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Ecology and Epidemiology

Infection Courts in Watermelon Plants Leading to Seed Infestation by *Fusarium oxysporum* f. sp. *niveum*

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ABSTRACT

Fusarium wilt incited by *Fusarium oxysporum* f. sp. *niveum* is a seed-transmitted disease that causes significant yield loss in watermelon production. The pathogen may infect watermelon seeds latently, which can be an important inoculum source and contribute to severe disease outbreak. However, information regarding infection courts of *F. oxysporum* f. sp. *niveum* leading to infestation of watermelon seeds is limited. To determine how seeds in watermelon fruit can be infested by *F. oxysporum* f. sp. *niveum* during the watermelon growing season, greenhouse and field experiments were conducted in 2014 and 2015 where watermelon flowers and immature fruit were inoculated with *F. oxysporum* f. sp. *niveum*. Seeds were extracted from mature watermelon fruit, and infestation of watermelon seeds was determined by isolation of *F. oxysporum* f. sp. *niveum* and further confirmed by real-time polymerase chain reaction (PCR) analysis. Inoculation of the pericarp of immature fruit resulted in 17.8 to 54.4% of infested seeds under field conditions and 0.6 to 12.8% of infested seeds under greenhouse conditions when seeds were not surface disinfested prior to isolation. Seed infestation was also detected in 0 to 4.5% of the seeds when seeds were surface disinfested prior to isolation. Inoculation of pistil resulted in 0 to 7.2% and 0 to 18.3% of infested seeds under greenhouse and field conditions when seeds were surface disinfested or not disinfested before isolation, respectively. Inoculation of peduncle resulted in 0.6 to 6.1% and 0 to 10.0% of infested seeds in the greenhouse and field experiments when seeds were surface disinfested or not disinfested before isolation, respectively. Seed infestation was also detected in all the experiments using real-time PCR assay when pericarp or pistil was inoculated, and in three of four experiments when peduncle was inoculated, regardless of whether seeds were surface disinfested or not disinfested. Pericarp and peduncle of immature watermelon fruit and pistil of watermelon flowers could be potential infection courts for *F. oxysporum* f. sp. *niveum* leading to infestation of seeds in asymptomatic watermelon fruit.

Fusarium wilt incited by *Fusarium oxysporum* f. sp. *niveum* is among the most economically important diseases on watermelon occurring in almost all watermelon-producing regions of the world. Occurrence of the disease was first reported by E. F. Smith in the southeastern United States (Smith 1894). Symptoms of the disease include damping-off and wilt of watermelon vines at any stage of plant growth (Egel and Martyn 2007). A common symptom of this disease is wilting of some vines of the plant while other plant parts remain symptomless. Internal vascular discoloration is apparent when the root or lower stems are cross sectioned.

Southeastern states in the United States, including Georgia, Florida, South Carolina, and North Carolina, are major producers of watermelon in the nation with more than 50% of national watermelon production in this region. Increasing damage in commercial watermelon fields in the southeastern United States caused by Fusarium wilt has been observed in recent years with disease incidence over 50% in many fields (P. Ji and A. Petkar, *unpublished data*). The disease is favored by cool wet conditions that are common in the southeastern states especially during the spring growing season. With the phase-out of methyl bromide, limited options are available for effective management of Fusarium wilt of watermelon. The only fungicide available, prothioconazole, was recently registered for the disease, and development of resistance by the pathogen to fungicides with single targeting site is a concern (Petkar et al. 2017). Using cover crops as soil amendments is a promising tactic for managing the disease (Keinath et al. 2010; Zhou and Everts 2004), though the approach has not been commonly adopted by growers in the southeastern United States.

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Another challenge in managing Fusarium wilt of watermelon is the seedborne nature of *F. oxysporum* f. sp. *niveum* that facilitates long distance dispersal of the pathogen and disease outbreaks. The seedborne nature of *F. oxysporum* f. sp. *niveum* has long been known. Fulton and Winston (1915) were the first to isolate *F. oxysporum* f. sp. *niveum* from watermelon seeds. Porter (1928) recovered *F. oxysporum* f. sp. *niveum* isolates from seeds of watermelon fruit developed on *F. oxysporum* f. sp. *niveum* infected vines and from commercial watermelon seedlots in Georgia and Texas. Later, Martyn (1987) isolated *F. oxysporum* f. sp. *niveum* from watermelon seeds used for the production of hybrid seedless watermelon in Texas. In a more recent study, Michail et al. (2002) obtained *F. oxysporum* f. sp. *niveum* isolates from five cultivars of watermelon and studied the relationship between level of seed infestation by *F. oxysporum* f. sp. *niveum* and Fusarium wilt development on watermelon plants grown from the seeds. Disease incidence was more than 45% when using seeds with moderate (8.5 to 9.5%) and high (23 to 31.5%) percentages of infestation, but less than 5 to 10% when seeds with a lower percentage of infestation (1.5 to 2.5%) were grown. These studies indicate that watermelon seeds latently infected by *F. oxysporum* f. sp. *niveum* play a significant role in development of Fusarium wilt.

Although it has long been demonstrated that infested watermelon seeds can be a significant source of *F. oxysporum* f. sp. *niveum* inoculum, knowledge about the mode of *F. oxysporum* f. sp. *niveum* infection leading to watermelon seed infestation by the pathogen is limited. Hence, the objective of this work was to determine potential infection courts of *F. oxysporum* f. sp. *niveum* leading to infestation of watermelon seeds. Information about *F. oxysporum* f. sp. *niveum* infection for seed infestation will not only advance our knowledge about pathogenicity and epidemiology of the pathogen, but also facilitate development of effective disease management approaches by protection of the infection courts to reduce seed infestation.

MATERIALS AND METHODS

***F. oxysporum* f. sp. *niveum* isolate and inoculum preparation.** The *F. oxysporum* f. sp. *niveum* isolate (race 1) used in the study was provided by H. Sanders at the University of Georgia. Single spore cultures were generated, and pathogenicity on watermelon seedlings was verified as described previously (Petkar et al. 2017). Mycelia of the single-spore isolates were transferred to fungal storage vials (Pro-Lab Diagnostics Inc., Round Rock, TX) and kept at −80°C for long-term storage. A single-spore isolate *F. oxysporum* f. sp. *niveum* R1 was used for all the greenhouse and field experiments. To prepare *F. oxysporum* f. sp. *niveum* inoculum, the isolate was grown on potato dextrose agar at 25°C for 7 days. Five mycelial plugs (7-mm diameter) from the edge of a growing colony were transferred to a flask (500-ml) containing 200 ml of liquid mineral salts medium (Esposito and Fletcher 1961). After incubating under light at 23 ± 1°C for 2 weeks with shaking (150 rpm), the liquid culture was filtered through sterile cheesecloth (three layers). Concentration of microconidia in the liquid culture was calculated using a hemocytometer, and sterile distilled water (SDW) was added to adjust the concentration to 10^6 spores/ml to be used in the study.

Greenhouse experiments. Two greenhouse experiments were conducted, one in fall 2014 and one in spring 2015. Watermelon seeds (Sugar Baby) were sown in seedling trays (with 3.5 × 3.5 cm cells) containing a commercial potting mix (Scotts Miracle-Gro, Marysville, OH). Seedlings were transplanted to 7.5-liter pots containing the same potting mix 3 weeks after seeding, one plant per pot. After anthesis, female watermelon blossoms (20 per inoculation method) were hand-pollinated and tagged for inoculation with *F. oxysporum* f. sp. *niveum*. Three potential infection courts were inoculated on separate plants: (i) swabbing of pericarp of immature fruit (approximately 1 × 2.5 cm in size); (ii) inoculation of peduncle of immature fruit; and (iii) inoculation of pistil. For pericarp inoculation, the pericarp of an immature watermelon fruit was swabbed with a microconidial suspension (10^6 spores/ml). For peduncle inoculation, the peduncle of an immature watermelon fruit was injected with a microconidial suspension (10^6 spores/ml, 10 µl/peduncle). This was to determine if *F. oxysporum* f. sp. *niveum* entering vascular tissues, through means like wounds that may occur during the growing season, might lead to seed infestation. For pistil inoculation, a female flower was inoculated by applying 10 µl of microconidial suspension (10^6 spores/ml) to the stigma (Fig. 1). A randomized complete block design was used with five replicates and 15 plants for each inoculation site (pistil, pericarp, and peduncle). The same numbers of plants treated with SDW were used as controls. The plants were maintained under greenhouse conditions with 22/30°C and 20/28°C night/day temperature for the experiments in fall 2014 and spring 2015, respectively. At maturity, fruit were harvested and surface sterilized with 70% ethanol, and seeds were extracted. To prevent cross contamination from seed to seed, watermelon fruit was cut with a sterilized knife and seeds were taken out individually using a sterile spatula. Seeds were dried on sterilized filter paper and evaluated for *F. oxysporum* f. sp. *niveum* infestation by plating as described below. For detection by a real-time polymerase chain reaction (PCR) assay, seeds from the same inoculation method in each replicate were pooled, maintained as a separate lot, and stored at 4°C until processed.

Field experiments. Two field experiments were conducted at University of Georgia Coastal Plain Experiment Station experimental farm in Tifton, GA, in fall 2014 and spring 2015. Field soil was loamy sand with no history of infestation by *F. oxysporum* f. sp. *niveum*. Raised beds (15 cm high, 76 cm wide) were prepared and centered 1.8 m apart. N-P-K (5-10-15) was applied (840 kg/ha) prior to laying plastic mulch, and the beds were covered by white or black plastic mulch in the fall and spring experiments, respectively. A single drip irrigation tape was installed approximately 2.5 cm below the surface in the center of the beds as the plastic mulch was applied. The drip tape used was Ro-Drip (John Deere Water, San Marcos, CA) with 30 cm spacing of emitters and a flow rate of 56 ml/m/min.

Watermelon plants ('Sugar Baby') were grown in seedling trays as in the greenhouse study. Four-week old seedlings were transplanted at 61 cm spacing within a row in the field beds. The experimental plots consisted of a single row that was 3 m long with 2.5 m buffer zones without plants maintained between plots. A randomized complete block design with five replicates for each inoculation site (pistil, pericarp, and peduncle) was employed. As described in the greenhouse experiments above, female watermelon blossoms (20 per inoculation method) were hand-pollinated after anthesis and inoculated at one of three sites. Mature fruit were harvested and surface sterilized with 70% ethanol, seeds were extracted and *F. oxysporum* f. sp. *niveum* was detected using plating and PCR analysis as in the greenhouse experiments.

Assessment of *F. oxysporum* f. sp. *niveum* seed infestation by plating assay. Watermelon seeds were tested for internal and external infestation by *F. oxysporum* f. sp. *niveum*. Sixty seeds were used per replicate and inoculation method to detect internal and external infestation, respectively. For internal infestation, seeds were soaked in 0.6% NaOCl solution for 3 min, followed by rinsing with SDW. The seeds were dried on sterile filter paper and placed on peptone PCNB agar (PPA) plates (Leslie and Summerell 2006), 10 seeds per plate. The plates were incubated for 2 weeks at 25°C and checked periodically for putative *F. oxysporum* f. sp. *niveum* colonies. For external infestation, seeds were rinsed with SDW, dried on sterile filter paper and placed on PPA plates. Putative *F. oxysporum* f. sp. *niveum* colonies were purified by subculturing hyphal tips on PPA plates and then transferring onto potato dextrose agar. The cultures were identified by morphological characteristics (Leslie and Summerell 2006) and PCR analysis using *F. oxysporum* f. sp. *niveum*-specific primers Fn-1/Fn-2 (Zhang et al. 2005) and Fon-1/Fon-2 (Lin et al. 2010). Total fungal genomic DNA was extracted using DNeasy Plant Mini kit (Qiagen, Valencia, CA). PCR was performed in 25 µl of reaction mixture containing 10 ng of DNA, 200 µM each dNTP, 0.5 µM each primer, 0.5 U of *Taq* DNA polymerase, and 10× PCR reaction buffer. The amplification was performed in a MyCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA) using the methods reported earlier (Lin et al. 2010; Zhang et al. 2005). PCR products were electrophoresed on agarose gel (1.5%) and visualized as described previously (Petkar et al. 2017). External and internal *F. oxysporum* f. sp. *niveum* seed infestation was quantified by percentage of seeds infested by *F. oxysporum* f. sp. *niveum*. To determine the effect of inoculation site on *F. oxysporum* f. sp. *niveum*

Fig. 1. Inoculation of watermelon plants with *Fusarium oxysporum* f. sp. *niveum*. **A,** Inoculation of pericarp; **B,** inoculation of peduncle; and **C,** inoculation of pistil.

seed infestation, data were analyzed with the PROC GLM procedure using the Statistical Analysis System (SAS Institute, Cary, NC) and Fischer's least significant difference test was used for mean separation at $P = 0.05$.

To further confirm the identity of the isolates, 11 representative isolates (one from each inoculation in the greenhouse and field experiments) were tested for pathogenicity on watermelon seedlings under greenhouse conditions. The isolates were grown and microconidial suspensions were prepared as described above. Watermelon seedlings ('Sugar Baby') were grown in 9-cm-diameter pots containing sand/peat/vermiculite mixture (4:1:1, vol/vol/vol). At the first true leaf stage, seedlings were inoculated by applying 5 ml of conidial suspension (10^6 spores/ml) to the base of each plant. Nine plants for each isolate, and nine plants treated with SDW were used as controls. A randomized complete block design was used with three replicates. The plants were incubated in a greenhouse (28/20°C day/night), and Fusarium wilt incidence was recorded 3 weeks after inoculation. Diseased plants were sampled for identification of the causal agent by isolation on PPA and PCR analysis using primer sets Fn-1/Fn-2 and Fon-1/Fon-2 as described above. The experiment was conducted twice under similar conditions.

Assessment of *F. oxysporum* f. sp. *niveum* seed infestation by real-time PCR assay. Watermelon seeds from the three inoculation sites (60 seeds/replicate/inoculation site) were either surface disinfested with 0.6% NaOCl or nondisinfested and crushed in a coffee grinder (Hamilton Beach Inc., Southern Pines, NC). Crushed seeds were transferred to 10 microcentrifuge tubes (1 ml of powder/tube), and the grinder was rinsed with SDW for three times after each sample processing. Crushed seed in each tube was weighted, and total genomic DNA was extracted using DNeasy Plant Mini kit. DNA of crushed seeds was assayed by real-time PCR with primers Fn-1 and Fn-2. For real-time PCR, 5 µl of DNA was amplified in a 25-µl volume of PCR master mix containing 25 µM each primer, and 12.5 µl of Sso Fast EvaGreen Supermix (Bio-Rad Laboratories). Each DNA sample (from1 ml of crushed seeds in one tube) was amplified in three repeated PCR reactions. Amplification was performed in a Cepheid SmartCycler (Cepheid, Sunnyvale, CA) using the protocol by Zhang et al. (2005).

To quantify *F. oxysporum* f. sp. *niveum* DNA from the seeds, genomic DNA of isolate *F. oxysporum* f. sp. *niveum* R1 was extracted as described above. A standard curve was established by plotting the log of known concentrations of DNA of *F. oxysporum* f. sp. *niveum* R1 from 10 ng to 100 fg, with 10-fold dilutions, against the cycle threshold (Ct) values. Ct values resulting from DNA samples of the watermelon seeds were plotted onto this curve, and the concentrations of *F. oxysporum* f. sp. *niveum* DNA and amount of *F. oxysporum* f. sp. *niveum* DNA per gram of seeds were calculated. Samples with Ct values larger than 35 were considered negative for *F. oxysporum* f. sp. *niveum*. Quantities of *F. oxysporum* f. sp. *niveum* DNA from seeds generated from the three inoculations were analyzed using SAS as described above.

RESULTS

***F. oxysporum* f. sp. *niveum* seed infestation detection by plating assay.** All isolates from seeds identified to be *F. oxysporum* f. sp. *niveum* by PCR using primers Fn-1/Fn-2 gave positive results when analyzed by PCR using primers Fon-1/Fon-2. In the greenhouse study in 2014, external infestation by *F. oxysporum* f. sp. *niveum* was detected in 0.6% of the seeds for both pericarp and pistil inoculations, and internal seed infestation was not detected (Fig. 2A). For peduncle inoculation, 0.6% of the seeds were positive for internal infestation by *F. oxysporum* f. sp. *niveum*, and external infestation by *F. oxysporum* f. sp. *niveum* was not detected. In the 2015 greenhouse experiment, external seed infestation was detected for all the three inoculation sites, and the percentage of infested seeds was significantly higher ($P = 0.02$) for pistil inoculation (18.3%) than pericarp (12.8%) and peduncle (6.1%) inoculation (Fig. 2B). For internal infestation, pericarp inoculation resulted in 4.5% seed infestation, which was significantly higher ($P < 0.01$) compared with peduncle (1.1%) and pistil (1.7%) inoculation. *F. oxysporum* f. sp. *niveum* was not detected from seeds, externally or internally, from watermelon treated with SDW using the three inoculation sites in both 2014 and 2015 experiments.

In the field experiment conducted in 2014, external seed infestation was only detected from pericarp inoculation (Fig. 3A). A higher level of internal seed infestation ($P = 0.02$) was detected for pericarp (2.8%) compared with peduncle (0.6%) inoculation. Neither external nor internal seed infestation was detected from pistil inoculation. In the field study in 2015, external and internal seed infestation was detected from watermelon inoculated at the three sites (Fig. 3B). Pericarp inoculation resulted in a significantly higher ($P < 0.01$) percentage of external seed infestation than pistil and peduncle inoculation. For internal infestation, significantly higher infestation was detected for pistil inoculation compared with peduncle and pericarp inoculation. External or internal seed infestation by *F. oxysporum* f. sp. *niveum* was not detected from watermelon treated with SDW using the three inoculation sites in either the 2014 or 2015 field experiments. In the pathogenicity assays, 11 representative isolates from the field and greenhouse experiments caused disease on watermelon seedlings with 100% disease incidence three weeks after inoculation. The noninoculated plants remained

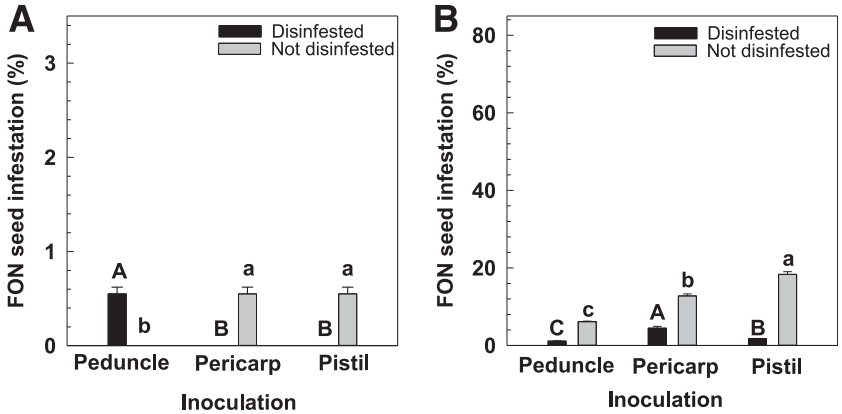

Fig. 2. Percentage of seed infestation by *Fusarium oxysporum* f. sp. *niveum* following peduncle, pericarp, and pistil inoculation under greenhouse conditions when detected by plating assay. **A,** Greenhouse study in 2014; and **B,** greenhouse study in 2015. Disinfested and not disinfested indicate the seeds were treated or not treated with 0.6% NaOCl, respectively, before isolation by plating. Error bars indicate standard errors of the means of five replicates. Means with the same uppercase or lowercase letters are not significantly different according to the least significant difference test ($P = 0.05$). *F. oxysporum* f. sp. *niveum* was not detected in seeds collected from plants treated with sterile distilled water, regardless of the inoculation sites used.

symptomless. *F. oxysporum* f. sp. *niveum* was reisolated from the inoculated plants but not the control plants.

***F. oxysporum* f. sp. *niveum* seed infestation detection by real-time PCR assay.** In the 2014 greenhouse experiment, *F. oxysporum* f. sp. *niveum* seed infestation was detected from all pericarp and pistil inoculated seedlots, but not from peduncle inoculated seedlots when the seeds were surface disinfested or not disinfested. More *F. oxysporum* f. sp. *niveum* DNA was detected in seeds from pericarp inoculation than seeds from pistil inoculation when seeds were not surface disinfested, and there was no significant difference in *F. oxysporum* f. sp. *niveum* DNA detected in seeds from pericarp and pistil inoculation when seeds were surface disinfested (Fig. 4A). In the 2015 greenhouse study, seed infestation was detected from all seedlots using the three inoculations when seeds were surface disinfested or not disinfested. Higher amount of *F. oxysporum* f. sp. *niveum* DNA was detected in seeds from pistil (P = 0.03) and peduncle (P = 0.02) inoculation compared with seeds from pericarp inoculation when seeds were not disinfested (Fig. 4B). For surface-disinfested seeds, significantly higher amount of *F. oxysporum* f. sp. *niveum* DNA was detected in seeds from pericarp (P = 0.01) and pistil (P < 0.01) inoculation than seeds from peduncle inoculation. *F. oxysporum* f. sp. *niveum* infestation was not detected by real-time PCR assay from seeds collected from plants treated with SDW, regardless of the inoculation sites used.

In the field study conducted in 2014, seed infestation by *F. oxysporum* f. sp. *niveum* was detected in all seedlots from the three inoculated sites. There was no significant difference (P = 0.4) in amount of *F. oxysporum* f. sp. *niveum* DNA detected in seeds from the three inoculated sites regardless of whether seeds were surface disinfested or not disinfested (Fig. 5A). In the 2015 experiment, *F. oxysporum* f. sp. *niveum* was detected from seeds generated through all three inoculations, when seeds were surface disinfested or not disinfested. Amount of *F. oxysporum* f. sp. *niveum* DNA was significantly higher in seeds from pericarp inoculation compared with pistil and peduncle inoculation when seeds were not disinfested (Fig. 5B). Similarly, higher amount of *F. oxysporum* f. sp. *niveum* DNA was detected in seeds from pericarp inoculation compared with pistil and peduncle inoculation when seeds were surface disinfested. *F. oxysporum* f. sp. *niveum*-infested seeds were not detected from watermelon treated with SDW with the three inoculations.

DISCUSSION

Infestation of watermelon seeds by *F. oxysporum* f. sp. *niveum* has been reported in a number of studies (Boughalleb and El-Mahjoub 2006; Fulton and Winston 1915; Martyn 1987; Porter 1928); however, none of the studies investigated potential infection courts for *F. oxysporum* f. sp. *niveum* seed infestation. In the present

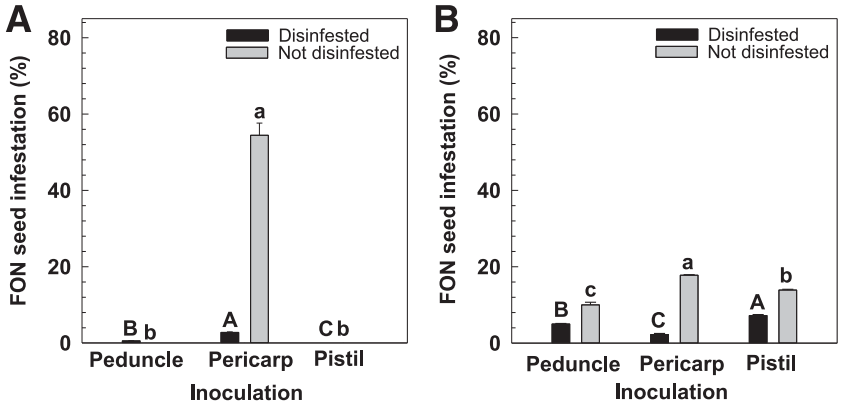

Fig. 3. Percentage of seed infestation by *Fusarium oxysporum* f. sp. *niveum* (FON) following peduncle, pericarp, and pistil inoculation under field conditions when detected by plating assay. **A,** Field study in 2014; and **B,** field study in 2015. Disinfested and not disinfested indicate the seeds were treated or not treated with 0.6% NaOCl, respectively, before isolation by plating. Error bars indicate standard errors of the means of five replicates. Means with the same uppercase or lowercase letters are not significantly different according to the least significant difference test (P = 0.05). *F. oxysporum* f. sp. *niveum* was not detected in seeds collected from plants treated with sterile distilled water, regardless of the inoculation sites used.

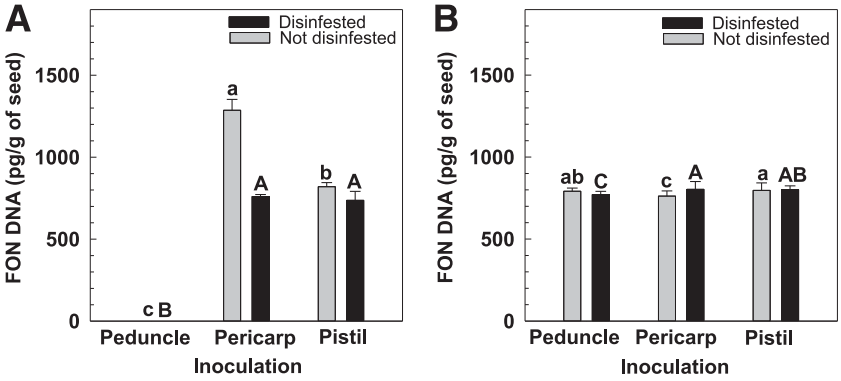

Fig. 4. Amount of DNA of *Fusarium oxysporum* f. sp. *niveum* detected in watermelon seeds following peduncle, pericarp, and pistil inoculation under greenhouse conditions when detected by real-time PCR assay. **A,** Greenhouse study in 2014; and **B,** greenhouse study in 2015. Disinfested and not disinfested indicate the seeds were treated or not treated with 0.6% NaOCl, respectively, before used for real-time PCR assay. Error bars indicate standard errors of the means of five replicates. Means with the same uppercase or lowercase letters are not significantly different according to the least significant difference test (P = 0.05). *F. oxysporum* f. sp. *niveum* was not detected in seeds collected from plants treated with sterile distilled water, regardless of the inoculation sites used.

study, inoculation of pistil, pericarp, and peduncle with *F. oxysporum* f. sp. *niveum* did not cause Fusarium wilt symptoms on watermelon under greenhouse and field conditions. *F. oxysporum* f. sp. *niveum* was detected on watermelon seeds, internally and externally, using plating and real-time PCR assays, suggesting pistil as well as pericarp and peduncle of immature fruit can be potential infection courts for *F. oxysporum* f. sp. *niveum* leading to infestation of seeds in asymptomatic watermelon fruit.

Inoculation of pericarp resulted in seed infestation in all the greenhouse and field studies, and internal seed infestation was detected in three of the four experiments. Inoculation of peduncle resulted in internal seed infestation in all the experiments. Inoculation of pistil resulted in seed infestation in three of the four experiments, and internal seed infestation was detected in two of the four experiments. Across the experiments, inoculation of peduncle, pistil and pericarp resulted in 0.6 to 10%, 0 to 18.3%, and 0.6 to 54.4% of infested seeds, respectively, when assayed by plating. Detection using real-time PCR further confirmed that seeds collected from watermelon plants inoculated with *F. oxysporum* f. sp. *niveum* were infested with the pathogen, regardless of the inoculation sites used. Unlike the plating assay, real-time PCR could amplify DNA of viable or nonviable *F. oxysporum* f. sp. *niveum* cells, so the PCR assay could not determine if *F. oxysporum* f. sp. *niveum* from the seeds were dead or alive. This could explain the discrepancy between the plating and PCR results. Limited information is available regarding potential infection courts of *F. oxysporum* f. sp. *niveum* leading to infestation of watermelon seeds. In a recent review, Martyn (2014) stated that nit mutants of *F. oxysporum* f. sp. *niveum* were isolated from watermelon seeds following inoculation of peduncles, though details were not reported.

Infection courts for seed infestation have been studied in other fungal pathosystems. Menzies and Jarvis (1994) reported that inoculation of peduncles with *F. oxysporum* f. sp. *radicis-lycopersici* resulted in tomato fruit rot and infested seeds. A series of studies conducted by Kuniyasu (1981) and Kuniyasu and Kishi (1977) demonstrated that the pathogen of Fusarium wilt of bottle gourd (*F. oxysporum* f. sp. *lagenariae*) could infest bottle gourd seeds by direct invasion through vascular bundles of mature fruit. *Alternaria brassicae* and *A. brassicicola* (causal agents of Alternaria black spot of cabbage and canola) can infest cabbage and canola seeds through invasion of ovary walls (Domsch 1957). Seed infestation through pistil invasion has been studied in *Ustilago* spp., causal agent of smut in cereal crops. It was reported that chlamydospores of *Ustilago* spp. of barley and wheat germinated on style surfaces and the resulting hyphae gained entry into the embryo through pollen tubes (Lang 1917). Studies indicated that conidia of *Alternaria alternata* can germinate on stigmas of pepper flowers, ingress the ovary through the style in the form of hyphae, and establish in pepper seeds (Meiri and Rilsky 1983). In other studies, infection of pearl millet by *Claviceps fusiform* occurred through the stigma (Luttrell 1977; Thakur and Williams 1980). To our knowledge, seed infestation by invasion through pistil or pericarp has never been reported in *Fusarium* spp.

Variability in the level of *F. oxysporum* f. sp. *niveum* seed infestation occurred in the field and greenhouse studies. Potential reasons for variability in seed infestation in the experiments could be due to a variety of factors. Infection of plants by pathogens is a complicated process, and minor changes in physiological status of host plants as well as biotic and abiotic environments associated with the plants may affect pathogen invasion and seed infestation. In addition, distribution of infested seeds in a seedlot may not be uniform and some seeds may have a level of inoculum below the detection threshold, which also contribute to variability in seed infestation detection when assayed by plating. Such variability has been observed with detection of infested seeds in other pathosystems. It was reported (Dutta et al. 2014) that detection of bacterial pathogens from seeds was nonuniform, and the observed variability was due to a number of factors including host susceptibility, distribution of inoculum within a seedlot, pathogen population per seed, and environmental conditions. Variation in seed infestation was also reported with other pathogens due to environmental factors (Jordan et al. 1992; Schuh 1992).

Results in our study were consistent with previous findings that *F. oxysporum* f. sp. *niveum* can be present on watermelon seeds externally or internally. Boughalleb and El-Mahjoub (2006) reported that *F. oxysporum* f. sp. *niveum* was isolated externally and internally from watermelon seeds. *F. oxysporum* f. sp. *niveum* was isolated from the seed coat of watermelon or the interior of the seed obtained from infected watermelon fruit (Martyn 1987; Taubenhaus 1935). In a study by Michail et al. (2002), *F. oxysporum* f. sp. *niveum* was isolated from the testa, embryo axis, and cotyledons of watermelon seeds with the pathogen most frequently from the testa. In the present study, *F. oxysporum* f. sp. *niveum* was isolated from watermelon seeds treated or not treated with NaOCl, and seed infestation was further confirmed by real-time PCR assay using *F. oxysporum* f. sp. *niveum*-specific primers. However, we did not determine in what internal tissues of the seed *F. oxysporum* f. sp. *niveum* was present, which could be interesting to investigate in future studies.

In summary, this study provides evidence that pistil, pericarp, and peduncle of watermelon can be sites of *F. oxysporum* f. sp. *niveum* infection leading to subsequent seed infestation in symptomless fruit. In considering the importance of seedborne inoculum in development and epidemics of the disease, protection of these infection courts by chemicals or biocontrol agents may contribute to reduction of Fusarium wilt in watermelon. Further studies can also be conducted to elucidate how inoculation of pistil, pericarp, and peduncle with *F. oxysporum* f. sp. *niveum* results in seed infestation, such as colonization, multiplication,

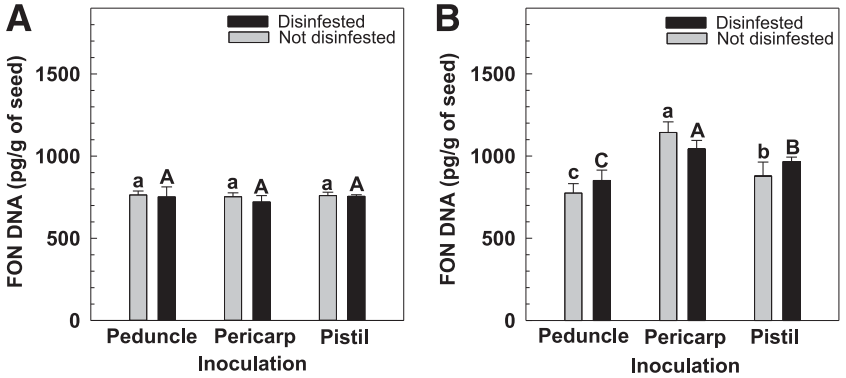

Fig. 5. Amount of DNA of *Fusarium oxysporum* f. sp. *niveum* (FON) in watermelon seeds detected following peduncle, pericarp, and pistil inoculation under field conditions when detected by real-time PCR assay. **A,** Field study in 2014; and **B,** field study in 2015. Disinfested and not disinfested indicate the seeds were treated or not treated with 0.6% NaOCl, respectively, before used for real-time PCR assay. Error bars indicate standard errors of the means of five replicates. Means with the same uppercase or lowercase letters are not significantly different according to the least significant difference test ($P = 0.05$). *F. oxysporum* f. sp. *niveum* was not detected in seeds collected from plants treated with sterile distilled water, regardless of the inoculation sites used.

and migration of the pathogen in different plant tissues associated with watermelon fruit. Information gained from these studies will help develop more effective management strategies to reduce loss caused by the disease through reduced seed infestation in watermelon production.

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