Floresta e Ambiente 2019; 26(2): e20170515
https://doi.org/10.1590/2179-8087.051517
ISSN 2179-8087 (online)

Original Article

Silviculture

Association of *Fusarium* and *Phomopsis* with Peroba Rosa Seeds

Edson José Mazarotto[1], Ida Chapaval Pimentel[2],
Daniela Cleide Azevedo de Abreu[3], Alvaro Figueredo dos Santos[4]

[1]Setor de Ciências Biológicas, Universidade Federal do Paraná – UFPR, Curitiba/PR, Brasil
[2]Universidade Federal do Paraná – UFPR, Curitiba/PR, Brasil
[3]Universidade Tecnológica Federal do Paraná – UTFPR, Dois Vizinhos/PR, Brasil
[4]Centro Nacional de Pesquisas Florestais, Empresa Brasileira de Pesquisa Agropecuária – EMBRAPA, Colombo/PR, Brasil

ABSTRACT

Peroba rosa (*Aspidosperma polyneuron*) is a native forest species endangered due to intense predatory exploitation. The objective of this study was to evaluate the association and fungi transmission in peroba rosa seeds from four regions of Paraná. Germination and vigor were evaluated using the paper roll method. Sanitary analysis consisted of the detection of endophytic and epiphytic fungi using potato-dextrose-agar medium and *Fusarium selective medium.* For transmission, non-disinfested seeds were sown in vermiculite and kept in greenhouse. Germination ranged from 9.3% to 60%. *Fusarium* sp. and *Phomopsis* sp. were found as epiphytic and also as endophytic. There was transmission of *Fusarium* sp. and *Phomopsis* sp. from seeds to seedlings, causing malformation and necrosis on roots and cotyledons.

Keywords: seed pathology, native forest seeds, endophytic fungi.

1. INTRODUCTION

Peroba Rosa (*Aspidosperma polyneuron* Muell). Arg.) is a native forest species endangered due to intense logging and fragmentation of original natural ecosystems (IPEF; EMBRAPA, 1981). This species is found in the semideciduous seasonal forest, in montane and submontane formations (Veloso et al., 1991; Carvalho, 2004). It occurs in the Southern, Southeastern and Midwestern regions of Brazil (Rizzini, 1990; Carvalho, 2004).

Peroba rosa presents excellent quality wood, being widely used in civil and naval construction, carpentry, furniture industry and reforestation. Due to its high commercial value, the species is endangered and is on the list for *ex situ* and *in situ* conservation, in both Brazil and Venezuela (Carvalho, 2004). It was also classified as a rare species in the semideciduous seasonal forest and included in the list of endangered plants of Paraná (Hatschbach & Ziller, 1995).

Few studies have been conducted in Brazil to determine the health and physiological quality of native forest species (Lazarotto et al., 2012). In general, seeds of these species present low germination, usually associated to the action of microorganisms that cause seed deterioration. Fungi are the main cause of diseases during seedling formation and germination (Vechiato & Parisi, 2013).

Some studies have been carried out to determine the main pathogens associated with seeds (Lazarotto et al., 2012; Maciel et al., 2012). In Cedar (*Cedrella fissilis*), Lazarotto et al. (2012) observed the presence of the following fungi: *Ascochyta* sp., *Aspergillus* sp., *Colletotrichum* sp., *Epicoccum* sp., *Penicillium* sp., *Rhizoctonia* sp., *Trichoderma* sp., *Phomopsis* sp., *Fusarium* sp., *Rhizopus* sp., and *Sphaaeropsis* sp. In angico-vermelho (*Parapiptadenia rigida*), the following fungi were detected: *Penicillium* sp ., *Aspergillus* sp., *Rhizopus* sp., *Epicoccum* sp., *Thielaviopsis* sp., *Cladosporium* sp., *Fusarium* sp., *Pestalotia* sp., *Alternaria* sp., *Phoma* sp. and *Nigrospora* sp. (Maciel et al., 2012). However, there is no previous research on fungi associated with peroba rosa seeds.

In this context, this study aims to evaluate the association of fungi with peroba rosa seeds from four regions of the state of Paraná and to verify the transmission of these fungi through seeds.

2. MATERIAL AND METHODS

2.1. Origin of seeds

Lots of peroba rosa seeds come from ten municipalities of four regions of state of Paraná (Table 1) and were provided by the Federal Technological University of Paraná (UTFPR - Campus Dois Vizinhos, PR). Physiological and sanitary quality tests were developed at "Laboratório de Sementes Florestais" (Laboratory of Forest Seeds) and at "Laboratório de Patologia Florestal – Embrapa Florestas" (Laboratory of Forest Pathology), in Colombo (PR).

2.2. Germination and vigor test

The germination test was performed using paper roll, with 150 seeds of each origin, divided into six replicates of 25 seeds each. Seed asepsis was carried out by immersing seeds in detergent solution (five drops of neutral detergent/100 ml of water) for five minutes and then washing in sterile distilled water. The substrate consisted of three "germitest"

Table 1. Peroba rosa seed collected in ten municipalities of four regions of the State of Paraná.

Lots	Region	Municipality	Weight of 1.000 seeds (g)
1	West	Capitão Leônidas Marques	70.3
11		Boa Vista da Aparecida	105.3
22		Corbélia	108.6
3	Southwest	Cruzeiro do Iguaçu	97.3
10		São Jorge do Oeste	89.5
7		Realeza	97.0
13		Nova Prata do Iguaçu	103.5
1M	South Central	Telêmaco Borba	79.7
8CM	Northwest	Terra Boa	81.3
9CM		Cianorte	83.5

paper sheets: two placed on the base and the other covering the seeds. Sheets were moistened with sterile distilled water at 2.5 times the paper weight. Subsequently, the material was placed in germination Chambers at 25 °C and continuous light for 24 hours (Brasil, 2013).

Two germination evaluations were carried out: the first germination count was performed 12 days after the test installation and the final count 26 days after test installation. The germination speed index (GSI) was determined with daily evaluations from the first day of test to stabilize germination, verifying the emission of the radicle in seeds. In the last evaluation, on the 26th day, the shoot and radicle length was measured to complement the vigor parameters of seedlings (Vieira & Carvalho, 1994).

2.3. Detection and isolation of endophytic fungi in Potato-Dextrose-Agar (PDA) and in Fusarium Selective Medium (FMS)

Seed asepsis was performed according to protocol described by Araújo et al. (2002): washing seeds in running water; immersing in 70% alcohol solution for 1 minute; immersing in 1% sodium hypochlorite solution for 4 minutes; immersing in 70% alcohol solution for 30 seconds; rinsing twice in sterile ultrapure water.

For the detection of fungi in PDA (Santos et al., 2011) and FMS media (Anderson, 1986), 100 seeds of each lot were used for each medium, divided into 20 replicates of 5 seeds. Disinfested seeds were plated in Petri dishes with PDA medium (39g commercial potato-dextrose-agar extract, 1000 ml ultrapure water) and FMS medium (15g peptone, 5g magnesium sulfate - $MgSO_4$, 1g potassium phosphate - $KH_2 PO_4$, 1g pentachloronitrobenzene PCNB, 20g agar, 1000 ml ultrapure water, 40 ppm chloramphenicol and 80 ppm ampicillin).

PDA and FMS plates were incubated at 20°C with 12 hours light/12 hours dark photoperiod for seven days.

2.4. Detection and isolation of epiphytic fungi in Fusarium Selective Medium (FMS)

Seed asepsis was carried out according to the following protocol: washing seeds in running water; immersing in 70% alcohol solution for 30 seconds; immersing in 1% sodium hypochlorite solution for 1 minute; rinsing twice in sterile ultrapure water. Seeds were plated in FMS and incubated at temperature of 20 °C, with 12 hours light / 12 hours dark photoperiod for seven days.

2.5. Test of transmission of fungi from peroba rosa seeds to seedlings

About 100 seeds without disinfestation from each lot were used, seeded in plastic tubes with vermiculite. The material was kept in greenhouse with daily irrigation. Evaluations began 21 days after the test was installed and ended after 60 days. The number of emergent symptomatic seedlings was determined. After 60 days, seeds that had not germinated were collected and placed in humid chamber in laboratory environment for 7 days. Phytopathogenic fungi were isolated in PDA medium.

2.6. Statistical analysis

A completely randomized design was used. Analysis of variance (ANOVA) and Tukey's test at 1% probability were performed. All analyses were performed using ASSISTAT software 7.7 (Silva & Azevedo, 2009).

3. RESULTS AND DISCUSSION

Lots with the highest germination percentage were 1M, 9CM and 3, with 56.6%, 58% and 60%, respectively, and there was no statistical difference ($p < 0.01$) between lots 8CM and 22 (Table 2). The average germination rate reported in literature for the species ranges from 35% to 70% (Carvalho, 2004). Due to the fact that it is a native forest species, peroba rosa presents irregular germination, as observed in this study. Similar values were found by Ramos et al. (1995), which obtained 67% germination rate for peroba rosa.

In Table 2, the average seed vigor values of peroba rosa are presented. Lot 1M presented the highest value in the first germination count (FGC), reaching 10.5% in 12 days, not different from lot 9CM, with 8.6%. Regarding the germination speed index (GSI), the highest percentages were obtained in lots 1M, 3, 8CM and 9CM, with 11.6%, 11.8%, 10.2% and 11.7%, respectively. Lot 9CM had the highest shoot length, with 66.8mm, while lot 22 presented the highest radicle length, 120.5mm, both differing only from lots 7 and 10.

According to Vieira & Carvalho (1994), seeds with the highest percentage of normal seedlings in the first count, higher germination speed and higher average shoot and root length of normal seedlings are the most vigorous. However, such values are still unknown for peroba rosa.

In all seed lots, the genus *Phomopsis* was found as endophyte in PDA medium, whose incidence reached 65% in lot 11 (Table 3). There was no significant difference among lots 3, 8CM, 10 and 11. The genus *Fusarium* was found in 70% of seed lots, reaching 7% in lot 7 (Table 3). There was no statistical difference among lots. Other fungi found were *Aspergillus*, *Penicillium*, *Rhizopus* and *Trichoderma*.

Garzonio & Mcgee (1983) and Hernández et al. (2015) reported that fungi of the genus *Phomopsis* colonize host tissues and establish relationships as endophytes, saprophytes or parasites. These fungi are associated with seeds, which are the main form of pathogen dispersal over long distances. The association of *Phomopsis* with seeds is evidenced by Walker et al. (2013), who verified 100% incidence of fungus in red angico seeds, and Lazarotto et al. (2012), who verified the presence of *Phomopsis* sp. in all samples of *Cedrela fissilis* seeds, which had incidence of up to 30.5%. This is the first report of *Phomopsis* sp. associated as endophyte in peroba rosa seeds.

In FSM, *Fusarium* was found as endophyte only in lots 1 and 1M, with 1% incidence (Table 3). There are reports of *Fusarium* sp. as endophyte in cowpea seeds (*Vigna unguiculata*), with frequency from 0.13% to 6.33% (Rodrigues & Menezes, 2002), which is similar to values obtained for peroba rosa.

The reports on the occurrence of fungi with pathogenic potential in seeds of forest species, both internally and externally to seeds, are scarce (Nascimento et al., 2006; Santos et al., 2011).

Table 2. Germination (G), first germination count (FGC), germination speed index (GSI), shoot length (SL) and radicle length (RL) obtained in germination and vigor tests for peroba rosa at 26 days, at 25 °C.

Lots	G (%)	FGC (12 days)	GSI (seeds/day)	SL (mm)	RL (mm)
1	26 cd**	3.3 cd**	5.1 bcd**	62.5 abc**	95.8 ab**
1M	56.6 a	10.5 a	11.6 a	65.7 ab	90.8 ab
3	60 a	6.3 bc	11.8 a	60.6 abc	111.9 ab
7	16.6 cd	1.8 d	3.16 cd	50.8 cd	87.3 b
8CM	50.6 ab	6.5 bc	10.2 a	57.8 abcd	92.9 ab
9CM	58 a	8.6 ab	11.7 a	66.8 a	95.8 ab
10	9.3 d	0.1 d	1.5 d	52.7 bcd	85.3 b
11	18 cd	0.3 d	2.9 cd	46.3 d	101.6 ab
13	34 bc	2.5 d	6.1 bc	58.9 abc	114.9 ab
22	48 ab	1 d	8.4 ab	54.8 abcd	120.5 a

**Means followed by the same letter in columns do not differ by the Tukey test at 1% probability.

Table 3. Incidence (%) of endophytic *Fusarium* spp. and *Phomopsis* spp. in peroba rosa seeds in PDA and FSM media at 7 days of incubation.

Lots	PDA		FSM	
	Fusarium spp.	*Phomopsis* spp.	*Fusarium* spp.	*Phomopsis* spp.
1	2^{ns}	17 c**	1^{ns}	0 b**
1M	0	23 bc	1	0 b
3	6	57 ab	0	16 a
7	7	23 bc	0	13 ab
8CM	6	28 abc	0	4 ab
9CM	1	18 c	0	2 ab
10	2	48 abc	0	6 ab
11	2	65 a	0	3 ab
13	0	25 bc	0	0 b
22	0	12 c	0	1 ab

**Means followed by the same letter in columns do not differ by the Tukey test at 1% probability. ^{ns} = not significant.

The niche occupied by microorganisms and their interaction with the host are the factors that classify them into endophytic, epiphytic and phytopathogenic. There is no clear boundary among these groups, but rather a gradient (Strobel et al., 2004). Endophytic fungi tend to remain latent while the environmental conditions are favorable to them; otherwise, endophyte fungi may assume a pathogenic function (Aly et al., 2010). However, several endophytic microorganisms establish an intimate association with the host, usually mutualistic, from which the hypothesis that endophytes may exert beneficial effects on their hosts emerged (Silva & Bettiol, 2009). Thus, while endophyte fungi obtains energy, nutrients and shelter, they protect the host against pathogens, herbivores and insects, and induce plant growth and defense mechanisms (Firáková et al., 2007; Alvin et al., 2014).

The genus *Fusarium* was found to be epiphytic in FSM in all seed lots, with the exception of lot 10. The fungus reached 52% incidence in lot 8CM and did not differ statistically from lot 9CM (Table 4). The genus *Phomopsis* was found at low percentages in only five lots and reached 25% incidence in lot 22. Other fungi found as epiphytic were *Aspergillus*, *Penicillium*, *Rhizopus*, *Trichothecium* and *Trichoderma*.

This is the first report of the association of fungi with peroba rosa seeds. There are reports of the Association of fungi with seeds of the family Apocynaceae with other forest hosts. There are reports of *Cladosporium* sp., *Colletotrichum* sp., *Epicocum* sp., *Pestalotia* sp., *Phoma* sp. and *Rhizoctonia* sp. associated with *Aspidosperma* sp. (Martins & Faiad, 1995). In mangaba (*Hancornia speciosa*), the presence of *Cladosporium* sp., *Fusarium* sp., *Pestalotia* sp. and *Rhizopus* sp. was verified (Barros et al., 2004).

Fusarium spp. has been found associated with seeds of several forest species (Lazarotto et al., 2012; Maciel et al., 2012, 2013). Carneiro (1990) assessed the sanitary quality of seeds of 11 native forest species and found association of *Fusarium* sp. with the following species: peroba amarela *(Aspidosperma ramiflorum*), aroeira *(Astronium urundeuva*), angico-do-campo *(Piptadenia macrocarpa*), algaroba (*Prosopis juliflora*), carvoeiro (*Sclerolobium paniculatum*) and ipê-amarelo (*Tabebuia serratifolia*).

There was transmission of fungus from seeds to seedlings in all lots. Lot 1 presented 18% of symptomatic plants, followed by lots 3 and 8CM, with 12% (Table 5). The symptoms identified were dark spots in roots, dark spots in cotyledons and seedling death. The presence of *Fusarium* spp. and *Phomopsis* spp. in symptomatic seedlings was observed.

The value for non-germinated seeds (NGS) ranged from 27% (lot 22) to 76% (lot 7) (Table 5). *Fusarium* spp. structures were observed in NGS from all lots, which reached 100% incidence in lots 3, 7, 8CM and 13 (Table 6). The lowest *Fusarium* incidence was verified

Table 4. Incidence (%) of epiphytic *Fusarium* spp. and *Phomopsis* spp. in peroba rosa seeds in FSM medium at 7 days of incubation.

Lots	1	1M	3	7	8CM	9CM	10	11	13	22
***Fusarium* spp.**	2 c**	17 bc	7 bc	12 bc	52 a	29 ab	0 c	13 bc	11 bc	2 c
***Phomopsis* spp**	0 b**	3 b	2 b	0 b	0 b	8 b	0 b	0 b	4 b	25 a

**Means followed by the same letter in rows do not differ by the Tukey test at 1% probability.

Table 5. Emergence percentage of normal and symptomatic seedlings and non-germinated peroba rosa seeds (NGS) at 60 days of seeding in the test of fungi transmission from seeds to seedlings.

Lots	Seedlings without symptoms	Seedlings with symptoms	Non-germinated seeds
1	18	18	64
1M	58	8	34
3	64	12	24
7	14	10	76
8CM	44	12	44
9CM	60	8	32
10	33	3	64
11	37	6	57
13	43	4	53
22	64	9	27

Table 6. Incidence (%) of *Fusarium* spp. and *Phomopsis* spp. on non-germinated peroba rosa seeds (NGS) in fungal transmission tests.

Lots	1	1M	3	7	8CM	9CM	10	11	13	22
***Fusarium* spp.**	56.25	55.88	100	100	100	84.37	98.43	94.73	100	59.25
***Phomopsis* spp.**	0	2.94	4.16	5.26	2.27	0	0	0	0	18.51

for lot 1M, with 55.88%. These high values for NGS indicate that the presence of *Fusarium* sp. prevented the germination of these seeds. Such pathogen behavior, which results in the embryo death before the emergence of seedling in the substrate, configures the pre-emergence damping-off (Mafia et al., 2007). *Phomopsis* sp. was found in 50% of NGS lots, with maximum percentage of 18.51% incidence in lot 22 (Table 6).

There are no reports in literature about the fungus transmission from seeds to seedlings of peroba rosa. However, Benetti et al. (2009) and Lazarotto et al. (2012) observed the transmission of *Fusarium* sp. from seed to seedlings in cedar. Lazarotto et al. (2010) also observed *Fusarium* transmission from seeds to seedlings of paineira (*Ceiba speciosa*). Maciel et al. (2012) obtained similar results for *Fusarium* sp. in red angico seedlings.

The action of *Fusarium* explains the high NGS levels in the assessed peroba rosa seed lots. According to Carneiro (1987), *Fusarium* spp. may be transmitted through infected seeds during germination. This can cause damage in pre-emergence, destroying seeds, or in post-emergence, damaging plants and causing lesions in the cervix, causing plant damping-off and death. Carneiro (1987) also points out that *Phomopsis* causes decrease in germination and seed rot disease.

4. CONCLUSIONS

Fungi *Fusarium* spp. and *Phomopsis* spp. were found both as endophytes and as epiphytic in peroba rosa seeds. *Fusarium* spp. and *Phomopsis* spp. transmission from seeds to seedlings was observed, which caused pre and post-emergence damping-off.

SUBMISSION STATUS

Received: 20 apr. 2017
Accepted: 19 jan., 2018

CORRESPONDENCE TO

Alvaro Figueredo dos Santos
Centro Nacional de Pesquisas Florestais, Empresa Brasileira de Pesquisa Agropecuária – EMBRAPA, Estrada da Ribeira, Km 111, CEP 83411-000, Colombo, PR, Brasil
e-mail: alvaro.santos@embrapa.br

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