Association of seed-borne *Fusarium* species on *Pinus ponderosa* with germination and seedling viability in Argentina

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Summary

Pinus ponderosa seedlots from eight seed orchards in Western Patagonia, Argentina, were evaluated for the presence of *Fusarium* species, and isolates obtained were subjected to pathogenicity tests. The following species were isolated: *Fusarium oxysporum*, *Fusarium equiseti, Fusarium verticillioides*, *Fusarium incarnatum*, *Fusarium acuminatum*, *Fusarium graminearum* and *Fusarium proliferatum.* With the exception of *F. proliferatum* and *F. graminearum* all *Fusarium* species were recovered from *P. ponderosa* seedlings with symptoms of damping-off. Five *Fusarium* species were re-isolated from necrotic roots of seedlings showing chlorosis (*F. oxysporum*, *F. equiseti*, *F. incarnatum*, *F. acuminatum* and *F. verticillioides*). High damping-off losses occurred in *Fusarium*-contaminated seed of *P. ponderosa* and low disease incidence occurred on less contaminated seed. The role of seed-borne inoculum in disease spread within forest nurseries is discussed.

1 Introduction

Pinus ponderosa Dougl. ex Laws. var. *ponderosa* (Ponderosa pine) is one of the most important exotic species planted in Western Patagonia, Argentina (sub-Antarctic region, adjacent to the Cordillera de los Andes) for production of both fibre and solid wood products. It is grown on 15- to 25-year rotations with yields higher than 30 m^3/ha/year (Mangieri et al. 1977).

Various seed-borne fungi affect the viability of seeds and cause damping-off. In many cases, the presence of fungi does not harm seed but hampers effective seedling production through loss of outplanted stock, as pathogenic fungi indirectly weaken seeds by predisposing them to attack by other soil-borne fungi (Mittal and Wang 1993). In Western Patagonia, several forest tree nurseries have experienced problems with damping-off and root rot caused by *Fusarium* spp. (Lori et al. 1999). Numerous species of *Fusarium* have been recovered from seed of *P. ponderosa* in Argentina as well as in the western United States (James 1985); some of these species can cause disease. In a previous communication, we provided information about fungal species associated with conifers (Lori and Salerno 2002). The aim of this study was to identify native *Fusarium* species associated with *P. ponderosa* seed in Argentina and to relate their occurrence to damping-off and root rot diseases that adversely affect nursery production.

2 Materials and methods

2.1 *Pinus ponderosa* seedlots

Seed samples were harvested in eight different orchards located in Western Patagonia (Table 1) and stored at 2–3°C for 40–60 days before each treatment. For stratification the seeds were soaked in running tap water for 48 h, air dried to a moisture content of 10–11%, and stored in double plastic bags at 2–3°C until processed. The viability of *P. ponderosa* seed was determined by plating untreated seed samples (no running water treatment) on filter paper, and incubating at 22–25°C in darkness, using the blotter method recommended by the Association of Official Seed Analysts, ISTA (Neergaard 1979). Seed germination was also monitored on the *Fusarium* selective medium (Nash and Snyder 1962) as well as on a substrate composed of perlite, vermiculite and field nursery soil (2 : 1 : 1 v/v). Ten seeds were sown in each pot, with 10 replicates.

2.2 *Fusarium* population associated with *Pinus ponderosa* seedlots

To detect *Fusarium* spp. 20 seeds per dish were plated on a modified Nash and Snyder medium (Nash and Snyder 1962; Lori and Wolcan 1996) in five replications. Before plating, seed were rinsed under running tap water for 24 h and/or 48 h, with periodic gentle agitation, to speed germination and allow growth of fungi present in the seed coats. Untreated control seeds were rinsed with sterile distilled water for a few min. All treatments were prepared at the same time and replicated five times. The dishes were incubated at 22–25°C in the dark and monitored for the presence of fungi after 7, 14, 21 and 30 days. Macroscopic examination of the seeds prior to initiating the experiments did not reveal visible fungal mycelium.

2.3 Seed-borne *Fusarium* associated with damping-off

A biological assay which involved the cultivation of ponderosa pine seedlings for 2 months in pots containing disinfected substrate comprising perlite, vermiculite and field nursery soil (2 : 1 : 1, v/v), was performed in the glasshouse under uncontrolled conditions. Ten untreated seeds of *P. ponderosa* were sown per pot in spring. Ten replicates (10 pots) were tested for each seedlot. Germination and emergence of pine seedlings were monitored at 2- to 3-day intervals in each pot. Post-emergence damping-off was determined by examining pots for fallen seedlings and disease incidence was expressed as the percentage of

Table 1. **Seedlots of *Pinus ponderosa* collected from different seed orchards in Argentina**

P. ponderosa seedlot	Place of collection	Storage period (years)[1]
1	Junín (39°57′S, 71°05′W)	2
2	Cuesta del Ternero (41°40′S, 71°30′W)	1
3	Mallín Ahogado (41°40′S, 71°28′W)	1
4	El Maitén (42°03′S, 71°10′W)	1
5	Las Golondrinas (41°58′S, 71°33′W)	1
6	Cerro Radal (42°05′S, 71°37′W)	1
7	Abra Ancha (39°15′S, 70°55′W)	1
8	Epuyén (42°14′S, 71°22′W)	0[2]

[1]After stratification, seeds were stored in double plastic bags with a moisture content between 10% and 11% at 2–3°C.
[2]Recently collected (non-stored).

damped-off seedlings. Damped-off seedlings were carefully removed and washed in sterile distilled water; the diseased roots were then surface sterilized by washing in 30% H_2O_2 for 5 s. Root fragments were rinsed with sterile distilled water, plated onto potato dextrose agar medium (PDA) and incubated at 23–25°C. The experiment ended when germination was considered complete (60 days after sowing).

2.4 Seed-borne *Fusarium* associated with root-rotted seedlings

Following the experiment described above, 3-month-old seedlings showing foliar chlorosis symptoms were lifted and roots examined using a dissecting microscope. Twenty seedlings emerging from each seedlot were harvested from their containers; two seedlings with symptoms of chlorosis were collected randomly from each pot and their roots analysed for the presence of *Fusarium* species as described above. For root isolations, fragments with necrotic symptoms were washed thoroughly to remove adhering soil particles; five or six root pieces per plant were surface sterilized, plated on PDA medium and incubated as described above. The percentage of plants with chlorosis, which yielded *Fusarium* species was calculated and expressed as root rot incidence.

The isolation frequency (Fq) and the relative density (RD) of *Fusarium* species were calculated for damping-off and root rot as follows: Fq (%) = Number of samples with occurrence of a *Fusarium* sp. (for each species separately)/Total number of samples × 100 and RD (%) = Number of isolates of a *Fusarium* species/Total number of all *Fusarium* spp. × 100.

2.5 Identification of *Fusarium* species

For identification, *Fusarium* colonies were transferred to PDA medium; single spores were then isolated from each colony, plated on PDA medium and incubated for 7–14 days at 22°C under fluorescent lamps supplemented with UV light for a 12-h photoperiod. The micromorphology and culture features of the isolates were examined and identified according to Booth (1971) and Nelson et al. (1983). To identify the *Fusarium*species within the section *Liseola*, the colonies were transferred to a ClK agar medium (Fischer et al. 1983). Representative cultures are kept in the CIDEFI (Plant Pathology Centre, University of La Plata) fungal collection which is accessible to other researchers.

2.6 Statistical analyses

Data taken as percentage, were arcsine-transformed prior to analysis. Transformed data were subjected to analysis of variance and mean values compared using LSD ($p < 0.05$). The analyses were performed using the statgraphics program (7.0).

3 Results

3.1 Seed germination of *Pinus ponderosa*

The germination percentage of *P. ponderosa* from different seedlots collected in Western Patagonia is summarized in Table 2. When seeds were plated on filter paper using the blotter method, germination rates varied between 3% and 44% (Table 2). Germination results obtained on filter paper did not correspond closely to those obtained in the agar medium selective for *Fusarium* species and on growth substrate in the glasshouse 1 month after sowing. In almost all untreated seed samples, germination rates obtained with the blotter method were significantly lower ($p < 0.05$) compared to the data obtained on Nash and Snyder medium. The effect on seed germination was not consistent when seeds were

Table 2. **Seed germination (%) of *Pinus ponderosa* in (i) filter paper (untreated seed), (ii) Nash & Snyder (N&S) agar medium selective for *Fusarium* [untreated seed and seed treated by a running water rinse (WR) for 24 and 48 h] and (iii) growing soil containing medium after 1 and 3 months**

Pinus ponderosa seedlot[1]	Filter paper untreated seed 1 month	N&S medium untreated seed 1 month	N&S medium WR × 24 h 1 month	N&S medium WR × 48 h 1 month	Growing medium untreated seed 1 month	Growing substrate untreated seed 3 months
1	44b[2]	56c	33a	32a	28a	48b B[3]
2	11b	13b	16b	0a	3a	48c B
3	7a	27c	15b	22bc	6a	76d D
4	10b	25c	13b	34d	4a	10b A
5	30c	10b	9b	14b	3a	46d B
6	3a	2a	10b	8b	4a	49c B
7	20a	60c	54c	46b	30a	65c C
8	22.5a	36b	34b	55c	29a	49c B

[1]See Table 1.
[2]Mean values within the same row followed by the same letter are not significantly different at $p < 0.05$.
[3]Mean values within the same column followed by same capital letter are not significantly different at $p < 0.05$.

Table 3. ***Fusarium*** **species isolated from** ***Pinus ponderosa*** **seeds collected from eight seed orchards in Western Patagonia**

Occurrence of *Fusarium* sp.	Place of seed collection																											
	Cerro Radal seedlot 6			Mallín Ahogado seedlot 3			Cuesta del Ternero seedlot 2			El Maitén seedlot 4			Las Golondrinas seedlot 5			Abra Ancha seedlot 7			Junín[1] seedlot 1			Epuyén[2] seedlot 8					
	C[3]	1	2	C	1	2	C	1	2	C	1	2	C	1	2	C	1	2	C	1	2	C	1	2			
F. acuminatum					x				x				x	x	x													
F. equiseti		x		x					x				x															
F. verticillioides																x	x											
F. oxysporum			x	x			x		x	x		x	x	x	x	x				x			x					
F. proliferatum																x												
F. incarnatum					x				x																			
F. graminearum					x																							

[1]All seed samples were stored for 1 year prior to examination except for this sample which was stored for 2 years.
[2]Seed sample collected just before examination.
[3]C, untreated seeds control; 1, seeds rinsed with water before plated for 24 h; 2, for 48 h isolation on Nash & Snyder medium.

treated with running water either for 24 or 48 h before sowing (Table 3). Germination on growth substrate in the glasshouse was lower compared to the blotter test (on filter paper) after 1 month, but after 3 months germination rates had increased significantly (46–78% except on lot El Maiten 10%).

3.2. Seed-borne *Fusarium* associated with damping-off

About 10–19% of the *P. ponderosa* seedlings showed symptoms damping-off, depending on seedlots (Table 4).

Six *Fusarium* spp. were isolated from damping-off seedlings from various seedlots (Tables 4 and 5). The most common species were *F. oxysporum*, *F. equistei* and *F. verticillioides*. *Fusarium proliferatum* and *F. graminearum* were present on the seeds but were not isolated from damped-off seedlings.

3.3 Seed-borne *Fusarium* associated with root-rotted seedlings

In 3-month-old seedlings with foliar chlorosis *Fusarium* spp. were isolated from necrotic roots. Root rot incidence associated with *Fusarium* species was 30% in seedlings from Cerro Radal, Epuyén and Las Golondrinas from Mallín Ahogado and El Maitén, from Abra Ancha, Junin and Cuesta del Ternero. *Fusarium* spp. recovered were similar to those observed in damping-off seedlings. *Fusarium oxysporum* was most frequently associated with symptoms (Table 4).

The isolation frequency and the RD of *Fusarium* species for damping-off and root rot are shown in Table 5. *Fusarium oxysporum* was the most frequently isolated fungus followed by *F. equiseti* and *F. verticillioides*. *Fusarium sambucinum* and *F. acuminatum* were both isolated less frequently.

4 Discussion

The results obtained here strongly suggested that seven *Fusarium* species carried on seed of *P. ponderosa* in Argentina served as inoculum sources for damping-off and root rot diseases in forest nurseries. These fungi are common seed-borne pathogens present on conifers around the world (Huang and Kuhlman 1990; Fraedrich and Miller 1995; Oak et al. 1999) and were previously reported on conifer seeds collected in the sub-Antarctic region (Lori and Salerno 2002). Some *Fusarium* spp. isolated from pine seed have been found to cause disease losses in forest nurseries in the USA (Pawuk 1981). This is the first report of the association of seed-borne *Fusarium* species on *P. ponderosa* with germination and seedling viability in Argentina.

Extensive *Fusarium* contamination was found in four of eight lots of *P. ponderosa*. This contamination was associated with reduced germination under controlled laboratory conditions and in the nursery.

According to these results, seed germination on soil containing growing substrate after 3 months gave the most reliable indication of seed viability. The *Fusarium* selective agar medium (Nash and Snyder 1962) gave a better indication of germination rates than the blotter test or filter paper. Seed germination is usually assessed by standard methods: incubation on blotter paper or soil for 7 days (Neergaard 1979); however, our results show that longer incubation periods should be taken into consideration for *P. ponderosa*. *Fusarium oxysporum*, *F. acuminatum* and *F. incarnatum* were the three main species associated with ungerminated seed. Apparently *F. subglutinans* was the main fungus isolated from *Pinus* seed in the United States (Dwinell and Fraedrich 1997). On *P. sylvestris* seed from Finland, *F. oxysporum* and *F. avenaceum* were pathogenic in growth substrates (Lilja et al. 1995).

Table 4. ***Fusarium*** **species associated with** ***Pinus ponderosa*** **seedlings with damping-off and root rot symptoms**

	Place of seed collection							
	Cerro Radal	Mallín Ahogado	Cuesta del Ternero	El Maitén	Las Golon-drinas	Abra Ancha	Junín	Epuyén
Damping-off								
% seedlings	10	19	12	10	15	13	13	17
% *Fusarium* sp.[1]								
F. oxysporum		16		30	20	31	7	
F. equiseti	10	11			7			12
F. verticillioides	10			20	13		7	
F. incarnatum		5						12
F. sambucinum			17					6
F. acuminatum					13			
Root rot incidence								
% chlorotic seedlings	30	20	10	20	30	10	10	30
% *Fusarium* sp.[1]								
F. oxysporum	7	10	3		20	10	3	10
F. equiseti		10		10				7
F. verticillioides	3					10		
F. incarnatum								10
F. sambucinum								3

[1]Percentage of affected seedlings with *Fusarium* sp.

Table 5. **Isolation frequency (Fq) and RD of *Fusarium* species on damped-off and root-rotted seedlings of *Pinus ponderosa***

	Damped-off seedlings		Root-rotted seedlings	
Fusarium spp.	Fq (%)	RD (%)	Fq (%)	RD (%)
F. oxysporum	21.2	47.2	10.0	40.0
F. equiseti	7.5	16.6	7.5	30.0
F. incarnatum	3.7	8.3	3.7	15.0
F. verticillioides	6.2	13.8	2.5	10.0
F. sambucinum	3.7	8.3	1.2	5.0
F. acuminatum	2.5	5.5	–	–

Fq, frequency (%); RD, relative density (%).

Fusarium oxysporum was the most prevalent species in most seedlots of *P. ponderosa*as well as in damped-off and root-rotted seedlings. This species causes damping-off in Ponderosa pine seedlings grown in containers in the United States (James 1985). However, in previous studies, *F. oxysporum* was only isolated in lots collected from Las Golondrinas (Lori and Salerno 2002), confirming that its presence varies between seedlots and from year to year, reflecting differences in environmental conditions.

Fusarium equiseti has been reported from seeds of *P. caribaea*, *P. merkusii*, *P. oocarpa*, *P. pseudostrobus, P. roxburghii* and *P. strobus* (Ocamb et al. 2002). It occurs also in different soils in Argentina (Lori et al. 1999, 2000) but the pathogenicity of *F. equiseti* is doubtful (Lori and Salerno 2003). Generally, it is not considered an important pathogen. However, in our study the isolation frequency and RD of *F. equiseti* were high, indicating that this species may be pathogenic to *P. ponderosa*.

Fusarium acuminatum was isolated from four of eight seedlots of *P. ponderosa* but it was previously found only once in a seedlot in the subtropical region of Argentina (Lori and Salerno 2002, 2003). The species was rarely isolated from damped-off seedlings in the present study. It is commonly reported on seed in the northern hemisphere (James 1986; James et al. 1989; Axelrood et al. 1995).

Fusarium proliferatum and *F. verticillioides* were isolated from one seedlot of *P. ponderosa*, but *F. proliferatum* was not associated with damping-off. Both fungi are the most frequent *Fusarium* species associated with conifer seeds and nursery diseases in the USA (James 1986; Fraedrich and Miller 1995).

Fusarium sambucinum was isolated from damped-off seedlings, but was not recorded on seeds. It is possible that this species was masked by other, more abundant or faster-growing *Fusarium* spp. previously isolated from Ponderosa pine seed (Lori and Salerno 2002).

The results reported in this paper support the role of seed-borne *Fusarium* spp. as a potential source of disease and shows how incomplete are disease control programmes carried out in forest nurseries when only the nursery soils are disinfected. To reduce the possibility of introducing seed-borne pathogens into the nursery, water rinses, which are regularly used in forest nurseries when infested seedlots are identified, have little effect on *Fusarium* spp. At present there is no operational fungi-free seed certification programme for conifer tree seed in Argentina and the detection of inoculum on seeds as well as the implementation of control practices prior to sowing represent important aspects of an integrated plant disease management programme.

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