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Full Length Research Paper

Pythium species and cold storage affect the root growth potential and survival of loblolly (*Pinus taeda* L.) and slash pine (*Pinus elliottii* Engelm.) seedlings

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When bareroot Southern pine (*Pinus*) seedlings are lifted in the fall and placed in cold storage for more than one week, survival is generally lower than when seedlings are lifted and stored during the winter. The combination of root wounding at the time of lifting with the presence of *Pythium* in the soil, the cool, moist conditions in cold storage encourage *Pythium* growth that results in seedling mortality after outplanting. Loblolly pine (*Pinus taeda* L.) and slash pine (*Pinus elliottii* Engelm.) seedlings were inoculated with either *Pythium dimorphum* or *Pythium irregulare*, cold stored for 3 weeks, and placed in a hydroponic system (aerated aquariums). Seedling Root growth potential (RGP was measured as the number, length, volume, surface area, and diameter of new roots and survival was recorded 4 months after outplanting. Both *Pythium* species reduced the number of new roots and root length, root surface area, and root volume in slash pine. *P. irregulare* reduced the number of new roots, root length, and root surface area and *P. dimorphum* reduced the number of new roots on loblolly pine. Although, *P. irregulare* lowered RGP, it did not reduce loblolly pine survival. In contrast, *P. irregulare* inoculation did reduce the survival of slash pine seedlings after outplanting.

Key words: *Pythium dimorphum*, *Pythium irregular*, root collar diameter; bareroot.

INTRODUCTION

Cold storage of pine (*Pinus*) seedlings is a common practice in forest tree nurseries of the Southern US when seedling demand is low or when weather conditions are poor for outplanting. Typically, seedlings are lifted from nursery beds and placed in cold storage (1 to 5°C) for 2 to 3 days if outplanted immediately ("hot planting") or for periods of one to several weeks (long-term storage). In some cases, lifting bareroot seedlings in the fall (before the winter solstice) has resulted in poor outplanting survival after long-term storage (Kahler and Gilmore, 1961; Dierauf, 1976; Hebb, 1982; Venator, 1984). However, seedling survival tends to improve when Seedlings are lifted and stored for the same duration during the winter months (Kahler and Gilmore, 1961; Hebb, 1982). The lifting season generally spans from October to the end of February, but can extend into March. The ability to cold store bareroot seedlings in the fall could allow seedlings to be outplanted earlier when field conditions are more favorable for root growth compared to the warmer, drier months of spring (Garber and Mexal, 1980).

Two theories exist to explain why bareroot pine seedling survival is poor following lifting and cold storage during the fall season. The first theory involves the lifting of seedlings that have non-dormant buds or that have not been exposed to a certain number of chilling hours. Chilling hours is defined as the exposure of seedlings to above-freezing temperatures (< 8°C) for a specific period of time, and after this time period, seedling buds may be classified as dormant (May, 1984). Carlson (1991) claimed that successful cold storage was dependant on meeting a chilling hour requirement and seedlings having

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Abbreviations: RGP, Root growth potential; **RCD,** root collar diameter.

dormant buds, which is why seedlings are generally “hot planted” after fall lifting and not stored. However, container-grown loblolly pine (*Pinus taeda* L.) (Boyer and South, 1985) and longleaf pine (*Pinus palustris* Mill.) (Pickens, 1998) have been successfully stored and outplanted in the fall without exposure to a certain number of chilling hours. These findings suggest that poor outplanting survival after storage may not be related to either chilling hours or bud dormancy but to another factor.

The second theory for poor seedling survival after storage may occur during the lifting process, where seedling roots can be torn and wounded as they are lifted from nursery beds, and as a result, provide infection sites for soil-borne pathogens such as *Pythium*. After seedlings are lifted, they are sprayed or dipped into a combination of water and superabsorbent gel to prevent desiccation in seedling storage bags (May, 1984). Excess water can pool in seedling bags and along with the cool storage temperatures (1 to 5°C), may provide an ideal environment for certain species of *Pythium* to multiply.

Jones et al. (1992) recovered *Pythium* on bareroot longleaf pine seedlings held in storage for 6 weeks. In their study, treating longleaf pine with combinations of metalaxyl and benomyl resulted in greater than 90% survival, while those that did not receive fungicides had less than 20% survival. Another study by Sun (1996) revealed that inoculating longleaf pine seedlings with increasing levels of *Pythium dimorphum* Hendrix and Campbell caused increases in seedling mortality after storage. This may have been the first study to show that a *Pythium* species could kill cold stored pine seedlings after outplanting.

Pythium is an oomycete or “water mold” commonly found in nursery soils that causes “fine feeder root disease” on young pine seedlings, which are important for nutrient and water absorption (Kelley and Oak, 1989). Seedling survival after outplanting depends on the production of new fine feeder roots. Root growth potential (RGP), is a measure of seedling quality based on new root production and can be defined as the ability of a seedling to initiate and grow new roots within a prescribed time period in an environment that is optimum for root growth (Simpson and Ritchie, 1996). Thus, root growth potential is often used to determine seedling performance potential before outplanting. Methods to determine RGP involve culturing seedling roots in either soil, hydroponic, or aeroponic systems (Rietveld, 1989), and the number and/or size of new white roots produced in these systems are then used to quantify RGP. A study has shown that RGP of loblolly pine steadily declined after 9 weeks of storage for fall-lifted seedlings, but improved after lifting and 9 weeks of storage in the winter (DeWald and Feret, 1988). Another study revealed that shortleaf pine (*Pinus echinata* Mill.) seedlings lifted in the winter had the greatest RGP after 4 weeks of storage compared to seedlings lifted and stored prior to the winter solstice (Hallgren and Tauer, 1989). In addition, the term “December dip” was coined by South (1999), which described unexplained seedling mortality observed in loblolly pine outplanted during the month of December. The “dips” in loblolly pine survival were more prevalent after cold storage and a link to decreases in seedling RGP was speculated as the reason (South, 1999).

The objective of this study was to quantify the effects that *P. dimorphum* and *Pythium irregulare* Buisman had on root growth potential, diameter growth, and survival of loblolly and slash pine (*Pinus elliottii* Engelm.) seedlings after long-term cold storage. *P. dimorphum* was chosen based on the results from inoculations on stored longleaf pine seedlings (Sun, 1996). *P. dimorphum* was first isolated from diseased loblolly pine roots in Louisiana (Hendrix and Campbell, 1971) and has since been isolated by Ho (1986) and Asiegbu et al. (1996). Root inoculations with *P. dimorphum* have reduced shoot height of Norway spruce (*Picea abies*) seedlings, and the pathogen has also been recovered from benomyl-treated seedlings (Borja, 1995). *P. irregulare* is not known as a storage pathogen but was chosen based on being one of the most common damping-off pathogens in nurseries (Hendrix and Campbell, 1973) and also to serve as a baseline for comparison to *P. dimorphum*. It was first discovered in the Netherlands on pea roots and cucumber seeds (Van der Plaats-Niterink, 1981) and has been associated with outplanting mortality of Mexican weeping pine (*Pinus patula*) seedlings in South Africa (Linde et al., 1994). This study was designed to test the null hypothesis that the root growth potential and survival of loblolly and slash pine seedlings are not affected by inoculations with *Pythium* species and cold storage. To date, the null hypothesis (that is, no effect on survival of stored seedlings) has not been rejected for any pathogen other than *Pythium* species.

MATERIALS AND METHODS

Pythium inoculum

P. dimorphum (ATCC 22843) and *P. irregulare* (ATCC 10951) were obtained from American Type Culture Collection (ATCC®, Manassas, VA). Both *Pythium* species were aseptically transferred from the ATCC vials to oatmeal agar (Kim et al., 2005). From the advancing margin of the fungal mycelium, three 0.5 cm disks of each *Pythium* spp. were again transferred to additional oatmeal agar plates to eventually use as seedling inoculum. Prior to inoculation, 1,190 g of oatmeal and 400 ml of distilled water were combined in two autoclavable bags, mixed thoroughly, autoclaved, and allowed to cool for 24 h. *Pythium* inoculum and agar were cut into pieces with one autoclaved bag receiving three agar plates of *P. dimorphum* while the other bag received three agar plates of *P. irregulare*. The oatmeal/*Pythium* inoculum was mixed every 12 h and stored at room temperature for 10 days prior to seedling root inoculations.

Seedling inoculations

Bareroot half-sib family loblolly and slash pine seedlings were obtained from Smurfit-Stone Corporation's Rock Creek Nursery near Brewton, AL on December 12, 2008. The soil texture at the nursery was loamy sand and the seedlings were grown in first year fumigated ground (2008). Prior to inoculations, loblolly and slash pine remained in cold storage (4 to 5°C) for 8 weeks at Auburn University.

On February 10, 2009, seedlings were subjected to four inoculation treatments: 50 and 200 g of *P. dimorphum* oatmeal inoculum and 50 and 200 g of *P. irregulare* oatmeal inoculum. Non-inoculated seedlings (controls) were dipped into a bucket of water without inoculum as the fifth treatment in the study. Inoculations began by weighing out the desired treatment amount of oatmeal inoculum on a scale and mixing in 11 L of water. Seedlings remained immersed in the bucket for approximately 5 s. Buckets were emptied, rinsed, and filled with a fresh inoculum mixture after each seedling bundle was inoculated. Inoculated seedlings were immediately placed in separate plastic bags (49 L) and put in cold storage (4 to 5°C) for 3 weeks. Three replications of each treatment were inoculated and the experimental unit consisted of 15 loblolly or slash pine seedlings (a replication or bag in storage). In all, 225 each of loblolly and slash pine seedlings were used in the study.

Root growth potential

After 3 weeks in storage, loblolly and slash pine seedlings were placed in an aerated hydroponic system as described by Palmer and Holen (1986) for 28 days. Fifteen aquariums (38 L) (5 treatments × 3 replications), which allowed seedling roots to be suspended in water contained an experimental unit of loblolly and slash pine seedlings each (15 of each) for each of the five treatments.

The aquariums were placed in a Randomized Complete Block Design (RCBD) on three greenhouse tables. Seedling root collar diameters (RCD) were measured on day 1 and 28 with calipers on the main stem. At day 28, seedling survival was recorded and RGP quantified by measuring the number, length, volume, surface area, and diameter of new white root tips using a WinRhizo™ root scanner and computer software (Regent Instruments, Inc., Quebec City, Quebec, Canada).

Seedling survival

After scanning, seedlings were returned to their appropriate location in the aquariums. Seedling roots were not allowed to dry out during the time they were removed from the aquariums for scanning and returned to the aquariums. On day 34 (April 9, 2009), the seedlings were removed from the aquariums and hand planted in a randomized complete block design at 0.3 × 0.3 m spacing on an outplanting site composed of sand at Auburn University. Seedling survival was monitored for 4 months.

Statistical analyses

Analyses of means were conducted using a General Linear Model (GLM) in SAS statistical software (9^{th} ed., SAS Institute, Cary, NC). Means of each experimental unit for each dependant variable were analyzed using Analysis of Covariance (ANCOVA), where initial root collar diameter (before placing seedlings in the hydroponic system) was included into the analysis to factor out any differences in seedling size (South et al., 1989). Orthogonal contrasts were performed using combined levels of *P. dimorphum* versus controls and *P. irregulare* versus controls. Data for each pine species were analyzed separately.

RESULTS

Loblolly pine

Inoculation with *P. dimorphum* reduced the number of new root tips on loblolly pine seedlings (Table 1). Non-inoculated seedlings had an average of 80 new roots, while seedlings inoculated with *P. dimorphum* had an average of 51 new roots. Aside from new root production, *P. dimorphum* did not affect any other RGP measurement (root length, surface area, volume, or diameter). *P. dimorphum* did not affect loblolly pine root collar diameter (Table 1).

Loblolly pine inoculated with *P. irregulare* had an average of 38 new white roots, which were 42 fewer roots than non-inoculated seedlings (Table 1). *P. irregulare* reduced root length and surface area by 15 cm and 2.84 cm^2, respectively, but did not affect root volume or diameter. Root collar diameter was also not affected by *P. irregulare*.

Seedling survival was not affected by root inoculation with *P. dimorphum* or *P. irregulare* after 28 days in the aquariums and 4 months after outplanting (Table 1). Despite reductions in new root growth by both *Pythium* species, only three of the 225 loblolly pine seedlings died during the RGP trial (data not shown).

Slash pine

P. dimorphum reduced the number of new white roots and root length, root surface area, and root volume of slash pine seedlings (Table 2). Non-inoculated seedlings had 174 new roots, whereas *P. dimorphum* inoculated seedlings averaged 81 new roots. *P. dimorphum* inoculated seedlings had an average reduction of 45 cm of root length, 11.64 cm^2 of root surface area, and 0.25 cm^3 of root volume when compared to non-inoculated seedlings (Table 2). Slash pine root collar diameter growth was not affected by *P. dimorphum*.

P. irregulare inoculated slash pine produced an average of 25 new roots, which were 149 fewer roots than non-inoculated seedlings (174 new roots) (Table 2). Compared to non-inoculated seedlings, *P. irregulare* inoculations reduced average root length, root surface area, and root volume by 70 cm, 17.38 and 0.39 cm^3, respectively. The effect from *P. irregulare* on all of the root growth potential variables was more severe than those for *P. dimorphum* with the exception of root diameter, which was unaffected by both oomycetes. *P. irregulare* inoculations reduced slash pine root collar diameter (Table 2). There was a two-fold reduction in RCD (- 0.17 mm) for *P. irregulare* inoculated seedlings when compared to non-inoculated seedlings (+ 0.15 mm).

During the 28-day RGP trial, *P. irregulare* inoculated slash pine survival was 85% as compared to 100% for *P. dimorphum* inoculated seedlings (Table 2). Four months following outplanting, seedling survival for *P. irregulare*

Table 1. Loblolly pine root growth potential (RGP), root collar diameter (RCD), and seedling survival as affected by *Pythium* treatments, analysis of covariance for RGP and RCD, and analysis of variance for survival.

Treatment	Root growth potential					Root collar diameter			Seedling survival	
	New roots (#)	Root length (cm)	Root surface area (cm^2)	Root volume (cm^3)	Root diameter (mm)	RCD before[z] (mm)	RCD after[y] (mm)	RCD growth (mm)	Survival 1[x] (%)	Survival 2[w] (%)
Control	80	24.67	5.06	0.09	0.71	4.38	4.55	0.16	97	43
P. dimorphum 50 g	52	11.16	2.31	0.04	0.77	4.33	4.34	0.00	97	46
P. dimorphum 200 g	51	18.81	4.88	0.11	0.84	4.64	4.75	0.11	100	15
P. irregulare 50 g	43	11.09	2.33	0.04	0.75	4.42	4.47	0.05	97	41
P. irregulare 200 g	33	8.33	2.12	0.05	0.80	4.53	4.52	-0.01	100	8
LSD[v]	(31)	(14.67)	(3.44)	(0.07)	(0.16)	(0)	(0.21)	(0.21)	(4)	(33)
Factor					P > F					
Covariate	0.8748	0.4191	0.2038	0.1078	0.2201	——[u]	0.0004	0.4879	——	——
Replication	0.1271	0.2717	0.3518	0.3788	0.0364	——	0.3964	0.3964	0.0256	0.0949
Treatment	0.0493	0.1381	0.2167	0.3407	0.7782	——	0.3108	0.3108	0.4609	0.3438
Control vs. *P. dimorphum*	0.0241	0.0766	0.1716	0.3759	0.2421	——	0.1366	0.1366	0.4774	0.3406
Control vs. *P. irregulare*	0.0053	0.0202	0.0397	0.0873	0.4209	——	0.0726	0.0726	0.4774	0.1774
P. dimorphum vs. *P. irregulare*	0.2159	0.2957	0.2527	0.2311	0.5895	——	0.6216	0.6216	1.0000	0.5841

[z] = RCD before the 28-day RGP trial, [y] = RCD after the 28-day RGP trial, [x] = survival after the 28-day RGP trial, [w] = survival 4 months after outplanting, [v] = least significant difference (α = 0.05), [u] = no P-value due to covariate factor.

Table 2. Slash pine root growth potential (RGP), root collar diameter (RCD), and seedling survival as affected by *Pythium* treatments, analysis of covariance for RGP and RCD, and analysis of variance for survival.

Treatment	Root growth potential					Root collar diameter			Seedling survival	
	New roots (#)	Root length (cm)	Root surface area (cm^2)	Root volume (cm^3)	Root diameter (mm)	RCD before[z] (mm)	RCD after[y] (mm)	RCD growth (mm)	Survival 1[x] (%)	Survival 2[w] (%)
Control	174	75.02	19.93	0.44	0.85	4.78	4.93	0.15	100	68
P. dimorphum 50 g	67	22.34	6.66	0.17	0.98	4.64	4.54	-0.10	100	26
P. dimorphum 200 g	95	36.79	9.92	0.22	0.85	4.75	4.80	0.05	100	64
P. irregulare 50 g	16	2.42	0.59	0.01	0.85	4.80	4.56	-0.24	82	0
P. irregulare 200 g	35	7.53	1.96	0.04	0.84	4.72	4.63	-0.09	88	13
LSD[v]	57	26	6.75	0.16	0.19	0	0.26	0.26	14	41
Factor					P > F					
Covariate	0.7595	0.6918	0.7592	0.8215	0.1072	——[u]	0.0068	0.4012	——	——
Replication	0.1209	0.0923	0.0550	0.0463	0.6994	——	0.5162	0.5162	0.1507	0.3759

Table 2. Contd.

Treatment	0.0022	0.0018	0.0017	0.0028	0.2262	——	0.0619	0.0619	0.0659	0.0168
Control vs. *P. dimorphum*	0.0039	0.0024	0.0031	0.0073	0.2100	——	0.1021	0.1021	1.0000	0.1716
Control vs. *P. irregulare*	0.0002	0.0002	0.0001	0.0002	0.9962	——	0.0126	0.0126	0.0298	0.004
P. dimorphum vs. *P. irregulare*	0.0124	0.0157	0.0095	0.0084	0.1346	——	0.1383	0.1383	0.0121	0.0158

[z] = RCD before the 28-day RGP trial, [y] = RCD after the 28-day RGP trial, [x] = survival after the 28-dayRGP trial, [w] = survival 4 months after outplanting, [v] = least significant difference (α = 0.05), [u] = no P-value due to covariate factor.

inoculated seedlings decreased to 7% as compared to 45% for *P. dimorphum* inoculated slash pine. The lack of root production on seedlings inoculated with 50 and 200 g was consistent with low seedling survival at each inoculum level (0 and 13%, respectively) after outplanting. However, even though RGP of *P. dimorphum*inoculated slash pine was less than non-inoculated seedlings, seedling survival was not affected.

DISCUSSION

Root growth potential is a measurement used to determine early seedling performance (survival) potential prior to outplanting. A seedling's ability to grow an adequate root system is important for good establishment in the field. Due to the need for soil moisture immediately after outplanting (Bronnum, 2005), the number of new roots produced by a seedling for the uptake of water may be the most important measure of RGP. Our results suggest that if loblolly or slash pine roots are infected with *P. dimorphum* or *P. irregulare* in the nursery, the production of new roots could be reduced after cold storage. The effects from *P. dimorphum* and *P. irregulare* inoculations on RGP were independent and differed depending on pine species. *P. irregulare* was consistently more virulent to loblolly (in terms of RGP) and slash pine (in terms of RGP and survival) than *P. dimorphum*. Slash pine RGP and survival was affected more by inoculations with both *Pythium* species than loblolly pine. Variations in genotype between loblolly and slash pine might account for these differences. Another study has shown independent effects from bacterial inoculations by demonstrating a decrease in root growth for loblolly and slash pine seedlings that received *Bacillus subtilis* compared to *Bacillus pumilus* and *Bacillus sphaericus* (Enebak et al., 1998). Their study also showed other response differences between the two pine species by loblolly pine experiencing increases in root length and biomass while slash pine did not.

Despite reductions in RGP of loblolly and slash pine seedlings from both *Pythium* species, only slash pine inoculated with *P. irregulare* experienced reductions in outplanting survival when compared to non-inoculated seedlings. Genotypic differences in pathogen susceptibility might also explain the ability of loblolly pine to recover from reduced RGP.

Seedling root collar diameter (RCD) is another indicator of seedling quality that is relatively quick to assess based on seedling morphology. A larger RCD usually indicates a healthier pine seedling that is correlated with higher survival and growth compared to smaller seedlings (South et al., 1985). Loblolly pine RCD was unaffected by either *Pythium* species, but *P. irregulare*-inoculated slash pine RCD was reduced during the 28-day RGP test by an average of 0.32 mm as compared to non-inoculated slash pine. This is the first report of a reduction in slash pine RCD after inoculations with *P. irregulare*, cold storage, and 28 days in a hydroponic system. The inoculation treatment may have reduced the ability of seedlings to uptake water, and this may have caused the RCD to shrink. A similar reduction in RCD due to inoculation with *Pythium* has been reported for longleaf pine (Jackson et al., 2008). In that study, the RCD of longleaf pine inoculated with *P. dimorphum* and *P. irregulare* decreased by 0.42 and 0.32 mm respectively, compared to non-inoculated seedlings. DeWald and Feret (1988) reported loblolly pine survival to be greater than 80% when outplanted with only 4 new roots. It could be that *P. irregulare* inoculated slash pine survival was affected more by reductions in RCD than the number of new roots (16 to 35) after outplanting.

Conclusions

We rejected the null hypothesis: root growth potential and survival of loblolly and slash pine seedlings is not affected by *Pythium* inoculation prior to cold storage. Both *P. dimorphum and P. irregulare* reduced loblolly and slash pine root growth potential, and after outplanting, only *P.*

irregulare inoculated slash pine experienced decreases in survival. In addition, the root collar diameter of *P. irregulare* inoculated slash pine was reduced during the RGP trial. This may be the first report of such effects from *Pythium* species on loblolly and slash pine seedlings after cold storage.

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