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Detection Methods of Fungi in *Ilex paraguariensis* Seeds

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Abstract

There has been an increase in the occurrence of fungal diseases in *Ilex paraguariensis* A. St.-Hill. (yerba mate) resulting from their domestication. Seed health tests may indicate pathogens prior to sowing, enabling their control. Our study sought to compare the efficacy of fungi detection methods on yerba mate seeds. Seeds from four lots were subjected to the following detection methods: BDA, V8 and filter paper; as well as the performance of asepsis with 1% sodium hypochlorite and 70% alcohol. The fungi found in the lots were: *Penicillium* spp., *Cladosporium* spp., *Aureobasidium melanogenum*, *Aspergillus* spp., *Pestalotia* spp., *Nigrospora* spp., *Trichoderma* spp., *Fusarium graminearum*, *F. oxysporum* and *F. guttiforme*. The asepsis in the seeds reduced the occurrence of contaminating organisms. There were differences between the evaluated lots regarding fungal incidence. In general, the BDA and V8 culture media showed better results in fungal detection.

Keywords: seed pathology, yerba-mate, sanity tests.

1. INTRODUCTION AND OBJECTIVES

Ilex paraguariensis A. St. Hill (Aquifoliaceae), known as yerba mate, is a characteristic species of Mixed Ombrophilous Montana Forest, associated with *Araucaria angustifolia* and found in some regions belonging to Semi-deciduous Seasonal Forest (Carvalho, 2003; Mazuchowski, 1989).

Yerba mate integrates one of the main Brazilian agroforestry systems, contributing to income generation for small and medium farmers in Southern Brazil (Sturion & Resende, 2010). Its use in the area of pharmaceuticals comprises a potential market and alternative to its commercialization as a beverage, in which it is widely used for preparing teas and *chimarrão* (Maccari, 2005; Sturion & Resende, 2010).

There have been great advances related to genetic improvement and management of yerba mate recently, with the main focus on increasing the productivity of herbs (Grigoletti & Auer, 2005). However, the occurrence of diseases in the crop has grown considerably due to its domestication, the increase in its planted area and the implemented management practices.

The most serious problems are registered in seedling nurseries of the species, favoring the development of pathogens due to the humidity conditions and high plant density (Grigoletti & Auer, 2005).

A potential source of disease-causing pathogens are the contaminated seeds used for seedling production due to the protection and nutrients they provide to micro-organisms (Machado, 2012).

One of the main genera of fungi considered as pathogenic to forest seeds is *Fusarium*, which can colonize fruits, branches, leaves and inflorescences (Poletto et al., 2006). This genus causes root rot in yerba mate, which can cause the death of plants and constituting a growing problem in the southern region of Brazil (Poletto et al., 2006; Poletto et al., 2010).

The filter paper method is one of the most used for fungi detection, characterized as an easy-to-conduct and low cost method. It enables the identification and quantification of the main fungi and can be used for all the seeds (Parisi & Santos, 2015).

When the filter paper method does not provide suitable conditions for the growth and sporulation of certain fungal

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groups, agaric media containing solutions and nutrients essential for their growth and reproduction are used, such as potato dextrose agar (BDA), known as universal culture medium (Henning, 2004; Zauza et al., 2007).

Thus, our study sought to compare the efficacy of different fungus detection methods on yerba mate seeds.

2. MATERIALS AND METHODS

The yerba mate seeds were harvested at four locations with distinct characteristics (Table 1). The seeds were harvested from several matrices at least 50 meters apart at each harvest location. The harvests were performed by means of fabric screens placed below the treetops. Fruits with dark purple coloring were considered mature, classified as color 2.5/1 F: 5Y on the Munsell Soils Table (2009), which consists of a color notebook initially used for characterizing soils. The seeds were extracted in a sieve and running water, and manually sorted to remove the impurities.

Determination of the moisture content of the seeds was carried out in a greenhouse with forced air circulation, regulated at 105 ± 3 °C for 24 hours. The results were expressed as a percentage based on the wet weight of the seeds, according to the Rules for Seed Analysis (Brasil, 2009).

Three methods of detecting pathogens in the seeds were tested: 1) BDA culture medium (200 grams of potato extract + 20 grams of dextrose + 20 grams of agar/L) + 0.02 grams of streptomycin; 2) diluted V8 culture medium (40 ml of V8 juice – vegetable juice "Campbell Soup Co" + 0.6 grams of $CaCO_3$ + 20 grams of agar + 1.0 grams of sucrose + 0.2 grams of yeast extract + 0.1 grams of neomycin sulfate + 0.02 grams of chlorofenicol + 1 L of distilled water); and 3) filter paper.

To prepare the BDA culture medium, 200 grams of peeled potatoes were boiled in 500 ml of water for at least 30 minutes, then the broth was filtered with the aid of gauze. The molten agar together with the dextrose was added to 500 ml of water, and the broth from cooking the potatoes was then added. The volume was then filled with 1,000 mL with distilled water and autoclaving at 120 °C for 20 minutes and adding 0.02 g antibiotic Streptomycin (Zauza et al., 2007). For preparing the V8 culture

medium, the molten agar was added to the other components (V8 juice, $CaCO_3$, sucrose, yeast extract and distilled water), completing the volume of 1,000 mL and autoclaving at 120 °C for 20 minutes (Zauza et al., 2007).

For the filter paper method, two sheets of paper were autoclaved at 1 atm (120 °C) for 20 minutes and then moistened with distilled and sterilized water, also autoclaved at 1 atm (120°) for 20 minutes.

Two hundred seeds of each lot and method (V8, BDA and Filter Paper) were divided into four replicates of 50 seeds. Each replicate was composed of four Petri dishes, disinfected in 1% sodium hypochlorite for 30 seconds and alcohol 70% before plating, then washed three times in sterile distilled water to remove the sodium hypochlorite residue. Subsequently, the seeds were put to dry on filter paper and then packaged in Petri dishes containing the different evaluated methods.

Another 200 seeds per lot and method were subjected to sanitary tests on agar medium and filter paper without asepsis. Four replicates of 50 seeds were used per lot for each method. Incubation was performed in a BOD (Biochemical Oxygen Demand) type growth chamber at a temperature of 22 ± 3 °C, with 12 hours of photoperiod.

The experimental design was completely randomized with four replicates for each performed test. The data arrangement of the seed health evaluation experiment was bifactorial composed of 4×3 replicates (four provenances × three methods of detection).

The fungi were evaluated by observing their reproductive structure under a stereoscopic and optical microscope, and the identification was performed with the aid of the identification key (Barnett & Hunter, 1972). Fungus incidence data were expressed as a percentage.

Identification for the *Fusarium* and *Aureobasidium* genera was conducted molecularly by the Biological Institute through isolates of all the origins in which they had incidence. *Fusarium guttiforme*, *Fusarium oxysporum* and *Aureobasidium melanogenum* were identified by the TEF region (Translation Elongation Factor) for *Fusarium guttiforme*, and ITS (Internal Transcribed Spacer, not deposited in GenBank) for the others.

Table 1. Seed collection locations of *Ilex paraguariensis* for sanity tests with different detection methods.

Lot	Altitude (m)	Average rainfall (mm/year)	Average rainfall during seed production (mm)[1]	Average temperature (°C)
Urupema – SC	1.324	1634	145.0	14.1
Porto União – SC	780	1667	161.25	17.7
Ajuricaba – RS	331	1768	153.75	19.8
Campo-Erê – SC	903	2045	180.5	16.7

[1] Period of seed production: October to December.
Source: Climate (2018).

The percentage data for the analysis of variance were transformed by the formula: Arcsen [(x + 0.5) / (100)] ½. The comparison of means was performed by the Tukey's test at 5% probability, while the SISVAR program was used for the statistical analysis.

3. RESULTS AND DISCUSSION

The asepsis decreased the incidence of contaminating microorganisms for all seed lots such as *Rhizopus* spp. and bacteria (Figure 1). These contaminants grow rapidly and interfere with the correct identification of pathogenic fungi.

High infestation of plaques with *Rhizopus* spp. was observed for all lots without asepsis, especially for the BDA method (Figure 2). The highest occurrence of infestation on

BDA plaques can be explained by the high concentration of carbon available to the fungi through the starch from potatoes present in the medium. Thus, we decided to only work with disinfected seeds based on these results.

Figure 1. Incidence of contaminating fungi in yerba mate seeds with asepsis (a) and without asepsis (b).
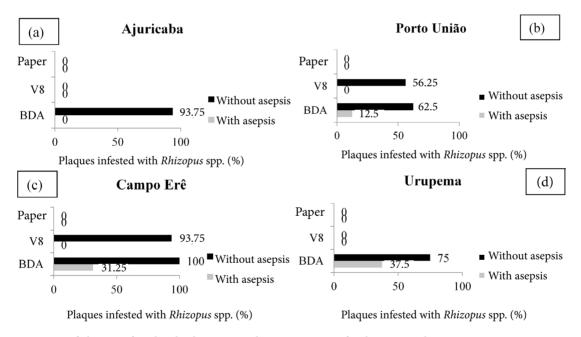

Figure 2. Percentage of plaques infested with *Rhizopus* spp. during sanity test of yerba mate seeds.

Regarding the fungal incidence, the following fungi were identified in yerba mate seeds: *Penicillium* spp. (identified morphologically), *Fusarium graminearum* (identified morphologically), *F. oxysporum* (molecularly identified by ITS region sequencing-Internal Transcribed Spacer), *F. guttiform* (molecularly identified by TEF region sequencing – Internal Transcribed Spacer), *Cladosporium* spp. (identified morphologically), *Aureobasidium melanogenum* (molecularly identified by ITS region sequencing – Internal Transcribed Spacer) and *Aspergillus* spp.

(morphologically identified). *Nigrospora* spp., *Bipolaris* spp., *Alternaria* spp. and *Trichoderma* spp. (all identified morphologically). These fungi were observed in lower incidence (Table 2).

The Polymerization Chain Reaction (PCR) technique has been used in several research areas, including fungi identification. One of the main regions used to identify pathogens is Internal Transcribed Spacer (ITS), located in the ribosomal DNA, with genetic variation that enables identifying pathogens (Jaccoud-Filho & Dabul, 2015).

Table 2. Incidence of fungi (%) in yerba mate seeds from four places, subjected to the sanity test on agar media and filter paper.

Fungus	Location	Substrate			
		BDA	V8	Paper	Average
Fusarium oxysporum	Porto União	12.5 bB[1]	3.0 abA	0.0 aA	5.2
	Campo Erê	22.5 cC	9.5 Bb	0.0 aA	10.5
	Ajuricaba	0.0 aA	0.0 aA	0.0 aA	0.0
	Urupema	12.0 bB	10.0 bB	0.0 aA	7.3
Average		11.7	5.6	0.0	CV(%): 29.7
Fusarium graminearum	Porto União	33.5 cB	47.5 cC	0.5 aA	27.2
	Campo Erê	0.0 aA	0.0 aA	1.5 aA	0.5
	Ajuricaba	0.0 aA	0.0 aA	0.0 aA	0.0
	Urupema	9.0 bA	12.0 bA	10.0 bA	10.3
Average		10.6	14.8	3.0	CV(%): 27.8
Fusarium guttiforme	Porto União	22.0 bB	32.5 bC	16.0 bA	23.5
	Campo Erê	30.5 bB	46.5 cC	13.0 abA	30.0
	Ajuricaba	2.0 aA	0.0 aA	0.0 aA	0.7
	Urupema	27.0 bB	32.5 bB	5.0 abA	21.5
Average		20.4	27.9	8.5	CV(%): 21.3
Penicillium spp.	Porto União	1.0 aA	0.0 aA	3.5 aA	1.5
	Campo Erê	9.0 bB	0.0 aA	1.5 aA	3.5
	Ajuricaba	1.5 aA	0.0 aA	0.0 aA	0.5
	Urupema	2.5 aAB	5.0 bB	0.0 aA	4.2
Average		3.5	1.3	1.3	CV(%): 49.8
Aspergillus spp.	Porto União	1.5aA	0.0 aA	2.5 aA	1.3
	Campo Erê	1.5 aA	44.5 bB	1.0 aA	15.6
	Ajuricaba	0.0 aA	0.0 aA	0.5 aA	0.0
	Urupema	0.0 aA	0.0 aA	0.0 aA	0.0
Average		0.8	11.1	1.0	CV(%): 91.5
Cladosporium spp.	Porto União	0.0[1] aA	2.5 aA	0.0 aA	0.8
	Campo Erê	0.0 aA	0.0 aA	0.0 aA	0.0
	Ajuricaba	0.0 aA	0.0 aA	0.0 aA	0.0
	Urupema	4.5 aA	17.0 bB	0.0 aA	7.2
Average		1.1	4.8	0.0	CV(%): 91.5

[1] Equivalent lowercase letters in the column and upper case in the row do not differ statistically from each other by the Tukey's test with 5% probability.

Poletto et al. (2015) observed the incidence of fungi genera *Verticillium*, *Penicillium*, *Paecilomyces*, *Phoma*, *Trichoderma*, *Alternaria*, *Cladosporium*, *Epicoccum*, *Mucor*, *Dactylella*, *Oidiodendron* and *Rhizopus* in yerba mate seeds sorted by nursery workers from different locations in Rio Grande do Sul. In evaluating lots of yerba mate seeds from Rio Grande do Sul and Santa Catarina, Oliveira et al. (2015) indicated the incidence of *Penicillium*, *Rhizoctonia*, *Fusarium*, *Trichoderma*, *Alternaria* and *Phomopsis* detected by the filter paper method and BDA culture medium. In addition to pathogenic fungi, the identification of rotting fungi such as *Penicillium* is of great importance for maintaining seed quality.

In general, the Campo Erê lot presented the highest fungal incidence among the evaluated lots, except for *F. graminearum*, which had a higher incidence for the Porto União lot, *Penicillium* spp. and *Cladosporium* spp., which had the highest incidence in the Urupema lot. The Ajuricaba lot presented the lowest incidence for all identified fungi (Table 2).

According to Marcos Filho (2015), the association of seeds with pathogens mainly occurs during the maturation process. Thus, the climatic conditions in this period can

significantly affect the sanity of seeds, favoring or hindering the survival and development of fungi.

For Bedendo (2011), factors such as humidity, temperature, light and wind may be responsible for the predisposition of plants to the attack of pathogens. Therefore, the results found in our study may be related to the occurrence of rainfall during the seed production period (from October to December); moreover, the Ajuricaba lot presented one of the lowest rainfall averages during the seed production period, while, in contrast, the Campo Erê lot presented the highest values of this climatic variable among the studied locations (Table 1).

Another factor that may influence seed health is the management performed on the matrices, such as distance from matrices with other individuals, fertilization and sanity. Thus, matrices with adequate distances between them with correct fertilization for their needs and which have sanitary control tend to produce seeds with better sanitary conditions. Poletto (2008) mentioned that infection of seeds by the *Fusarium* genus in most cultivated grains is favored by the supply of nitrogen to the plants. However, these relationships are determined by the form and content of nitrogen available, in addition to the vegetable/plant species.

In the detection methods, BDA showed greater efficiency in identifying *Fusarium oxysporum* and *Penicillium* spp. The V8 medium showed greater sensitivity in the detection of *Fusarium graminearum*, *Fusarium guttiforme*, *Aspergillus* spp. and *Cladosporium* spp. On the other hand, the filter paper method was considered the least efficient in detecting fungi identified in our study (Table 2). This is because the agar media are rich in nutrients, mainly carbon and nitrogen, favoring the development of microorganisms (Lazarotto et al., 2012; Zauza et al., 2007).

Rego (2005) considers that the filter paper method presents limitations for the detection of slow-growing and internally located fungi due to the absence of these nutrients in their composition. Oliveira et al. (2015) reported that BDA culture medium as well as the filter paper method are efficient in identifying fungi in yerba mate. Lazarotto et al. (2012) also found satisfactory results in detecting fungi using BDA as well as filter paper for *Cedrela fissilis* seeds. Fantinel et al. (2015) found that agar medium was more effective in detecting fungi in *Acca sellowiana* seeds.

The *Fusarium* genus, which presented considerable incidences in our study, is one of the main fungi associated with the tipping of yerba mate seedlings registered in the pre or post-emergence phase of the seedlings. Another problem that can be caused by the genus is root rot, which occurs in both sowing and seedlings, with yellowing and falling leaves,

evolving to death of the branches and total drought in the plants (Grigoletti & Auer, 1996).

Poletto et al. (2006) concluded that five species of *Fusarium* (*F. oxysporum*, *F. solani*, *F. decemcellulare*, *F. tricinctum* and *F. tabacinum*) are pathogenic and associated with root rot in yerba mate and may present different degrees of severity. Also, according to Grigoletti et al. (1998), the *Colletotrichum, Fusarium* and *Rhizoctonia* genera are potentially pathogenic to yerba mate.

The incidence of rotting fungi such as *Penicillium* spp., *Aspergillus* spp. and *Cladosporium* spp. were considered low for all evaluated seed lots. This fact can be explained by the moisture content of the seeds, which were from 6.93 to 12.52% (Table 3). According to Berjak (1995), these fungal genera have their proliferation and metabolic activity favored with humidity levels between 12 and 16%. Marcos-Filho (2015) reports that the development of microorganisms occurs when the water content of the seeds is above 20%, under relative humidity higher than 95%.

Aspergillus spp. and *Penicillium* spp. are storage fungi, as their occurrence increases in the post-harvest period. They can cause physicochemical changes in the tissues of the seeds, causing loss of lipids, carbohydrates, proteins and increased fatty acid (Kruppa & Russomanno, 2011; Lazarotto et al., 2010).

According to Cherobini et al. (2008), these fungal groups may cause seed rot and germination reduction in several forest species, in addition to *Penicillium*, and cause lesions in seedlings, decreasing their development. The results of *Aureobasidium melanogenum* were compared considering the two asepsis situations, seeking to illustrate their predominance in seeds from Ajuricaba, especially for the agar medium (Table 4).

The *A. melanogenum* species is an ascomycete belonging to the Dothioraceae family, presenting smooth, fast-growing colonies with viscous masses of conidia (asexual spores) that initially have a light color and are brown or black in color (Figure 3). The hyphae of this fungus are hyaline and septate, and the conidia are also of hyaline staining with smooth walls, ellipsoidal of variable sizes (Russel et al., 2016).

Table 3. Degree of moisture of yerba mate seeds from four harvesting locations.

Lot	Degree of moisture (%)
Porto União – SC	6.93
Campo Erê – SC	8.73
Ajuricaba – RS	9.78
Urupema – SC	12.52

Table 4. Incidence (%) of *Aureobasidium melanogenum* in yerba mate seeds from four collection locations with and without asepsis, and subjected to the sanity test on agar medium and filter paper.

Fungi	Location	With asepsis				Without asepsis				Average
		BDA	V8	Paper	Average	BDA	V8	Paper	Average	
Aureobasidium melanogenum	Porto União	0.0aA	4.0 bB	0.0 aA	1.3	0.0 aA	3.0 aA	0.0 aA	1.0	
	Campo Erê	0.0 Aa	0.0 aA	0.0 aA	0.0	0.0 aA	0.0 aA	0.0 aA	0.0	
	Ajuricaba	1.5Aab	0.0 aA	4.0 bB	0.5	66.0 bC	35.5 bB	5.5 bA	35.6	
	Urupema	0.0 Aa	0.0 aA	0.0 aA	0.0	0.0 Aa	0.0 aA	0.0 Aa	0.0	
Average		0.37	1.0	1.0		16.5	9.6	1.37		
CV%		46.23				120.79				

¹ Equivalent lowercase letters in the column and upper case in the row do not differ statistically from each other by the Tukey's test with 5% probability.
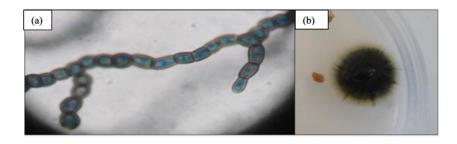

Figure 3. Characteristics of *Aureobasidium melanogenum*: (a) hyaline and septate hyphae; (b) colony in BDA medium.

Albiero et al. (2015) indicated *Aureobasidium pullulans* as one of the main yeasts found in leaves of *I. paraguariensis* from Vargeão, SC. Although reported as saprophytic and poorly pathogenic, the same species was considered pathogenic for mangaba (*Harconia speciosa*) (Anjos et al., 2009).

We emphasize the need for future studies seeking to evaluate the pathogenicity of *A. melanogenum* in *Ilex paraguariensis* seeds due to the little information found in the literature regarding the action of this fungi on forest seeds.

4. CONCLUSION

(i) The asepsis considerably reduces the occurrence of contaminants in sanity tests of yerba mate seeds;

(ii) There are differences between the evaluated lots of yerba mate seeds regarding the fungal incidence;

(iii) The detection methods in BDA and V8 culture medium showed higher efficiency in fungi detection associated with yerba mate seeds.

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