla* (S, WD and D), it was possible to determine that WD was superior to the other methodologies, since the area under the mycelial growth curve (AUMGC), which has been used to compare mycelial growth between different fungal isolates or treatments [31], showed higher values in comparison with the other two methods, with R showing intermediate results. This performance can be explained by the absence of disinfectants, which, besides removing any contaminants, can also unduly disrupt the target fungus [32]. In addition, since inoculations and pathogenicity tests were conducted under strictly aseptic conditions, the absence of disinfectants did not result in contamination. In the rare occurrence of this event, contaminated samples were discarded. In addition, the low values of the coefficients of variation (%) found for AUMGC, demonstrate accuracy and reliability of the data obtained.

Table 3. Average number of *P. grandis-urophylla* spores per milliliter after obtaining purified colonies on PDA medium from the three tested methodologies for isolate recovery(†)

Isolate	Methodology used for the re-isolation(‡)			
	S	D	WD	Average
E-72-02	0 cA	0.6 x 10^5 cA	0.6 x 10^5 cA	0.4 x 10^5 d
E-72-03	9.2 x 10^5 bC	13.7 x 10^5 aB	16.6 x 10^5 aA	13.2 x 10^5 a
E-72-04	0 cB	11.2 x 10^5 bA	0 cB	3.7 x 10^5 c
E-72-06	12.8 x 10^5 aC	1.6 x 10^5 cA	7.0 x 10^5 bB	7.4 x 10^5 b
Average	5.5 x 10^5 A	6.8 x 10^5 A	6.0 x 10^5 A	-
CV	21.60 %	27.09 %	41.22%	31.43%

(†) Means followed by the same lowercase letters in columns and capitals letters in lines do not differ statistically among themselves, according to Scott-Knott test (P≤0.05)

(‡) S: Scraping injuries using platinum needle; D: Disinfection of infected vegetal tissue, with subsequent transplanting to PDA medium; WD: infected vegetal tissue transplanting directly to PDA medium (without disinfection)

When comparing the isolates through the general average of AUMGC, in relation to the methodologies used in this work, isolates E-72-02 and E-72-03 were superior to the others. This can be credited to the isolates genetic variability, which showed different behaviors when in contact with each technique. In general, it is assumed that these two isolates mentioned would respond well to the application of any isolation technique. In fact, as expected, AUMGC is a useful variable in the evaluation of isolates [26]. In addition, it is important to note that the methodologies tested in this work (S, WD and D) met an important requirement cited by [12], which is the maintenance of the original characteristics of the phytopathogens, such as pathogenicity, colony growth and sporulation.

The sporulation was the same for all isolates, even when submitted to different re-isolation methodologies. This can be explained by the fact that the conditions favoring fungal growth are not always the same for sporulation. In this case, disinfestation factors that inhibit AUMGC did not impair sporulation [25]. Moreover, the mechanisms that potentiate the sporulation of Hyphomycetes fungi are distinct from the techniques evaluated in the present work, which aimed at the recovery of pathogenicity and mycelial growth [18].

Contrary to what was observed for sporulation in relation to each isolation technique, a difference among the isolates regarding this physiological characteristic was observed. The difference in sporulation of isolates of the same species of *Pestalotiopsis* has already been observed in several studies [26,33,34]. According to [18], sporulation is an isolate-dependent characteristic, which may be related to several factors, such as influence of the technique used for obtainment of pure colonies, virulence of the pathogen and its reaction to physical factors that induce sporulation, such as continuous light.

It is important to take into consideration the isolates performance on the applied tests, specially the isolate E-72-03, which showed good results in all steps. Although laboratory conditions do not perfectly simulate natural conditions [24], the reaction of phytopathogens isolates to different physical factors, such as *in vitro* culture or any other factor that can influence the pathogenicity power, are the main tools that subsidies studies related to epidemiology of diseases under field conditions [10].

4. CONCLUSION

The differences regarding sporulation in relation to each obtained isolate, shows that this is an isolate-dependent characteristic. Besides, all evaluated techniques (S, WD and D) allow the recovery of *P. grandis-urophylla* sporulation after submission to a 14 months storage periods.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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