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Scientific article

Seed pretreatment for control of powdery mildew infection on purple ipe micropropagation

Talita Cristina Mamedes[1], Amanda Abdallah Chaibub[2], Kellen Cristhina Inácio Sousa[2], Maria Tereza Faria[3], Letícia de Almeida Gonçalves[3], Marta Cristina Corsi de Filippi[4], Leila Garcês de Araújo[2*], Sérgio Tadeu Sibov[1]

[1] Universidade Federal de Goiás, Escola de Agronomia, Laboratório de Cultura de Tecidos Vegetais, Goiânia-GO, Brazil.
[2] Universidade Federal de Goiás, Instituto de Ciências Biológicas, Laboratório de Genética de Microrganismos, Goiânia-GO, Brazil.
[3] Universidade Federal de Goiás, Instituto de Ciências Biológicas, Laboratório de Anatomia Vegetal, Goiânia-GO, Brazil.
[4] Embrapa Arroz e Feijão, Laboratório de Microbiologia Agrícola, Santo Antônio de Goiás-GO, Brazil.

Abstract

Purple ipe (*Handroanthus impetiginosus*) is an important tree species in Cerrado biome conservation and very popular at the landscaping and urban afforestation. However, its micropropagation is affected by pathogens, such as *Oidium* sp. The aim this study was evaluate the efficiency of seed treatments in the control of powdery mildew of purple ipe obtained by micropropagation. The symptoms were observed during *in vitro* germination, a Koch's postulates were performed for confirm the pathogenicity, colonization of the pathogen on the leaves was analyzed in optical and scanning microscopes and a scale to evaluate severity was proposed. Two experiments were realized to powdery mildew control using a completely randomized design, with 30 replicates. First experiment: Seeds were treated with ethanol (Et), chlorothalonil + thiophanate-methyl (C+TM), and sodium hypochlorite (NaOCl); second experiment: Seeds were treated with Et, NaOCl, C+TM, and neem oil. Disease severity and area under the disease progress curve (AUDPC) were assessed in both experiments. Disease symptoms and typical pathogen structures were observed, and the pathogenicity was confirmed. The disease severity was reduced by 30.78% in 1.5% neem oil for 10 min when compared with C+TM for 15 min. We conclude that neem oil can be a strategy sustainable for the control of powdery mildew in purple ipe in tissue culture.

Keywords: alternative control, fungicide, *Handroanthus impetiginosus*, neem oil, tissue culture.

Resumo

Pré tratamento de sementes para controle de oídio em micropropagação de ipe roxo

O ipê-roxo, *Handroanthus impetiginosus*, é uma espécie arbórea importante na conservação do bioma Cerrado e muito procurada no paisagismo e arborização urbana. No entanto, sua micropropagação é afetada por patógenos, como *Oidium* sp. O objetivo deste estudo foi avaliar a eficácia dos tratamentos em sementes no controle do oídio do ipê roxo por micropropagação. Os sintomas foram observados durante a germinação *in vitro*, os postulados de Koch foram realizados para confirmação da patogenicidade, a colonização do patógeno nas folhas foi analisada em microscópios ópticos e de varredura e uma escala para avaliação de severidade foi proposta. Foram realizados dois ensaios para controle do oídio em delineamento inteiramente casualizado, com 30 repetições. Primeiro experimento: sementes foram tratadas com etanol, clorotalonil + tiofanato-metílico (C+TM) e hipoclorito de sódio; segundo experimento: sementes foram tratadas com etanol, NaOCl, C+TM e óleo de neem. A severidade da doença e a área abaixo da curva de progresso da doença foram quantificadas. Os sintomas da doença e estruturas típicas do patógeno foram observados e a patogenicidade foi confirmada. A severidade da doença foi reduzida em 30,78% com 1,5% de óleo de neem por 10 min quando comparado com C + TM por 15 min. Concluímos que o óleo de neem pode ser uma estratégia sustentável para o controle do oídio em ipê roxo na cultura de tecidos.

Palavras-chave: controle alternativo, cultura de tecidos, fungicidas, *Handroanthus impetiginosus*, óleo de nem. Abstract

*Corresponding author: leilagarcesaraujo@gmail.com

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Introduction

Handroanthus impetiginosus (syn. *Tabebuia avellanedae*), popularly known as purple ipe, is a tree found in several Brazilian biomes; the conservation of this species is crucial since it is included in the official list of endangered flora species (Cncflora, 2012).

The purple ipe has great beauty when in bloom, so it is one of the most popular species in Brazilian landscaping and in urban afforestation (Bassegio et al., 2017). Due to its importance, it was the first Cerrado tree to have its genome completely sequenced (Silva-Júnior et al., 2018). The purple ipe is widely used in the reforestation of degraded areas and urban landscaping owing to its recognized beauty (Chaves, 2018; Máximo, 2020). Furthermore, it is an arboreal species of economic value because of its wood presents good durability and resistance in addition to its ornamental and medicinal uses (Santos et al., 2020) and a source of *β*-lapachone, one medicinal component (secondary metabolite), present both in stem periderm and woody part, which has anti-inflammatory, analgesic, antibiotic and antineoplastic properties (Bang et al., 2016; Campanholi et al., 2018).

Oidium sp., a biotrophic pathogen is the causal agent of powdery mildew in horticultural, fruit, ornamental, and forest species, including purple ipe and causing significant losses (Liyanage et al., 2017; Li et al., 2020). The disease occurs in all regions of the world and causes physiological changes such as reducing photosynthesis and perspiration (Suthaparan et al., 2016; Zhai et al., 2020;).

In vitro propagation is highlighted as a tool for the deployment of clonal forests, because enables to rapid production of standardized plants of selected genotypes on large scale (Mushtaq at al., 2017). The contamination by microorganisms and pathogens represents one of the major problems of tissue culture and may become a limiting factor in establishing protocols for some species (Orlikowska et al., 2017; Kim et al., 2017).

One of the pathogens of purple ipe is powdery mildew which occurs in natural habitat and conventional system. The pathogen is recognized by the plasma membrane, overcoming the initial and later defense responses. Subsequently, they secrete effector proteins that alter the resistance and manifestation of defense responses in the cytosol through the haustoria, their feeding structures (Li et al., 2020), and the analysis of the colonization of the interaction becomes important to reveal the forms of control.

In conventional agriculture the disease control is performed only with fungicides such as chlorothalonil + thiophanate-methyl (C+TM), which have a systemic and contact action (Ihara, 2019). Alternative control, such as the use of oils and plant extracts of neem (*Azadirachta indica* A. Juss), is widely used in organic crops, with acaricide, nematicide, and fungicide effects (Mishra et al., 2017), however, no reporting of your use in micropropagation has been reported.

Therefore, the aim this study was evaluate the efficiency of seed treatments in the control of powdery mildew of purple ipe obtained by micropropagation.

Material and methods

Seeds of purple ipe from a matrix located in the Leolídio di Ramos Caiado municipal park in Goiânia, Goiás State, Brazil (16°37'46"S 49°15'23"W) were used.

In one previously experiment well-formed seeds with no visible signs of injury or disease had their membrane wings removed. For first cultivation the seeds were disinfested or not with ethanol and NaOCl for 10 and 20 min. After the treatment of seeds in a laminar flow cabinet, they were rinsed three times with autoclaved H_2O and inoculated in sterile flasks of 300 mL containing 30 mL of MS medium (Murashige and Skoog, 1962), supplemented with inositol (100 mg L^{-1}) and sucrose (30 g L^{-1}), and solidified with agar (7 g L^{-1}). The pH of the medium was adjusted to 5.8 before autoclaving at 120 °C for 20 min. The cultures were kept in a growing room at a temperature of 25 ± 2 °C, photoperiod of 16 h light, and irradiance of 36 mmol m^{-2} s^{-1} (Abbade et al., 2007; Paiva et al., 2007). The design was entirely randomized, with 50 replicates and one seed per experimental plot.

After the presence of a pathogen in the *in vitro* plants was detected in all plants, the identification was performed on the basis morphological characters (type and form of conidiophores and conidia) using the mycological key by Barnett and Hunter (2003). At 60 days, 12 leaf samples of purple ipe with or without symptoms of the disease were collected and fixed in FAA_{70} (Formalin –70% alcohol – acetic acid) (Johansen, 1940) for 24-h and conserved in 70% ethanol for further observation using optical microscopy and scanning electron microscopy (SEM).

For observation under optical microscope, transversal sections were clarified with 6% NaOCl, washed in distilled water, and subjected to double coloration with 0.1% basic fuchsin and 0.3% Astra blue in the ratio 1:3 for 3 min. Then, they were mounted between the slide and a coverslip with hydrating glycerin. The observation was performed using a Leica DM 500 optical microscope with a digital camera attached. For observation using SEM, transversal and longitudinal sections approximately 1 cm in length were dehydrated in increasing ethanolic series (70%, 80%, 90%, and 99.1% ethanol, 15 min each); adapted from Chaibub et al. (2020) subjected to CO_2 critical point drying, and covered with gold films in a Denton Vacuum film deposition system. The images were obtained using a Jeol (JSM-6610) scanning electron microscope equipped with EDS System (Thermo Scientific NSS Spectral Imaging), operated at 4 Kv, and installed at the High-Resolution Microscopy Multiuser Laboratory (LABMIC).

In this case, in which powdery mildew is a mandatory and biotrophic parasite, in which it needs its living host and cannot be cultivated, Koch's postulate is accomplished by exposing a sick plant to a healthy plant. A Koch's postulate was performed on the healthy leaves, detached from purple ipe cultivated *in vitro* for 15 days, to determine and confirm the host-pathogen association. Three detached healthy leaves were exposed to a diseased leaf and incubation in a germination chamber at 25 ± 2 °C, photoperiod of 16 h light. The evaluation of the presence of powdery mildew symptoms was performed at 10 days after inoculation.

Two experiments were performed in a completely randomized design, with 30 replicates to evaluate the efficiency of other seed disinfectants in the control of powdery mildew and each experimental plot consisted of one seed. In the first experiment, the seeds were subjected to the following treatments: control (no treatment), 70% ethanol for 2 min, C+TM for 5 min, and 2% NaOCl for 10 min; 70% ethanol for 2 min, C+TM for 10 min, and 2% NaOCl for 10 min; 70% ethanol for 2 min, C+TM for 15 min, and 2% NaOCl for 10 min. The fungicide (C+TM) was used at the dose recommended by the manufacturer to control powdery mildew.

In the second experiment, the following treatments were tested: control (no treatment), 70% ethanol for 2 min and 2% NaOCl for 10 min; 70% ethanol for 2 min, C+TM for 5 min, and 2% NaOCl for 10 min; C+TM for 15 min, and 1.5% neem oil for 10 min. In both experiments, after the treatments, the seeds were inoculated in MS medium, kept under the same conditions for *in vitro* germination as described in the previous experiment.

In the first experiment, powdery mildew severity was measured at 60, 80, and 100 days and in the second experiment from 14 to 28 days with two-day intervals between the evaluations to determine the area under the disease progress curve (AUDPC), according to Shaner and Finney (1977). In both experiments, severity evaluation was performed using a scale adapted from Paz Lima and Café Filho (2004) developed for this work (Table 1), ranging from 0 (no symptoms) to 5 (very severe infection) and the data were transformed using arcsen $\sqrt{x}/100$. Subsequently, the data were subjected to analysis of variance and means compared using the Tukey test at 5% probability with the R software.

Table 1. Severity assessment scale developed for powdery mildew in purple ipe plants obtained by *in vitro* germination.

Grade	Infection	Description
0	no symptoms	Leaves without signs of injury
1	mild	Sporulation with less than 25% of affected leaf area, without deformation, without necrosis, and falls from primary leaves
2	average	Sporulation with less than 25% of affected leaf area, with deformation, with or without necrosis, and falls from primary leaves
3	moderate	Sporulation with more than 25% and less than 50% of affected leaf area, with deformation, with necrosis, folding and falls of the primary leaves
4	severe	Sporulation with more than 50% of affected leaf area, with deformation, with necrosis, rolling and falls of the primary leaves
5	very severe	Total coverage of the affected leaf area, with deformation, with necrosis, folding and falls of the primary leaves

Results and Discussion

In the present study, the occurrence of powdery mildew in purple ipe plants may have been favored by *in vitro* culture conditions such as controlled temperature, light intensity, and the culture medium used. According to Rana et al. (2018) and Gu et al. (2020) these variables influence spore production. For biotrophic parasites like *Oidium* sp. the sporulation is high when conditions favorable to photosynthesis (long photoperiods, high luminous intensity, and broad light spectrum) during the colonization period. Such advantages for the reproduction and development of the fungus are found during the process of *in vitro* seed germination.

This is the first report of the occurrence and control of powdery mildew in plants obtained by *in vitro* germination of purple ipe seeds. The scale adapted from Lima and Café Filho (2004) developed for this work (Table 1, Figure 1), ranging from 0 (no symptoms) to 5 (very severe infection) was efficient to evaluate of disease.

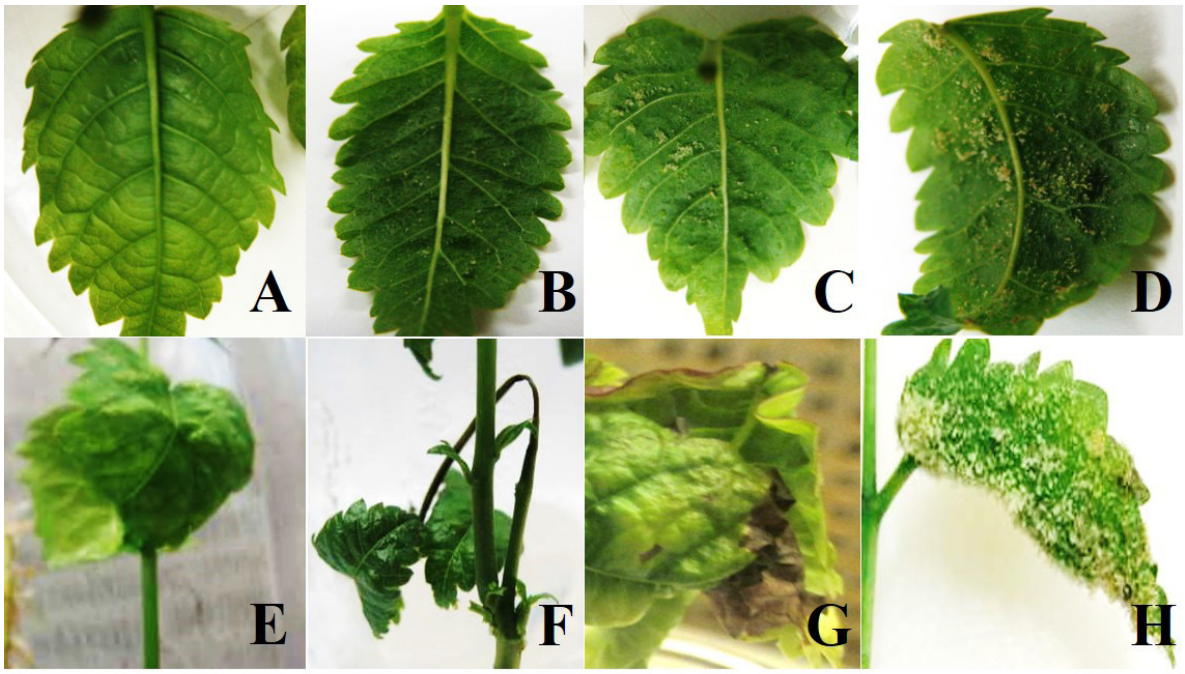

Figure 1. Symptoms observed in purple ipe plants during *in vitro* germination used to adapt the scale proposed by Lima and Café Filho (2004). A) leaves without symptoms, B) sporulation with less than 25% of affected leaf area, C) sporulation with more than 25% and less than 50% of affected leaf area, D) sporulation with more than 50% of affected leaf area, E) rolling, F) stem necrosis, G) leaf necrosis, and H) sporulation with total coverage of the affected leaf area.

The main symptoms observed and described on the scale that were illustrated are leaves without symptoms (Figure 1A), leaves with different degrees of sporulation (Figures 1B, 1C, 1D, 1H), deformations, curling and leaf fall (Figures 1E, 1F), necrosis on the stem (Figure 1F) and on the leaves (Figure 1G).

After the symptoms description and observed, Koch's postulates were carried out to confirm the association of the pathogen to the host and with this we confirm this association. When selecting a diseased plant (Figure 2A) and placing it in contact with a healthy plant (Figure 2B), we observe the reproduction of symptoms (Figure 2C) and therefore, we confirm the association of the pathogen with the host.

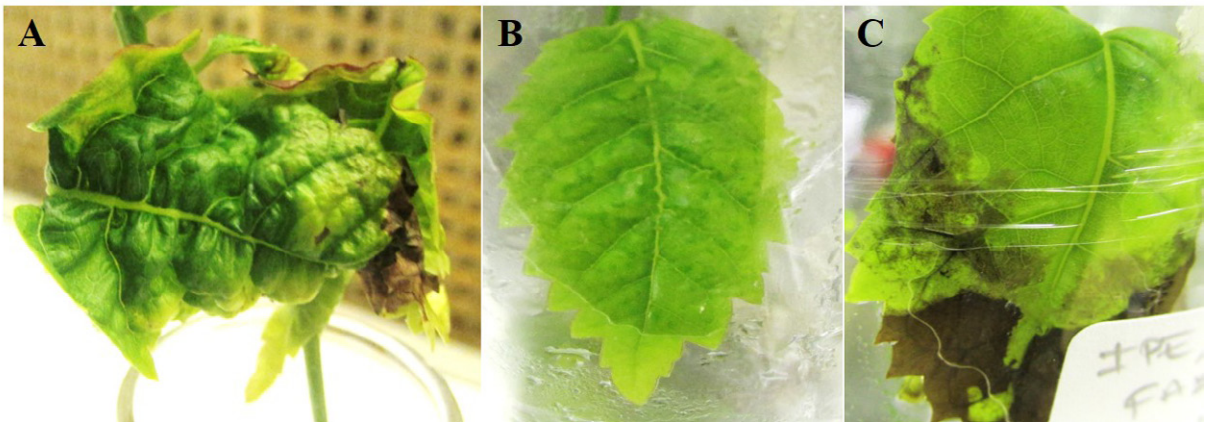

Figure 2. Koch's postulates performed to confirm the powdery mildew association in purple ipe plants during *in vitro* germination. A) symptomatic leaf, B) leaf without symptoms, and C) leaf with symptom after contact with first symptomatic leaf.

Botelho et al. (2008) highlighted the importance of pathogens associated with purple ipe seeds since they are harmful to the production of this species' plants. Our study demonstrated and confirmed that powdery mildew is a potential pathogen for purple ipe and is carried by the seed.

Transverse and longitudinal sections of the leaves with symptoms observed under optical microscope (Figures 3A, 3B) and SEM (Figures 3E, 3F), respectively showed the presence of typical conidiophores of the genus *Oidium*: simple, erect, and increased in length as the conidia are formed in the chain.

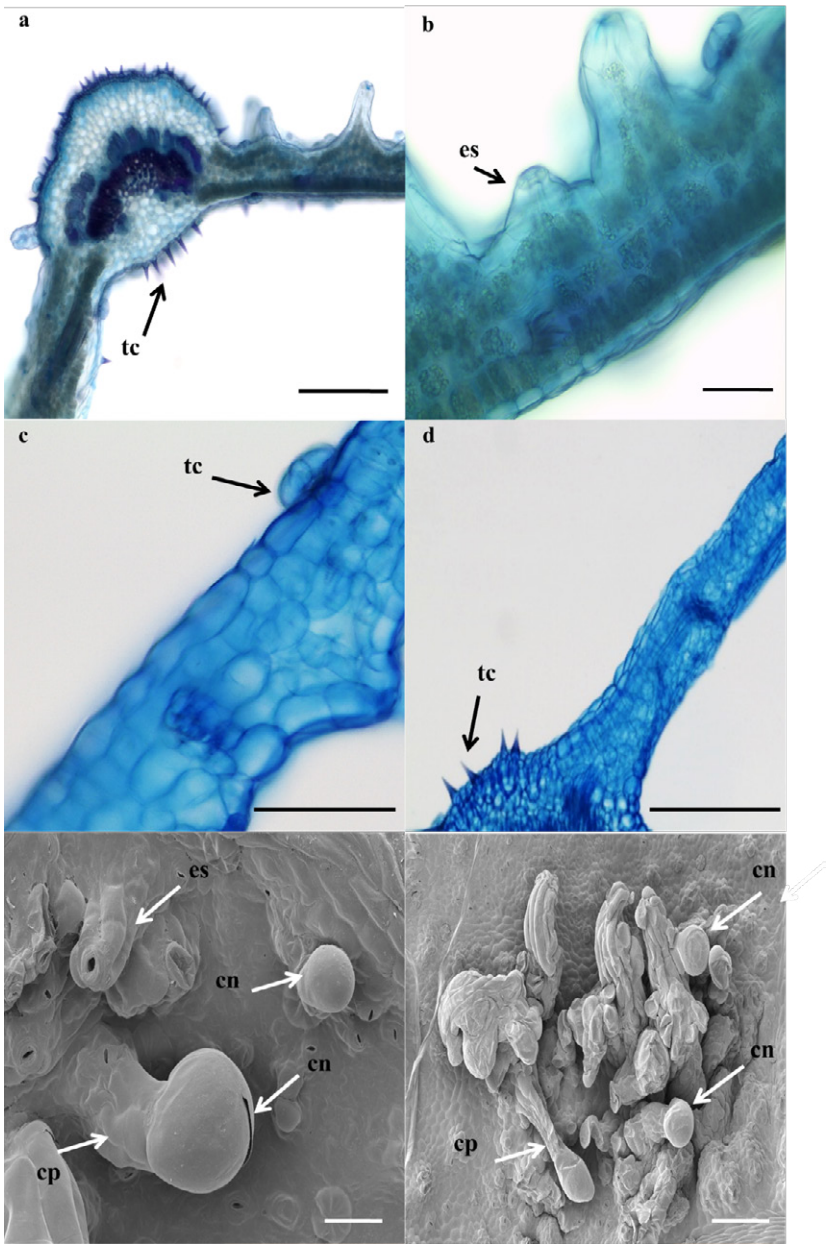

Figure 3. Description of the occurrence of powdery mildew in purple ipe plants during *in vitro* germination. A and B) optical microscope of purple ipe plants sections with conidiophores of powdery mildew (cp) and typical structures of leaves as trichomes (tc) and stomata (es) (Bars = 100 and 50 µm, respectively), C and D) leaves without symptoms with typical structures of leaves as trichomes (tc) and without conidiophores of powdery mildew (Bars = 50 and 500 µm, respectively), E and F) elevation of the epidermal region under the stomata seen in scanning electron microscopy (es) and formation of conidia (cn) and conidiophore (cp) of powdery mildew on leaves (Bars = 50 and 100 µm, respectively).

The conidia are typical of the genus *Oidium*: cylindrical, hyaline, and produced in basipetal chains (older conidia at the apex and younger at the base of the chain). Transverse and longitudinal sections of leaves without symptoms observed under optical microscope demonstrated absence of powdery mildew structures and the presence of characteristic leaf structures such as trichomes (tc) (Figures 3C, 3D) also observed in symptomatic leaves (Figures 3A, 3B). The colonization of the pathogen altered the development of the stomas on the abaxial side of the leaf. Although the stomata normally develop at the same height as the other epidermal cells, i.e., on the symptomatic leaves, the stomas near the regions of colonization developed above the other cells (Figure 3E). In the samples analysed using SEM, conidiophores that formed from the differentiation of hyphae in regions near the stomas were also identified (Figure 3E and 3F).

In both experiments the treatment control without seed treatment not germinated because the high occurrence of powdery mildew, others fungi, and bacteria.

In the first experiment at 60, 80 and 100 days after the *in vitro* establishment, severity was high in plants subjected to 2 min in 70% ethanol, 15 min in C+TM, and 10 min in 2% NaOCl) by 2.92, 2.92 and 3.15, respectively when compared with 70% ethanol for 2 min, C+TM for 5 min, and 2% NaOCl for 10 min (1.57, 1.57 and 1.65); and 70% ethanol for 2 min, C+TM for 10 min, and 2% NaOCl for 10 min (2.75, 2.75 and 2.95)

Table 2. Severity at 60, 80, and 100 days of powdery mildew in purple ipe plants during *in vitro* germination in seed treatments: 70% Ethanol for 2 min, C+TM for 5 min, and 2% NaOCl for 10 min; 70% Ethanol for 2 min, C+TM for 10 min, and 2% NaOCl for 10 min; and 70% Ethanol for 2 min, C+TM for 15 min, and 2% NaOCl for 10 min.

	Powdery mildew severity*		
Treatments	**60 days**	**80 days**	**100 days**
70% Ethanol for 2 min, C+TM for 5 min, and 2% NaOCl for 10 min	1.57 ab	1.57 ab	1.65 b
70% Ethanol for 2 min, C+TM for 10 min, and 2% NaOCl for 10 min	2.75 ab	2.75 ab	2.95 ab
70% Ethanol for 2 min, C+TM for 15 min, and 2% NaOCl for 10 min	2.92 a	2.92 a	3.15 a

Means followed by the same letters in column were not significantly different from each other according to Tukey's test ($P < 0.05$).

* Scale ranging from 0 (no symptoms) to 5 (very severe infection).

The aim of the chemical treatment of seeds of a forest species is to control or reduce diseases efficiently. Besides ethanol and NaOCl, some treatments used in this study included systemic and contact fungicide (C+TM). This product is recommended for the control of powdery mildew in field conditions in several crops such as beans, melon, watermelon, and roses (IHARA, 2019).

Treatment with 2 min in 70% ethanol, 5 min in C+TM, and 10 min in 2% NaOCl promoted the lowest values of severity. In general, plants from seeds exposed for a less time to the disinfecting agents were less fragile, presenting lower severity than that of other treatments. The shorter exposure time of fungicide (C+TM) meant that the tissues of the seeds were less degraded, leading to less physiological alterations. Thus, the higher severity rates observed in in other treatments may be related to the toxic effects of the exposure time to the disinfecting agents used.

In the second experiment, from 14 (disease emergence) to 22 evaluation days, the treatment with 1.5% neem oil for 10 min was the only one that differed from the others from 16 days to the last evaluation day. The treatments with 70% ethanol for 2 min and 2% NaOCl for 10 min); 70% ethanol for 2 min, C+TM for 5 min, and C+TM for 15 min presented 3.56, 3.43, and 3.8 of severity on the last evaluation day, respectively, while treatment with 10 min in 1.5% neem oil presented 2.63

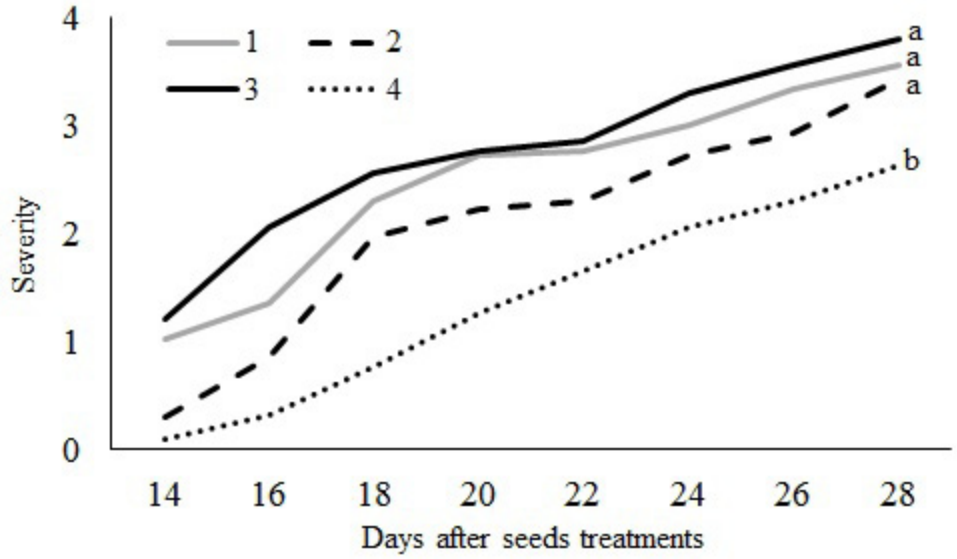

Figure 4. Area under disease progress curve of powdery mildew in purple ipe plants during *in vitro* germination at 14, 16, 18, 20, 22, 24, 26, and 28 days of seed treatments 1 (70% Ethanol for 2 min and 2% NaOCl for 10 min), 2 (70% Ethanol for 2 min, C+TM for 5 min, and 2% NaOCl for 10 min), 3 (C+TM for 15 min), and 4 (1.5% neem oil for 10 min). Means followed by the same letters were not significantly different from each other according to Tukey's test ($p < 0.05$).

The AUDPC in the treatment 10 min in 1.5% neem oil was 19.63, and was significantly lower than 70% ethanol for 2 min and 2% NaOCl for 10 min; 70% Et for 2 min, C+TM for 5 min and C+TM for 15 min with 36.90, 29.90, and 40.46 respectively (Figure 4). The disease severity was reduced by 30.78% in the treatment with 1.5% neem oil for 10 min when compared with C+TM for 15 min.

Cardoso et al. (2012) aimed to control *Oidium anacardii* by using C+TM, and observed a reduction in the severity and AUDPC in cashew; however, the authors found that it was not the most efficient treatment for disease control, as also observed in our study. In the second experiment (A2), the best treatment was with the neem oil, which showed a significant difference in severity of powdery mildew and AUDPC compared to those of other treatments.

Alternative products, such as neem oil, probably leave no residues on plant tissues and have low production costs, and thus are more advantageous. Neem oil as an alternate control agent has proven to be efficient, including for powdery mildew. The neem oil reduced powdery mildew severity in rose (Ramos et al., 2020), garden pea (Mishra et al., 2017) and in *Cannabis sativa* L. of over 50% (Scott and Punja, 2020). Spraying neem oil on *C. sativa* with powdery mildew was also efficient and the results did not differ statistically from that fluopyram treatment (Scott and Punja, 2020).

There are four registered products based on neem oil in Brazil, however, it has an indication for the control of powdery mildew in cashew and beans. Our results, thus, show potential use of the product registered also for the purple ipe (Agrofit, 2021).

The effects that contributed to the efficiency of neem oil in the control of powdery mildew may have been direct through the action of toxic compounds in the death and inactivation of the pathogen and indirect through the induction of resistant plants eliciting pathways signaling defense responses.

In our study will contribute to future micropropagation for this specie on the selection of superior genotypes, including the evaluation of *in vitro* resistance in many genotypes, with improved environmental control and in less physical space. In addition, this study provides a promising method of seed treatment that could be used *ex vitro*.

Conclusions

We conclude that *Oidium* sp. is a potential pathogen to the purple ipe and the neem oil can be a strategy sustainable for the control by seed treatment of powdery mildew in tissue culture.

Author contribution

TCM: Preparation, implementation, data collection, evaluation, and scientific writing; **AAC:** Statistical analysis of sample data and intellectual contributions to scientific research; **KCIS**: Implementation, data collection and evaluation; **MTF**: Performed optical microscopy of the anatomical sections; **LAG**: Performed scanning microscopy of the anatomical sections; **MCCF**: Intellectual contributions to scientific research and scientific writing; **LGA**: Research co-orientation and scientific writing; **STS**: Research orientation and scientific writing.

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