

## Seed Treatment Prevents Vertical Transmission of *Fusarium moniliforme*, Making a Significant Contribution to Disease Control

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*Fusarium moniliforme* is a widespread facultative endophyte, primarily associated with corn, where it causes extensive crop damage. *F. moniliforme* can be toxigenic, the carcinogenic fumonisins being accumulated predominantly when the fungus colonizes corn plants. The pathogen is transmitted both through contaminated seeds and through environmental inoculum. This study utilized marked *nit*-mutant *F. moniliforme* inoculum in order to evaluate the quantitative significance of seedborne disease transmission. Greenhouse and field trials demonstrated that seedborne isolates were responsible for up to 50% of *F. moniliforme* disease. Seed treatment with the fungicide prochloraz was found to control seedborne transmission and to protect against *F. moniliforme* seedling blight. The elimination of seedborne inoculum resulted in reduced incidence of kernel rot and avoided the increment in soil inoculum accumulation associated with the introduction of infected seeds.

KEY WORDS: *Fusarium moniliforme*; corn disease; seedborne; *Zea mays*.

### INTRODUCTION

*Fusarium moniliforme* Sheldon is a toxigenic, nonobligate plant pathogen that colonizes a range of host crops (4,18,33). It is associated primarily with corn (*Zea mays* L.), in which it causes seedling blight as well as root, stalk and kernel rots (8,13,20). Serious economic losses associated with *F. moniliforme* have been recorded worldwide (3,18,21). The fungus often persists as a symptomless endophyte, systemically colonizing all plant tissues including kernels. *F. moniliforme* may remain undetected in kernels until germination, when it infects the emerging seedlings (1). Incidence of kernel infection has been reported to reach 100% in some seed lots (19). When colonizing sensitive host cultivars, under certain conditions *F. moniliforme* becomes aggressive and causes severe crop damage (2,13,17,18). The pathogen has been reported to exhibit highly variable pathogenicity in the presence of additional pathogens and other stress factors (7,29,34). Yields are reduced in non-symptomatic infected plants, due to deterioration of the stalk parenchyma tissue and gradual dehydration of the plant (12).

*Fusarium moniliforme* colonization of crop plants may present a serious health risk as the fungus is associated with the production of five different mycotoxins, including fusaric acid and fumonisins (1,4,23,30). Fumisin production occurs predominantly on

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corn (22,27), continuing during the passive endophytic phase (1). Fuminosins have been associated with human esophageal cancer and a variety of animal diseases, and persist in processed foods and animal feeds (6,22,32).

An understanding of the pattern of disease dissemination is essential for the development and implementation of effective disease control strategies. In addition to transmission by infected seeds, *F. moniliforme* is also spread to uninfected plants by inoculum present in the field. Infected stalks partially buried in the field are known to be major overwintering sites (8,15,17,24). Airborne conidia are abundant in corn fields in the growing season, and may penetrate stalk tissues through wounds (29), or infect developing kernels through the silks (10,14). Mature corn kernels may also be infected after sowing, by soilborne inoculum penetrating fissures in the pericarp; or at germination, where the pericarp is torn by the emerging seedling (27). Use of easily detectable *nit*-mutants enabled quantitative evaluation of those isolates colonizing corn plants derived from seedborne inoculum separately from those which were derived from environmental inoculum (15–18). Assessment of the proportion of isolates in the plant derived from seedborne inoculum also provides an indication of the potential contribution of effective seed treatments to overall disease control.

The present study was initiated after the observation of a phenomenon in northern Israel whereby extensive stands of corn became parched and fell before harvest, resulting in the loss of entire yields, while no visible symptoms of disease were evident (personal observations). During 3 years of surveillance, *F. moniliforme* was detected in all affected plants, although the quantity of pathogen was not directly correlated with the extent of crop damage. The aims of this study were to determine quantitatively the importance of seedborne transmission of *F. moniliforme*; to develop an effective fungicide seed treatment; and to assess the efficiency of seed treatment on disease control in short-term tests and in field trials.

## MATERIALS AND METHODS

**Generation and characterization of *nit*-mutants** Generation and characterization of *nit*-mutants was achieved according to the methods described by Correll *et al.* (9) and Kedera *et al.* (18). Mycelial discs were placed on medium containing 3% KClO<sub>3</sub> for mutant selection. Hyphal tips from fast-growing sections were transferred to minimal medium for classification, with *nit* phenotype reconfirmation based on differential growth on media containing NaNO<sub>3</sub> as the sole nitrogen source.

**Preparation of fungal inoculum** *nit*-mutant isolates of *F. moniliforme* grew on basal medium (9,31) for 7 days at 25°C in 12 h dark/light cycles prior to the collection of  $2 \times 10^6$  conidia ml<sup>-1</sup> and suspended in sterile distilled water.

**Fungicides** Prochloraz (*N*-propyl-*N*-[2-(2,4,6-trichlorophenoxy) ethyl] imidazole-1-carboxamide) (Octave or Mirage); propiconazole (1-[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl] methyl] -1*H*-1,2,4-triazole); and imazalil (1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl]-1*H*-imidazole) were used. Standard commercial preparations of the fungicides or their water suspensions were used, with a concentration of 10 mg a.i. per gram of seeds. Each fungicide was applied independently by shaking kernels for 1 h in a closed vessel containing 100 seeds (ratio of 10 mg of a.i. of fungicide to 1 g of seeds).

**Plant material** Corn cultivars Ht0, Ht1, Ht2, Ht3 and 'Jubilee' were used in the initial greenhouse trials; the commercial cultivar Jubilee was used in the field trials. The corn seeds were disinfected either by hot water eradication at 55°C for 10 min according to the method described by Daniels (11), or by fungicide application as described above.

**Recovery of *F. moniliforme* from corn plants and seeds** Tissue sections from each plant and/or seed were surface-sterilized by immersion in a solution of 5% NaClO and 0.5% Tween 20 for 20 sec, rinsed twice in sterile water, and transferred either to Komada medium or to the selective NaNO<sub>3</sub>-containing medium (18,31). Identification of *F. moniliforme*-wild type or *nit*-mutants was performed after about one week's incubation at 25°C in 12 h dark/light cycles.

**Quantitative examination of colony-forming units (cfu) of *F. moniliforme* in corn seedlings** Corn seeds were hot-water-disinfected according to the method described by Daniels (11), and artificially inoculated with *nit*-mutant *F. moniliforme* by adding a conidial suspension at  $1 \times 10^6$  conidia ml<sup>-1</sup> for 18 h at 25°C (Table 2). The inoculated seeds were then sown in sterile perlite, using a total of 30 seeds per cultivar. Disease incidence was evaluated 4–5 weeks after sowing. After qualitative disease evaluation, a quantitative assessment of *F. moniliforme* colonization was carried out using plate counts. Leaves were discarded, and the seedlings were surface-sterilized (as described above), and blender-homogenized in sterile distilled water. Aliquots of the resultant suspension, at two dilutions per sample, were spread in petri dishes on modified minimal medium – standard MM medium (18,31) in order to identify the *nit*-mutants with restricted supplements (Oxgell) and antibiotics (streptomycin and chloramphenicol). The number of fungal colonies per plate was counted after one week's incubation, enabling the calculation of cfu of *F. moniliforme* per gram of plant material.

**Field trials** These trials were carried out over three growing seasons in commercial corn fields in northern Israel. Commercial seed batches tested for low rates (~2%) of *F. moniliforme* contamination were divided into treatment groups. Control lots were commercial seeds with no further treatment. Test batches were either hot-water disinfected, or treated with prochloraz as described above. Half of each batch of disinfected seeds was re-infected with *nit*-mutant *F. moniliforme*. Seeds from each of the treatments were sown in five different plots. Each plot was sown in four rows of 4 m, with eight plants grown per meter, giving 128 plants per plot, and a total sample size of 640 plants for each experiment.

**Quantitative estimation of cfu of *F. moniliforme* in corn plants** Plant tissue samples were collected after 5 and 8 weeks, by cutting transverse slices from the four lowest nodes. In mature fields before harvest (after 12 weeks' growth) both stalk cross-sections from the four lowest nodes, the stem section below kernels, and kernels were collected. After recovery of *F. moniliforme* as described above, all samples underwent quantitative analysis.

**Quantitative estimation of cfu of *F. moniliforme* in the soil** A plate count method was devised for the isolation and determination of the cfu of *F. moniliforme* in the soil using a selective Komada agar (28). Soil samples were diluted with one 10-g soil sample shaken in a bottle with 200 ml 0.1% agar for 20 min at room temperature. The resulting suspension was then plated out, with two 10-g samples tested per plot. Fungal colonies were counted after incubation for approximately one week at 25°C. *F. moniliforme* identification was performed according to Nelson *et al.* (28).

All experiments were repeated at least three times. Results from all experiments were combined and the effects of fungus and fungicide treatment were analyzed by Super ANOVA. Using Fisher's protected least significant difference (LSD) test, the greatest difference was found in all experiments.

## RESULTS AND DISCUSSION

**Detection of *F. moniliforme* contamination in seeds** During the first phase of the investigation, corn seeds from the commercial cv. Jubilee were surveyed for *F. moniliforme* contamination. It was of interest to note that 7% of the seeds from apparently disease-free ears were infected with *F. moniliforme* (Table 1). This agrees with previous findings that symptomless seed may in fact be infected (2). *F. moniliforme* was isolated from more than 90% of seeds displaying disease symptoms (Table 1). Data accumulated over 4 years' screening of commercial corn seeds had shown infection rates with *F. moniliforme* to vary from 2% to 50% (data not shown). In order to achieve seeds infected exclusively with *nit*-mutants, a reliable method of seed disinfection with no residual activity was required. Hot-water treatments were found to be over 90% effective. Exposure to *nit*-mutants for 18–24 h gave 94% efficiency of re-infection (Table 2).

TABLE 1. Recovery of *Fusarium moniliforme* from corn seeds (cv. Jubilee), taken from symptomatic and symptomless ears collected from commercial cornfields in northern Israel

Batch no. <sup>z</sup>	Symptomless ears		Symptomatic ears			
	Seeds (symptomless)		Seeds (symptomless)		Seeds (symptomatic)	
	Number of seeds	% Infected	Number of seeds	% Infected	Number of seeds	% Infected
1	48	6.2	49	18.4	6	83.3
2	53	9.4	50	8.0	4	100
3	52	5.8	40	7.5	11	100
	Total: 153	Avg. 7.2 a <sup>y</sup>	Total: 139	Avg. 11.5 a	Total: 21	Avg. 95.2 b

<sup>z</sup>Each batch included five symptomless and five symptomatic corn ears collected from commercial corn fields in northern Israel.

<sup>y</sup>Figures followed by the same letter do not differ significantly at  $P=0.05$ .

TABLE 2. Effect of length of inoculation time on the percent of seeds successfully inoculated with *nit*-mutant *Fusarium moniliforme*

Time (h)	Seeds infected (%)
1	11
2	22
4	39
18	94
24	94

**Effects of seed treatments on emergence and disease severity in short-term greenhouse trials** Greenhouse experiments were carried out to test the effects of seed treatments on disease incidence. The commercial cv. 'Supersweet' Jubilee seeds showed low emergence rates in naturally infected seeds (60%), as reported by Wilson *et al.* (35) and Styer and Cantliffe (33), and this was slightly reduced after disinfection by hot-water treatment to

TABLE 3. Effects of hot water seed treatment, prochloraz seed treatment, and artificial infection on emergence and disease incidence in corn seeds (cvs. Jubilee and Ht2)

Seed treatment	Seed emergence (%)		Disease incidence (%)		Density of fungal propagules/g/plant	
	Jubilee	Ht2	Jubilee	Ht2	Jubilee	Ht2
None (control) <sup>z</sup>	60 a <sup>v</sup>	78 b	5 a	96 c	1,120 b	1,229 b
Hot water <sup>y</sup>	47 a	77 b	8 a	22 b	290 a	64 a
Inoculated with <i>nit</i> -mutant <sup>x</sup>	37 a	57 a	0 a	94 c	3,250 c	30,000 d
Prochloraz <sup>w</sup>	53 a	77 b	0 a	0 a	73 a	6 a

<sup>z</sup>Naturally infected seeds.

<sup>y</sup>Naturally infected seeds disinfected with hot water (treatment at 55°C for 10 min; ref. 11).

<sup>x</sup>As for footnote y, re-infected with *nit*-mutant *Fusarium moniliforme*.

<sup>w</sup>As for footnote x, and then treated with the fungicide prochloraz at 10 mg a.i. g<sup>-1</sup> seeds.

<sup>v</sup>Within columns, figures followed by the same letter do not differ significantly at *P*=0.05.

TABLE 4. Comparative effect of prochloraz, propanazole and imazalil seed treatments on disease incidence in 4-week-old corn seedlings grown from seeds naturally infected or (*nit*-mutant) artificially inoculated with *Fusarium moniliforme*

Seed treatment	Density of fungal propagules / g / plant	
	Naturally infected kernels from ears showing disease symptoms	Kernels artificially infected with <i>nit</i> -mutant <i>F. moniliforme</i>
Control	2470 c <sup>z</sup>	800 c
Prochloraz	21 a	8 a
Propiconazole	305 b	274 b
Imazalil	372 b	85 ab

<sup>z</sup>Within columns, figures followed by a common letter do not differ statistically at *P*=0.05.

an average of 47% emergence. After *nit*-mutant inoculation this was further reduced to an average of 37% emergence (Table 3). Jubilee is also disease-tolerant, with symptoms appearing only in later developmental stages, if at all. Additional cultivars were therefore assessed for use in short-term trials.

TABLE 5. Effect of prochloraz seed treatment on disease incidence associated with *Fusarium moniliforme* at different developmental stages of corn in the field

Seed treatment	Six-leaf stage	Initiation of kernel development	Mature plants prior to harvest			
	% infected, disc samples <sup>x</sup>	% infected, disc samples	% Infected disc samples	% Disc infected, <i>nit</i> -mutant	% Infected kernels	% Kernels infected, <i>nit</i> -mutant
Seeds inoculated with <i>nit</i> -mutants <sup>z</sup>	4 a <sup>w</sup>	16 a	62 b	17 b	11 a	5 b
Prochloraz-treated seeds inoculated with <i>nit</i> -mutants <sup>y</sup>	0 a	2 a	29 a	0 a	4 a	0 a

<sup>z</sup>Naturally infected seeds, inoculated with *nit*-mutant.

<sup>y</sup>As footnote z, treated with prochloraz.

<sup>x</sup>Percent of cross-sectional discs cut from the bottom four nodes of the plants and found infected with *F. moniliforme*.

<sup>w</sup>Within columns, figures followed by the same letter do not differ statistically at *P*=0.05.

TABLE 6. Effect of prochloraz seed treatment on the development of *Fusarium moniliforme* in the soil of a corn field

Treatment applied to seeds sown in soil plot	cfu of <i>F. moniliforme</i> per gram of soil		
	5 weeks after sowing	8 weeks after sowing	12 weeks after sowing
Seeds inoculated with <i>nit</i> -mutants <sup>z</sup>	57 a <sup>x</sup>	37 a	194 b
Prochloraz-treated seeds inoculated with <i>nit</i> -mutants <sup>y</sup>	21 a	13 a	29 a
Naturally infected seeds (~2% contamination rate)	62 a	50 a	130 ab
Naturally infected seeds (~2% contamination rate) treated with prochloraz	30 a	17 a	53 a

<sup>z</sup>Naturally infected seeds inoculated with *nit*-mutant.

<sup>y</sup>Naturally infected seeds treated with prochloraz.

<sup>x</sup>Within columns, figures followed by a common letter do not differ statistically at  $P=0.05$ .

Among the cultivars tested the super-susceptible cv. Ht2 proved to be most suitable, with good germination rates, and disease symptoms strongly evident at the seedling stage (Table 3). Hot-water treatment did not significantly alter emergence rates in Ht2, despite a reduction in fungal propagule density from ~1200 propagules per g plant tissue in controls to ~60 propagules per g plant tissue after hot-water disinfection. Symptomless infection of seeds up to a certain threshold therefore does not appear to reduce emergence rate, although higher inoculum loads, as found in re-inoculated Jubilee (~3000 propagules per g plant tissue) and re-inoculated Ht2 (>30,000 propagules per g plant tissue), did reduce emergence (Table 3). The percent of Ht2 plants displaying disease symptoms was reduced after hot-water seed disinfection from 96% before treatment to 22% after treatment; Jubilee seedlings did not display symptoms at this stage (Table 3). As reduction of inoculum load is critical in preventing the fungus from entering the aggressive pathogenic phase, hot-water seed disinfection may be of interest as an alternative to chemical seed treatments.

Conventional agricultural practice is still oriented toward highly efficient, fast acting chemical fungicides. Preliminary trials indicated that prochloraz compared favorably with other fungicide seed treatments (Table 4). Following prochloraz treatment, the emergence rate was similar to that after hot-water disinfection, *i.e.*, reduction in emergence caused by the *nit* inoculation was prevented, with no detrimental effect noted due to the fungicide application (Table 3). Seedlings of both Jubilee and Ht2 cultivars grown from artificially inoculated seeds treated with prochloraz showed no disease symptoms, quantitative analysis confirmed that there was minimal or no infection (Table 3). The fungicide seed treatment was thus found to be effective in short-term disease control under the conditions used.

**Field trials investigating the effect of seed treatments** The longer-term effect of prochloraz seed treatments on disease incidence and on inoculum levels in the soil was assessed in commercial agricultural fields of cv. Jubilee corn. Jubilee seeds from a batch with an unusually low level of *F. moniliforme* contamination (~2%) were sown following normal agricultural practices. Test seed lots were disinfected using prochloraz and then, where applicable, re-infected with *nit*-mutant *F. moniliforme* before sowing. Plant tissue and soil samples were collected and analyzed for *F. moniliforme* propagules at 5, 8 and 12 weeks after sowing. Plant tissue samples underwent quantitative analysis for *nit*-mutant

and wild-type *F. moniliforme* isolates.

After 5 weeks, with plants at the six-leaf stage, no *F. moniliforme* was detected in plants grown from prochloraz-treated seeds (Table 5). There was little difference in inoculum load between soils sown with differently treated seeds at both the 5-week and 8-week stages (Table 6). After 8 weeks plants were at the beginning of the reproductive growth stage, with kernel development initiated. By this time some plants grown from prochloraz-treated seeds were found to be infected in the lowest four nodes, although significantly fewer than for the plants grown from non-fungicide-treated seeds (Table 5). After 12 weeks plants were mature, with ears ready for harvest. Plants grown from prochloraz-treated seeds had 50% fewer infected nodes than non-treated plants (Table 5). The source of inoculum could not be specifically determined, but wild-type *F. moniliforme* was present in the fields used. Quantitative analysis of *F. moniliforme* isolated from plants grown from non-treated seeds revealed that ~30% of contaminated nodes carried *nit*-mutant *F. moniliforme*, while the remainder was infected with wild-type isolates. This implies that ~30% of the *F. moniliforme* inoculum carried in the plant nodes was derived from seedborne inoculum.

Disease incidence was assessed also in the kernels produced in plants sown from *nit*-infected seeds, and from *nit*-infected-treated seeds. In the case of plants grown from the *nit*-infected-treated seeds, ~50% fewer kernels were infected (Table 5). In plants grown from the *nit*-infected seeds (not treated with prochloraz), ~50% of the *F. moniliforme* isolated from the infected kernels was *nit*-mutant (Table 5). This demonstrates clearly for the first time that up to 50% of *F. moniliforme* contamination of infected corn kernels is derived from systemic seedborne inoculum.

The highest rates of *F. moniliforme* colonization in soils at 12 weeks after sowing (immediately before harvest) were found in those plots sown with *nit*-mutant artificially inoculated seeds, this being compatible with the introduction of additional inoculum on contaminated seeds (Table 6). At this stage soils sown with prochloraz-treated seeds had at least 50% fewer propagules than soils sown with non-treated seeds. This finding emphasizes the importance of seed treatment for long-term disease control, as soilborne *F. moniliforme* inoculum also contributes to crop disease (8,17,18).

In sweet corn, as opposed to field corn used for animal feed, many varieties contain the *shrunk-2* gene, which interferes with starch synthesis, allowing sugars to accumulate in the kernel. These *shrunk-2* hybrids also tend to be particularly susceptible to microbial colonization of the seed after planting, in part due to thin, cracked pericarps (35). Infected seeds have poor field performance with seedling blight being strongly associated with *F. moniliforme* (26). Genetic resistance in corn to seedling blight caused by *F. moniliforme* is reported to be due to additive gene action, with the pericarp being the site of gene action. Resistant cultivars were found to have measurably thicker pericarps (26). Commercial sweet varieties, such as Jubilee, with thin pericarps have high susceptibility to *F. moniliforme*, making effective seed treatments vitally important. Seed treatments presently applied to commercial seed lots are inadequate for the control of seedborne *Fusarium*, as demonstrated by the persistent retrieval of contaminated seeds (35). Recently seed companies have changed the standard treatment from captan to Maxim XL, a combination of fludioxonil and mefenoxam. While it is reported that treatment with the newer fungicides results in improved control of seedling blight (25), this does not confer long-term effectiveness, as symptoms appear only at later stages in cv. Jubilee. We found that prochloraz completely eradicates *F. moniliforme* from seeds of all corn cultivars tested.

Our results indicate that prochloraz provides efficient control of seedling blight, and that widespread implementation of prochloraz seed treatments would also significantly reduce crop damage by *F. moniliforme*.

Bacon *et al.* (5) claimed that endophytic infections transmitted through systemically infected seeds are not controlled by seed applications of fungicides. Our results show that prochloraz seed treatment is effective in controlling fungal inoculum derived from *nit*-mutant infected seeds. The artificial inoculation with *nit*-mutant *F. moniliforme* closely models transmission of the fungus by seeds infected with environmental inoculum prior to germination, as the fungus was unlikely to have penetrated deeply into sub-pericarpal tissue. Our results confirm that half of the inoculum in contaminated seeds is derived from seedborne infection (whether of systemic or environmental origin). We also demonstrated that where seedborne inoculum is controlled by prochloraz (as was found with the artificially inoculated *nit*-mutants), the benefit derived from reduced inoculum is conserved until kernel formation. It would be of interest to treat seeds systemically infected with *nit*-mutants with prochloraz, to determine whether the fungicide is equally effective in controlling *F. moniliforme* from the systemically infected seeds. Notwithstanding the possibility that some small quantity of deep-seated inoculum may persist following prochloraz treatment, the benefit derived from the eradication of the vast majority of seedborne inoculum remains.

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