THE EFFECTS OF USING POLLEN CONTAMINATED WITH CONIDIA OF FUSARIUM MONILIFORME VAR. SUBGLUTINANS ON CONTROL-POLLINATED STROBILI OF SLASH PINE

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Abstract.--Strobili of slash pine were inoculated at the time of pollen receptivity with a mixture of pollen and conidia of Fusarium moniliforme var. subglutinans (FMS). FMS was subsequently isolated from apophysis, ovule, and axis tissues of relatively few sampled strobili. Mean strobilus losses through 10 months were greater in FMS-inoculated treatments than in treatments with pollen alone or wind-pollinated controls. FMS was not isolated from living conelets sampled at 10 months, and the fungus was recovered from only 1 of 64 conelets collected after 11 months. The pathogen was not cultured from seeds of cones that survived to maturity. Under the conditions of this research, seed yields and seed infections did not appear to be influenced by exposure of strobili to conidia of FMS at the time of pollen receptivity.

Additional keywords: Pinus elliottii var. elliottii, pitch canker, inoculations, seed damage, seed orchards.

In the mid-1970's, an investigation of the causes of poor seed germination of slash pine (Pinus elliottii (Engelm.) var. elliottii) seed lots from the Arrowhead Seed Orchard (ASO) of the Georgia Forestry Commission (GFC) and an industrial orchard in Georgia resulted in the isolation of fungi from the affected seeds. Subsequent research in the ASO determined that the pitch canker fungus, Fusarium moniliforme Sheld. var. subglutinans Wollenw. & Reinke (FMS), and a second fungus, Lasiodiplodia theobromae (Pat.) Griff. & Maubl., were the principal pathogens associated with the seeds' deterioration. Inoculation tests confirmed that both organisms were pathogenic on maturing cones and seeds of slash pine, and that FMS could cause abortion of conelets following spray application of aqueous suspensions of the fungus to female strobili at the time of pollen

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receptivity (Miller and Bramlett 1979). In 1980 and 1981, Anderson et al. (1984) cultured slash pine seeds collected from seed orchards throughout the southern United States and found up to 30% of seeds from certain orchards to be infected with FMS. Additional studies on FMS suggested the possibility that the pitch canker disease was being introduced into nursery beds via infested or infected seeds (Barnard and Blakeslee 1980, Blakeslee et al. 1981). During this nursery research, FMS was isolated from a stored collection of pollen, suggesting that FMS-contaminated pollen may be one of the means by which this pathogen becomes established in the strobili and seeds of slash pine.

The objective of the present research was to determine whether the use of pollen artificially contaminated with conidia of FMS would result in infection of female strobili and seeds of slash pine following controlled pollination.

METHODS

In February 1982, 35 strobilus-bearing branches were tagged on a single ramet of GFC clones 17, 60, 68, 111, and 174 in the ASO. Twenty-five of these branches were bagged for controlled pollination using pollen with and without spores of FMS. The remaining 10 branches were wind-pollinated controls.

Inoculum of FMS, as conidia, was collected from cultures of two isolates of FMS (AH-2 and PM-7-5-2) that were isolated from slash pine seeds collected earlier in the ASO.

The pollen used was collected, vacuum dried, and stored in 1980. A five-clone mixture was made just prior to inoculum preparation and rehydrated overnight in a water-saturated atmosphere. The pollen was determined to be free of FMS through isolation tests prior to use.

Two different combinations of pollen and FMS conidia were prepared, one containing primarily microconidia (MIC) and the other containing mostly macroconidia (MAC). The MIC mixture was prepared by washing conidia from 8- to 10-day-old cultures of FMS, decanting excess water, mixing with talc into a slurry that was desiccated for 4 days at 2C. The mixture was then sieved, mixed with the pollen, and dispensed into plastic test tubes, 0.5 cc per tube. Inoculum concentration for the MIC mixture was approximately 5.3 X 10 conidia per cc of pollen. The MAC inoculum was prepared by growing FMS for 8 to 10 days on sterile carnation leaves placed on the surface of 1.5% water agar. The leaves were removed from the culture dishes, air dried overnight, and the conidia were scraped from the leaves into ca 2 cc of talc. The talc-conidia mixture was vigorously agitated on a mechanical vibrator, mixed with the pollen, vibrated again, and dispensed into plastic tubes at 0.5 cc per tube. Inoculum concentration for the MAC mixture was approximately 2.7 X 10 conidia per cc of pollen. The uncontaminated pollen was also dispensed into plastic tubes, 0.5 cc per tube. All pollen and pollen-spore mixtures were refrigerated immediately after preparation and transported to the ASO, where they were retained on ice in a cooler until used.

Treatments were applied to the selected, pollen-receptive strobili on February 18-19, 1982. The treatments were randomly assigned to the bagged branches on each ramet. Nine branches received each of the pollen-FMS combinations and seven branches received pollen only. Pollen combinations were applied using cyclone-type pollinators (Matthews and Bramlett 1981). Individual pollinators were used for each mixture and were kept separated. The person doing the pollinating rinsed his hands with 95% ethanol after each contaminated mixture was applied. Samples of the pollen and pollen-spore mixtures that were not used in the seed orchard were returned to the laboratory and tested for viability of both conidia and pollen 1 week after the treatments were applied.

Bags were removed 30 days after pollination. Strobili were examined for survival and symptoms of pitch canker or other damage at the time the bags were removed and at 2, 5, and 10 months after treatment. Sample strobili were collected at each observation period, except at 5 months, for cultural isolation to determine whether FMS was present. In addition, a sample strobilus from each treatment on each of the five clones was collected after 1 month for ovule dissection to determine in vivo pollen germination and indications of possible infection by FMS (Matters and Blalock 1981).

Strobili collected for isolation were surface sterilized individually and split longitudinally. Six scales were selected per strobilus, three from each half distributed in the center portion of the strobilus. Each selected scale was cut in half midway between the apophysis and the ovules. A segment of tissue was also taken from the axis of each strobilus half. All tissues were placed on a culture medium selective for the growth of FMS. All cultures of FMS recovered from the sample strobili were tested for pathogenicity by inoculating 1- to 2-year-old seedlings of slash pine in a greenhouse and observing symptom development typical of the pitch canker disease. The strobili collected at the first observation period, 30 days after treatment, were shaken for 1 minute in 5 ml of sterile water with one drop of 10% Tween 20; the water was decanted and plated onto the FMS-selective medium.

The strobili collected for ovule observations were dissected and 10 ovules on 5 scales per strobilus were examined under a microscope. The total number of pollen grains and the number germinated were recorded for each ovule.

In January 1983, the seed orchard suffered severe damage from an ice storm. Shortly after the ice breakage occurred, 64 strobili that were still attached to broken, tagged branches were collected and subjected to the isolation procedures described above. The substantial losses from storm damage precluded additional sampling planned between 10 and 19 months.

All surviving cones were collected from the trees in mid-September 1983. Cones were placed individually in labeled, kraft paper bags, dried, and seeds were extracted. Seeds from each cone were placed on adhesive paper and radiographed to determine the condition of each seed (filled, empty, or damaged by fungi or insects).

After the seeds were radiographed, five seeds were randomly selected from each cone for isolation procedures. If a cone had five or less seeds, all were used. Selected seeds were surface sterilized for 15 minutes in 0.05% aqueous sodium hypochlorite, rinsed for 5 minutes in sterile distilled water, and cut in half longitudinally with a surface-sterilized razor blade. Seed coat segments were placed onto acidified potato dextrose agar (PDA). Gametophyte segments were surface sterilized for an additional 5 minutes in a sodium hypochlorite solution, rinsed for 2 minutes in sterile water, and placed onto the culture medium. Fungi growing from the seed parts were transferred to normal PDA for growth and identification. Isolates suspected to be FMS were transferred onto sterile carnation leaves on 1.5% water agar to confirm the identification.

RESULTS

Viability tests made on residual pollen and pollen-spore mixtures 1 week after the treatments were applied indicated a pollen germination of 86% and spore germination of both MIC and MAC sources of >95%.

Ovule Dissection--The mean number of pollen grains per ovule ranged from 1.5 for pure pollen to 2.9 for the MIC treatment, with the MAC treatment and wind pollination at 2.4 and 2.2 respectively. Pollen germination in the ovules was excellent, averaging 94% for the sources with FMS and 96% for sources with pure pollen and wind pollination. No obvious differences were observed in ovule appearance or abortion at this observation period in any of the four treatments. Macroconidia were observed in a resin droplet at the micropylar opening of one ovule from one MIC and one MAC scale of clone 17. In no ovules, however, were conidia observed in the pollen chambers.

Isolations from Sample Strobili--Cultures of FMS developed from the water used to rinse all of the MIC-treated strobili collected 1 month after treatment, but none developed from the MAC-treated, pure pollen, or wind-pollinated control samples.

Isolations from 33 sample strobili collected 1 month after pollination from the four treatments and five clones yielded cultures of FMS only from strobili receiving the MIC-pollen mixture. The tissues of the infected strobili from which FMS was isolated are shown in Table 1. All of the recovered FMS isolates were pathogenic to slash pine seedlings inoculated in the greenhouse.

Isolations from 15 strobili collected 2 months after treatment yielded a single isolate of FMS from an apophysis of a single scale from an MIC-treatment strobilus of clone 17. This isolate of FMS was not pathogenic on slash pine seedlings.

No cultures of FMS were isolated from three MIC, one MAC, and one pure pollen cones collected 10 months after treatment.

Following the ice storm in January 1983, a total of 64 strobili were collected from fallen branches that retained identification tags: 6 MIC, 14

MAC, 16 pure pollen, and 28 wind pollinated. FMS was isolated from the axis of a single MIC strobilus from clone 174. The pathogenicity test on slash pine seedlings was positive.

Table 1--Distribution of Fusarium moniliforme var. subglutinans (FMS) in tissues of slash pine strobili from five slash pine clones 1 month after MIC pollination.

GFC clone	Infected strobili	Strobilus tissue: Axis[1/]	Apophysis[2/]	Ovule[2/]
17	A	-	-	1
	B	-	-	1
	C	1	-	-
60	A	1	-	-
111	A	1	1	4

MIC = FMS microconidia + pollen.

1/ Number of FMS cultures from two axis samples, one per each strobilus half.

2/ Number of FMS cultures from three scales cut into an apophysis and an ovule half, six tissue segments per half.

The total losses of strobili from tagged branches, exclusive of those collected, from initiation of the research in February 1982 through early December 1982, are summarized in Table 2.

Unexplained losses of treated strobili were greatest in the MIC and MAC treatments on clone 17 and the MIC treatment on clone 174, where losses exceeded 20%. Within the remaining three clones, strobilus losses on the pollen only and wind controls were nearly equal or exceeded those of the pollen-spore mixtures. Losses for all treatments averaged across clones were highest for the MIC treatment (14.3%) followed by MAC (11.9%), pollen only (10.1%), and wind pollination (7.5%) (Table 2).

Seed Analysis--The average number of seeds within each of the different condition classes, by treatment, is shown in Table 3. The wind-pollinated controls produced more total seeds and more filled seeds per cone than the controlled-pollination cones, but they also had the highest average number of seeds classified as fungus damaged by radiographic analysis.

Table 2--Percentage of unexplained losses of treated strobili on five slash pine clones recorded during observations between February and December 1982. 1/

GFC clone	Treatment			
	MIC 2/	MAC 2/	Pollen 2/	Wind
17	27.6	23.1	4.8	3.3
60	6.1	3.1	12.5	0.0
68	3.7	14.8	10.0	15.2
111	13.6	14.3	16.7	11.5
174	20.7	5.0	6.3	6.9
Mean	14.3	11.19	10.1	7.5

1/ Exclusive of strobili collected for isolation.
2/ MIC = FMS microconidia + pollen.
MAC = FMS macroconidia + pollen.
Pollen = Pollen only.

Table 3.--Total cones and mean number of seeds per cone of different condition classes extracted from surviving cones of five slash pine clones 19 months after strobili were pollinated with pollen with and without conidia of Fusarium moniliforme var. subglutinans (FMS).

Treatment	Total cones (No.)	Seed per cone	Mean Number of Seeds 1/			
			Filled	Empty	Damaged by fungi	Damaged by insects
MIC 2/	51	55	45	6	3	<1
MAC 2/	48	47	35	8	4	<1
Pollen 2/	31	32	24	6	1	<1
Wind	59	90	70	10	10	<1

1/ Classified by radiography.
2/ MIC = FMS microconidia + pollen.
MAC = FMS macroconidia + pollen.
Pollen = Pollen only.

Isolation--FMS was not recovered from any of the 841 seeds that were tested for the presence of the pathogen. A total of 126 cultures were isolated from either the seed coats or the internal seed tissues. The only known pathogen recovered was L. theobromae which was present in 14 seeds (1.7%). Other fungi isolated were Fusarium sp., Pestalotia sp., Penicillium sp., and several as yet unidentified fungi.

DISCUSSION

The combined results of the viability tests made on pollen and pollen-spore mixtures 1 week after treatment, the presence and viability of pollen grains observed in the dissected ovule samples 1 month after treatment, and the number of filled seed in mature cones indicate that the pollination techniques used in this study were successful. Also, the isolation of FMS from the surface and from internal tissues of MIC-treated strobili 1 month after treatment demonstrated that infection can occur during the time of pollination. The failure to recover FMS from any of the MAC-treated strobili is difficult to interpret, but it may be related to differences in type of conidia used (microconidia vs. macroconidia) or inoculum density; conidia of FMS/cc of pollen in MIC was nearly double that of the MAC (5.3 million vs. 2.7 million spores/cc of pollen).

The greatest amount of unexplained loss of strobili during the first 10 months after treatment occurred in the MIC and MAC treatments in clone 17, and the MIC treatment in clone 174. It may be significant that clone 17 was the one from which FMS was recovered most frequently in the samples collected after 1 month, and the source of the only FMS isolate from the samples collected after 2 months. We have no explanation, however, for the failure to isolate FMS from the MAC-treated strobili of clone 17 or the MIC-treated flowers of clone 174 where, in both cases, the mortality after 10 months was considerably higher than in other treatments.

Mortality of slash pine strobili inoculated with aqueous suspensions of FMS mycelium and conidia at the time of pollen receptivity has been reported previously by Miller and Bramlett (1979), based on research in the same seed orchard. The higher infection rate obtained in this earlier research may reflect differences in environmental conditions at the time of inoculation or it may have resulted from the fact that the inoculum containing mycelium was applied in water, which could create a more favorable environment for spore germination and infection of the strobili.

In this current study, neither the radiographic analysis nor the cultural isolations of seeds extracted from the mature, treated and control cones indicated any effect of the inoculation of pollen-receptive strobili with FMS on the average number of filled or fungus-damaged seed per cone. The average number of fungus-damaged seeds per cone in the wind-pollinated controls was either approximately the same or greater than that from either of the two pollen-spore mixtures.

The overall results of this research confirm the previous research by Miller and Bramlett (1979) that, under conditions of artificial inoculation, FMS can infect female strobili of slash pine, and at least in certain

clones, may cause a significant loss of strobili. There was no indication, however, of a direct relationship between inoculation of female strobili with FMS and subsequent seed damage at maturity, at least based on the approximately 50% of the experimental cones that survived the ice storm.

Based on the results of this study and previous research (Miller and Bramlett 1979, Anderson et al. 1984), there can be little doubt that cone and seed infection by FMS does occur in slash pine and can cause significant losses, but it appears unlikely that strobilus infection at the time of pollen receptivity is a major source of seed infection. Further research is needed to determine when and how FMS and other fungi gain access to cones and seeds of slash pine.

CONCLUSIONS

1. FMS can infect strobili of slash pine when conidia are introduced at the time of pollen receptivity.
2. FMS can be isolated from asymptomatic tissues (ovules, scales, axis) of inoculated strobili.
3. Seed yields and seed infections did not appear to be significantly affected by inoculations with FMS at the time of pollen receptivity.
4. Infection of seeds by FMS apparently occurs sometime during the second season of cone growth and maturation.

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