Pythium Root Rot of Flue-Cured Tobacco Seedlings Produced in Greenhouses: Factors Associated with its Occurrence and Chemical Control

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Abstract
Pythium root rot is an economically important disease of tobacco seedlings produced in greenhouses. In a survey conducted in 1998 and 1999, five Pythium species were found to cause Pythium root rot symptoms in tobacco seedlings. In pathogenicity studies the most aggressive species were Pythium volutum and P. myriotylum, although these were not the most frequently isolated species. We conducted studies with potting media from different commercial sources. Media were not found to be a source of primary inoculum for the disease in those studies. Infested trays had the potential to carry inoculum from one season to the next. The fungicides etridiazole, mefenoxam, and azoxystrobin along with acibenzolar-S-methyl and Joy detergent were evaluated for controlling Pythium root rot. Etridiazole and azoxystrobin applied to the float water provided the best control.

Introduction
Tobacco transplants in the USA are almost exclusively produced in greenhouses using a float tray system (Fig. 1A). Pythium root rot is an economically important disease of seedlings under greenhouse conditions that is difficult to manage (8). Pythium root rot affects the vigor of seedlings, resulting in poor quality of transplants. Yellowing of lower leaves of seedlings resembles nitrogen deficiency, but symptoms caused by Pythium ssp. differentiate from nitrogen deficiency by the wilting of leaves (Figs. 1B and C). A light brown discoloration of the roots observed in early stages of the infection becomes a few days later dark brown with a slime looking appearance (Fig. 1D). As the disease progresses infected roots fell off, leaving seedlings without a root system (Fig. 1E). This symptom is an indication of severe Pythium root rot incidence and results in seedlings that are not usable. A seedling is considered usable when it has white roots and > 7 cm stem length from the soil line to apical growth point and stem diameter > 5 mm. Non-usable seedlings, if transplanted, die or grow slowly in the field. Also they may be more susceptible to other diseases such as collar rot and Rhizoctonia stem rot and less tolerant to hot and dry conditions (8). Losses due to Pythium ssp. are difficult to quantify, because they are not usually reflected in final yields; however, diseased seedlings may soon die after transplanted in the field or result in slower than normal growth of plants and thus indirectly increase the cost of production.
Several species of *Pythium* that affect tobacco roots have been reported worldwide. *Pythium* species that affect tobacco transplants are: *P. aphanidermatum* (Edson) Fitzp. (7,10,11,13); *P. ultimum* Trow. (5); *P. irregulare* Buisman; *P. spinosum* Sawada; *P. oligandrum* Drechs.; *P. splendens* H. Brun (3); and *P. myriotylum* Drechs. (2,4,7). However, comprehensive studies that report the species infecting tobacco seedlings in southeast USA and their relative aggressiveness are not available.

The importance of commercial potting media as a primary source of *Pythium* spp. has been reported (3). It is suggested that particles of potting media may be contaminated with *Pythium* spp. (14). No other sources of primary inoculum have been reported. Once the pathogen has been introduced into the float system it appears that little can be done to reduce the disease incidence except for the use of fungicides. Etridiazole (Terramaster 4EC) is the only registered fungicide for prevention and control of Pythium root rot in tobacco greenhouses.
Little information is currently available on other effective fungicides or their application method to control Pythium root rot in the tobacco float water system.

Sample Collection, Pythium spp. Isolation, and Identification

Samples were collected from symptomatic tobacco seedlings in 1998 and 1999. A total of 129 samples were collected from commercial tobacco greenhouses where Pythium root rot was diagnosed or from tobacco seedlings submitted to the Plant Disease and Insect Clinic at North Carolina State University in Raleigh, NC. All samples were processed within 48 hours upon arrival at the Plant Disease and Insect Clinic or collection in greenhouses. Pieces of roots were taken from symptomatic tobacco seedlings and placed on petri dishes containing a modified PARP medium (1). Dishes were placed in the dark at room temperature (approximately 25°C). After two days of incubation, hyphal tips from each colony were transferred into 1.7% Corn Meal Agar (CMA) to initiate a pure culture of a Pythium isolate. Isolates were maintained in test tubes containing sterile water and stored in the dark at room temperature. Pythium isolates were identified on the basis of morphological characteristics of sexual and asexual structures produced on tall fescue grass, following the protocol described by Abad (1). Eighty-nine Pythium isolates were obtained. The species were identified as Pythium myriotylum, P. dissotocum, P. irregulare, P. volutum, and P. spinosum (Fig. 2). No Pythium spp. was isolated from 32 percent of decayed root samples (Fig. 1).
Fig. 2. Frequency of Pythium species isolated from tobacco seedlings in North Carolina during 1998-1999. A total of 129 samples were collected from infected seedlings in the greenhouse and from root samples that arrived at the Plant Disease and Insect Clinic at North Carolina State University, Raleigh, NC.

Pathogenicity Tests

Tobacco seeding production. One-hundred-ninety-two cell polystyrene trays were filled with the soilless potting medium Metro-mix 200 (Scotts, Marysville, OH) and seeded with pelletized tobacco seeds of cultivar K 326. Each tray was placed into a black plastic container (Kadon Corp., Dayton, OH) filled with 11 liters of water to simulate the float tray system used in tobacco greenhouses. Water soluble fertilizer (Peter's 20-20-20) was added 7 days after seeding to adjust the nutrient solution to 150 ppm of nitrogen. Fifty ppm of nitrogen was added two weeks later. Tobacco seedlings were clipped weekly, starting 28 to 30 days after seeding. Tobacco seedlings were kept in the greenhouse for 60 to 65 days.

Inoculation. Two isolates per Pythium species were used. Each Pythium isolate was transferred to a plate of 1.7% CMA medium. Four days later, six discs of 5 mm in diameter from each culture were transferred into 60 × 15-mm petri dishes. Deionized water was added to make a 3 to 5 mm film of water, and then

Frequency

P. myriotylum
P. dissotocum
P. irregular
P. volutum
P. spinosum
No pythium
20 to 25 pieces of sterile tall fescue leaves, approximately 1 cm in length, were added. Petri dishes were incubated for 5 to 7 days at room temperature and 12 hours of photoperiod. For the inoculation of the seedlings 3 to 5 pieces of infested fescue leaves were placed into the float water. Seedlings were twenty-five-days old when inoculated. There were three replications (i.e., trays) per Pythium isolate. Twenty seedlings per replication were randomly selected from each tray and evaluated 60 days after seeding. Root rot incidence defined as percent of seedlings with discolored roots, percent of usable plants, stem length defined as the distance between the soil line and the bud, and root length defined as the distance between the soil line and the tip of the longest roots were evaluated for each seedling and then averaged. The experiment was a complete randomized design conducted between 9 May and 30 June 1999 and again between 3 September and 25 October 1999.

Data were subjected to analysis of variance using SAS (SAS Institute Inc., Cary, NC) and significant differences in pathogenicity among Pythium species were determined by the Fisher's least significant difference (LSD) test at the 5% level. Pathogenicity tests indicated that P. myriotylum and P. volutum were the most aggressive species because there were the two species that caused the highest root rot incidence (Fig. 3). Seedlings inoculated with these two species were stunted, with few healthy roots, and the lowest percent of usable transplants at the end of the experiment (Fig. 3). Root rot incidence was significantly lower for the other three Pythium spp. and a significantly higher percent of usable transplants was obtained. Symptoms, such as root discoloration, were restricted to the root system but above ground symptoms were not observed with any Pythium spp.
Fig. 3. Effect of Pythium species on the incidence of root rot of tobacco seedlings (A), percentage of usable seedlings (B), stem length (C), and root length (D). P.d. = *P. dissotocum*, P.m. = *P. myriotylum*, P.i. = *P. irregulare*, P.s. = *P. spinosum*, and P.v. = *P. volutum*. Means followed by the same letter are not significantly different according to Fisher’s least significant difference (LSD), *P* ≤ 0.05.
**Seedling Age and Pythium Root Rot Incidence**

To determine if the age of tobacco seedlings at the time of infection significantly affects the incidence of Pythium root rot, seedlings of cultivar K 326, grown on 45-cell polystyrene trays as described above, were inoculated at 14, 21, or 28 days after seeding. Two species of *Pythium* were used for inoculation: *P. myriotylum* (isolate #1) and *P. volutum* (isolate #26). Inoculum was prepared and plants were inoculated as described above. There were three replications (i.e., trays) per *Pythium* species and inoculation date. The experiment was a complete randomized design conducted two times, between 12 April and 31 May 2000 and again between 4 September and 30 October 2000. Root rot incidence was evaluated 7, 14, and 21 days after inoculation as described above. Data were analyzed separately for each date of evaluation with analysis of variance by SAS (SAS Institute Inc., Cary, NC). Means were separated by Fisher’s LSD test at the 5% level. Disease progress in tobacco seedlings was similar for both *P. myriotylum* and *P. volutum*. Root rot incidence varied upon the date of inoculation. Root rot developed faster on seedlings inoculated at 21 and 28 days than at 14 days after seeding (Fig. 4).
Fig. 4. Disease progress curves. Dotted line represents *P. volutum* (Pm), solid line represents *P. myriotylum* (Pm), ♦ = inoculation at 14 days, ● = inoculation at 21 days, and ■ = inoculation at 28 days after seeding. ○ = nontreated control. Root rot incidence was evaluated for three weeks after the inoculation. Incidence was compared within an evaluation date. Means within the same date followed by the same letter are not significantly different according to Fisher's least significant difference (LSD) test, $P \leq 0.05$.

**Sources of Inoculum**

Two potential sources of inoculum were investigated: (a) commercially available potting media used to grow tobacco seedlings and (b) trays contaminated with *Pythium* spp. Commercial potting media were assayed by growing tobacco seedlings of cultivars K 326 and NC 71, two of the most planted commercial cultivars, in them. New polystyrene trays with 192 cells were filled with each of the three predominant in the market commercial potting media: Carolinas Choice (Carolina Soil Company, Kinston, NC); Sunshine (Sun Gro...
Seeded trays were placed into 12-liter black plastic containers filled with 11 liters of water. Treatments were arranged in a split plot design with soil mix as main plot and tobacco cultivar as subplot. Three replications per treatment were used. Root rot incidence was evaluated as described above 60 days after seeding. A root sample was collected after the end of the experiment evaluation from each seedling used in the evaluation and cultured in PARP medium to isolate *Pythium* spp. as described above. The experiment was a complete randomized design conducted two times between April and 31 May 2000 and between 4 September and 30 October 2000.

The potential of *Pythium* spp. inoculum to overwinter and carry over from one season to the next on trays was also evaluated. Treatments were: (i) new trays, no inoculated seedlings; (ii) new trays, inoculated when seedlings were 25 days old; and (iii) trays contaminated with *Pythium* spp. Contaminated trays were obtained from greenhouse experiments where tobacco seedlings were inoculated with *Pythium* species and subsequently developed Pythium root rot symptoms. Contaminated trays remained stored for 10 months in a greenhouse. Before contaminated trays were used for the experiment, roots trapped in the crevices of these trays were plated on PARP medium and *Pythium* colonies were recovered. Cultivar K 326 was used for this experiment. Root rot incidence, and percent of usable seedlings were evaluated 60 days after seeding as described above. The experiment was conducted two times between April and May 2000. Data were subjected to analysis of variance using SAS (SAS Institute Inc., Cary, NC) and significant differences were determined by Fisher’s LSD test at the 5% level. Pythium root rot was not observed in seedlings grown in any commercial potting media tested (*data not shown*). A faint root discoloration was observed on seedlings growing in all three potting media tested reminiscent of root rot symptoms caused by *Pythium* spp.; however, *Pythium* was not isolated from discolored root samples incubated for 48 hours on PARP medium. Root rot developed on seedlings growing in trays that were either new but artificially inoculated or previously contaminated with *P. myriotylum* (Fig. 5).
Chemical Control

Experiments were conducted using 192-cell polystyrene trays with 3 replications (trays) per treatment. *P. myriotylum* was used to inoculate seedlings of cultivar K 326 25 days after seeding. Experiments were repeated once. Root rot incidence, and percent of usable seedlings were evaluated 65 days after seeding. Fungicides were: (i) mefenoxan at the rate of 0.75 ppm ai; (ii) etridiazole at the rate of 144 ppm ai; (iii) acibenzolar-S-methyl at the rate of 5 mg ai/ha; (iv) azoxystrobin at the rate of 63 ppm ai; and (v) Joy dishwasher (Procter & Gamble) detergent, 260 ppm. Etridiazole is the only registered fungicide for prevention and control of Pythium root rot in tobacco greenhouses. The other three fungicides are registered for control of other diseases on tobacco and the rates tested for these fungicides were based on the rates available on the label for use on tobacco (8). Joy dishwasher was included to evaluate anecdotal
evidence among tobacco growers that the product controls Pythium root rot. Treatments were applied 7 days after seeding in the float water. Seedlings were inoculated 24 days after seeding. Subsequently three methods of applications of etridiazole were tested: (a) mixing the fungicide with the potting media; (b) drenching over the top; and (c) applying to the float water. Treatments were: (i) non-inoculated control; (ii) inoculated; (iii) non-treated control; and (iv) etridiazole at the rate of 116.6 g ai/m³ when mixed with potting medium; (v) etridiazole drench at the rate of 286 mg ai/liter, (drench volume used was 5.1 liter/m²); (vi) etridiazole at the rate of 144 mg ai/liter applied to the float water. Products were applied before seeding in the case of mixing with potting medium, one week after seeding for the float water applications, and one day after inoculation for the drench application. Data were subjected to analysis of variance using SAS (SAS Institute Inc., Cary, NC) and significant differences were determined by Fisher’s LSD test at the 5% level.

Significant differences in disease incidence and percent of usable plants were observed among treatments (Fig. 6A). Etridiazole and azoxystrobin had the highest percentage of usable plants per tray, and least root rot incidence. Acibenzolar-S-methyl, mefenoxam, and Joy detergent had high percentage of root rot incidence that was not significantly different than the inoculated, untreated control (Fig. 6A). Applying etridiazole into the float water was the most effective method for controlling Pythium root rot (Fig. 6B). Mixing with the potting soil mix or drenching the product onto the tobacco seedlings provided less effective control. Moreover, the drench application caused phytotoxic symptoms on tobacco leaves.
Fig. 6. Efficacy of fungicides in Pythium root rot incidence and production of usable tobacco seedlings (A). All fungicides were applied to the float water. Black column = Root rot incidence, Clear column = Usable seedlings. Means within root rot or usable seedlings followed by the same letter are not significantly different according to Fisher's least significant difference (LSD) test, \( P \leq 0.05 \); Efficacy of the method of application of etridiazole in Pythium root rot incidence and production of usable tobacco seedlings (B). Black column = Root rot incidence, Clear column = Usable transplants. Means within root rot or usable seedlings followed by the same letter are not significantly different according to Fisher's least significant difference (LSD) test, \( P \leq 0.05 \).
Discussion

*Pythium myriotylum* and *P. volutum* were identified as the most aggressive species causing root rot of tobacco seedlings. *Pythium myriotylum* has been previously reported as the principal species causing this disease (2,4,7). In our study *P. volutum* was the second most frequently observed species. This is the first report of this *Pythium* species affecting tobacco seedlings. *P. dissotocum*, *P. irregulare*, and *P. spinosum* were also isolated from seedlings that developed root discoloration, but these species did not significantly reduce the production of usable transplants. The pathogen was not recovered in a large percentage of seedlings with visual root discoloration. These last two findings suggest that (a) root discoloration may not a safe diagnostic symptom for *Pythium* root rot on tobacco seedlings and (b) some *Pythium* spp. may cause infection but no severe disease development and thus remain undetected. It was also rather unexpected that *P. aphanidermatum*, commonly found in tobacco fields (8) was not isolated from tobacco greenhouses, suggesting that this *Pythium* species is not transferred from tobacco greenhouses to tobacco fields.

The difference in the amount of root tissue present at the time of infection could explain the faster development of root rot of seedlings inoculated at 21 and 28 days than at 14 days after seeding. Particularly, 28-day-old seedlings have a much larger and well-developed root system expanding into the float water; the amount of roots present at this stage give to the pathogen a much higher probability to encyst, penetrate, and infect tobacco roots. Production of secondary inoculum likely is higher on 21 to 28 days old seedlings, due to the larger root volume, than 14 days old seedlings.

Most potting media used in the production of tobacco are made of peat moss with various combinations of vermiculite and perlite which provide a relative clean substrate, but the different sources of raw material used to prepare them vary from location to location, increasing the likelihood to introduce *Pythium* into soil-less potting media. Thus although Pythium root rot did not occur in our experiments when different potting media were tested one cannot exclude their importance as a source of inoculum based on findings from a previous study (3). Other sources such as contaminated water (12,15) or infested particles (dust and soil mix particles) from walkways, floors, and beds within the greenhouse that blow in the seed beds (15,16), could also be potential primary sources of inoculum. Contaminated trays that harbor pieces of infected roots are important sources of *Pythium* inoculum as has been observed with other tobacco greenhouse diseases (6,16). Therefore, sanitation of trays before re-use is strongly recommended (8). Potential sources of *Pythium* can be anywhere in and around tobacco greenhouses, and for these reasons routine sanitation procedures should be considered as the first step to control this disease (15).

Once the pathogen is introduced into the float tray system it will be favored by uniform temperatures and a favorable medium (water) for rapid dispersal. Once *Pythium* is introduced, the use of a fungicide is necessary to eliminate the presence of the pathogen. Etriazole applied in the float water is an effective fungicide and thus has been the standard recommended chemical control for Pythium root rot in tobacco greenhouses (8). Azoxytrobin provided a similar to etriazole level of control. On the other hand percentage of usable plants and Pythium root rot incidence in seedlings treated with mefenoxam, acibenzolar-S-methyl, and Joy detergent were not statistically different than in the untreated, inoculated seedlings.

Literature Cited