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# Frequency of *Fusarium oxysporum* F. sp. *niveum* and *F. solani* F. sp. *Cucurbitae* from Watermelon Seeds and Their Effect on Disease Incidence*

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**Abstract:** Isolation from watermelon seeds from six commercial seed companies demonstrated the presence of *F. oxysporum* f. sp. *niveum* (*Fon*) *and F. solani* f. sp. *cucurbitae* (*Fsc*). Both fungi were detected internally, but predominantly externally. Isolation results showed that seed infestation level had a significant effect on subsequent disease incidence. Some seedlots were heavily infested with *Fsc* and *Fon*, resulting in a combined disease incidence of more that 50%. However, seed isolations resulted in a poor prediction of the resulting disease incidence when the seeds were planted. This is the first documentation that *Fsc* is seed-transmitted in watermelon.

**Keywords:** Watermelon seed, *Fusarium oxysporum* f. sp. *niveum, F. solani* f. sp. *cucurbitae*, infestation level, Fusarium crown and root rot, Fusarium wilt

## INTRODUCTION

Fusarium wilt of watermelon (*Citrullus lanatus* (Thunb.) Matsum and Nakaï) is incontestably one of the most serious diseases on this cucurbit in the world (Holliday, 1998). Recently, severe outbreaks of the disease have been observed in Tunisia also (Boughalleb and El Mahjoub, 2005). The disease is caused by the fungus *Fusarium oxysporum* Schlechtend.: Fr. f. sp. *Niveum* (E.F.Sm.) W.C. Snyder and H.N. Hans. (*Fon*). Differences in virulence among isolates of *Fon* have been recognized for many years with three races being identified and designated as races 0, 1, or 2. It has been suggested that the *Fon* population does not fit the strict definition for races and should more appropriately be considered a continuum from weakly-virulent to highly-virulent (Bruton, 1998; Bruton and Damicone, 1999). Although seed transmission has been clearly demonstrated, there is little quantitative data on percentage infestation within seedlots from different seed companies. However, Michail *et al*. (2002) reported that seed infestation levels of 10-30% could result in a wilt incidence of about 50%.

A new crown rot disease, caused by *F. solani* (Mart.) Sacc. f. sp. *cucurbitae* W.C. Snyder and H.N. Hans. (*Fsc*), has been recently described by Boughalleb *et al*. (2005) that further threatens watermelon production in Tunisia. Historically, this fungus has been associated to root and crown rot of cucurbits including species of *Benincasa*, *Cucurbita*, *Cucumis*, *Citrullus*, *Lagenaria* and *Luffa* (Toussoun and Snyder, 1961; Vannacci and Gambogi, 1980; Paternotte, 1987; Kinjo and Tokashiki, 1989). Until recently (Boughalleb *et al*., 2005), Fusarium crown rot had never been reported as causing disease of watermelon in the field. Two races of the pathogen (race 1 and 2) are recognized and are based on host tissue specificity (Toussoun and Snyder, 1961), but only race 1 attacks the root, stem and fruit of cucurbits. The incidence of Fusarium crown rot on muskmelon and squash appears to be increasing in recent years (Bruton, 1998). Since the first report of the disease in South Africa (Doidge and Kresfelder, 1932), the disease has become worldwide in distribution (Boughalleb *et al*., 2005), although not necessarily widespread within specific regions (Bruton, 1998). Several research groups have reported seed transmission (internal and/or external) of the fungus in *Cucurbita* spp. (Vannacci and Gambogi, 1980; Paternotte, 1987; Fantino *et al*., 1989; Armengol *et al*., 2000).

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The purposes of this research were to (I) determine seed infestation level of *Fon* and *Fsc* in commercial watermelon seedlots and (ii) determine the influence of seed infestation level on disease incidence.

## MATERIALS AND METHODS

### Seedlots

Fifteen watermelon seedlots from six commercial seed companies were obtained from the Laboratory of Seed and Plant Control (*Direction Générale de la Protection et du Contrôle de la Qualité des Produits Agricoles, Ministère de l'Agriculture et des Ressources Hydrauliques*). These seedlots represent different watermelon cultivars: Charleston Gray types L1, L3, L10, L12, L16; Crimson Sweet types L6, L7, L8, L11, L13, L14, L18, L19 and L20; and Jubilee type L9.

### Determination of Infestation Level of Watermelon Seedlots

### Isolation of *Fusarium* Spp. From Seeds

The isolation method adopted for this paper was proposed by Littke (1996). For each seedlot, 600 seeds were distributed as follows: three replications of both 100 seeds with seed coat, rinsed in Sterile Distilled Water (SDW), 100 seeds with coat were plated on the Fusarium selective Komada's medium (Komada, 1975), 10 seeds per Petri dish. The Petri dishes were incubated in the laboratory for 14 days at 22-25°C with a 12 h photoperiod. Fungal colonies exhibiting characteristics consistent with *Fusarium solani* or *F. oxysporum* that developed around individual seeds, were transferred to PDA medium (Potato-dextrose-Agar) for purification and identification. The infection level of each seedlot was evaluated as percent of infected seed.

### Purification of *Fusarium* Spp. Isolates

Single-spore strains were obtained by transferring a simple conidium germinating on 2% water-agar to a petri dish on PDA (Hansen and Smith, 1932). Strains were stored in glycerol 50% at -20°C until used.

### Identification of *Fusarium* Spp.

Identification of each *Fusarium* colony was based on morphological criteria proposed by Booth (1971). The macroscopic features were determined on PDA adjusted to pH 6.5-7, whereas the microscopic determination was done on Spezieller Nährstoffarmer agar (SNA) (Summerell *et al.*, 2003). Cultures on PDA and SNA were incubated 4 days at 22-25°C under 12 h light/darkness alternation. The diameter and coloration of the colonies and the aspect of the mycelia were noted. The microscopic features were examined after 7 days of incubation. The isolates were stored on PDA in tubes maintained at 5°C or in glycerol 50% at -20°C until their utilization.

### Pathogenicity Tests

190 and 66 strains of *F. solani* and *F. oxysporum*, respectively, were tested for pathogencicity on susceptible watermelon cultivar 'Giza'. Inoculum consisted of microconidial suspensions harvested from cultures grown on Potato-Dextrose-Broth (PDB) on a rotary shaker at room temperature (22°C) for 14 days and adjusted at a concentration of $1\times106$ conidia $mL^{-1}$ using a hematocymeter. Seeds were surface disinfected in 5% sodium hypochlorite solution for 5 min, rinsed with SDW and sown in vermiculite. When the first true leaf was evident (about 2 weeks after seeding), the seedlings were uprooted and the roots gently washed under flowing water. Seedlings were root-dipped into the respective inocula for 15-20 sec, swirled several times and transplanted into 7.5 cm diameter pots, containing vermiculite, three seedlings per pot and five pots per strain). All plants were maintained in the greenhouse and fertilized regularly after one week with a fertilization solution composed of Macro-

elements (mg $L^{-1}$): $KNO_3$ (0.225); $KH_2PO_4$ (0.3); $NH_4Cl$ (0.225); $MgSO_4 7H_2O$ (0.45); $(NH_4)_2SO_4$ (0.3); $Ca(NO_3)_2$ (1.2) and $C_{10}H_{14}N_2FeO_8$ (0.045) and of Oligo-elements (mg$L^{-1}$): KCl (3); $H_2BO_3$ (3.3); $MnSO_4 7H_2O$ (1.8); $ZnSO_4 7H_2O$ (0.3); $(NH_4)_6Mo_2O_{24}$ (0.3) and $CuSO_4 5H_2O$ (0.15). The average air and soil temperatures during the experiment were 27 and 24.7°C, respectively. The assay was conducted for more than four months.

**Association of Seed Infestation with Disease Incidence**

Culture in pots. To determine the effect of seed infestation level on disease incidence caused by *Fon* and *Fsc*, we used two culture substrates. The first was a disinfected perlite and peat mixture (1:1v); The second one was a disinfected sand used for cropping vegetables. This assay was carried out under 500 $m^2$ greenhouse divided in two blocks: the first half included 20 pots×15 seedlots×3 replications. Thus, 900 pots were filled with peat and perlite and additional 900 pots were filled with sand. In each pot, one seed was planted. The assay was conducted on seedlots in two successive years; seeding was done in the January-February period. The percentage of diseased plants was recorded every two days until the end of the study. In addition, at the end of the assay, each surveying plant was uprooted to observe the roots and crown and to determine xylem discoloration. To identify the causal agent, we made isolations on PDA with subsequent microscopic examination.

**Statistical Analysis**

To characterize watermelon seedlots on the basis of their infestation level by *Fon* and *Fsc* and the subsequent effect on disease incidence in pots, an analysis of variance of the data was performed by SPSS Software program (SPSS Inc. Headquarters, Chicago, Illinois). Treatment means were compared using the Duncan's multiple range test ($p \leq 0.01$).

## RESULTS

**Isolation, Pathogenicity and Identification of *Fusarium* Spp. From Watermelon Seed**

After three days on Komada's medium, colonies of *Fusarium* spp. began to develop from intact seed and shelled seeds. The predominant colony from each petri dish was transferred to PDA for further purification. Single spore isolates were subsequently transferred to PDA for colony color and growth characteristics and to SNA for microscopic examination. Macroscopic features of *F. solani* and *F. oxysporum* were confirmed by microscopic identification. The isolates were identified either as *F. oxysporum* using the taxonomic description of Booth (1971) and Nelson *et al.* (1983) for *F. solani* based on the description of Tousson and Snyder (1961).

To determine pathogenicity, 190 isolates identified as *F. solani* and 66 isolates identified as *F. oxysporum* were inoculated to the cv. Giza at $1 \times 10^6$ conidia $mL^{-1}$. The cv. Giza is susceptible to all three races of *Fon*, although, no attempt was made to determine the specific race/races involved. Watermelon seedlings inoculated with pathogenic *F. solani* isolates exhibited linear cortical lesions in the hypocotyls about 14 to 21 days after inoculation and eventually plant death. 180 out of 190 *F. solani* strain were tested pathogenic. The isolates identified as *F. oxysporum* caused a severe stunting, wilt, or plant death within 28 days. 58 out of 66 strains identified as *F. oxysporum* were pathogenic. There-isolation from symptomatic plants inoculated with *F. solani* or *F. oxysporum* (the identify of the inoculated) satisfying the Koch's postulates. Based on taxonomic descriptions and pathogenicity on watermelon, the fungi were further classified as *F. oxysporum* f. sp. *niveum* or *F. solani* f. sp. *cucurbitae*. Other fungi that were determined to be saprophytes were eliminated.

**Determination of Seed Infestation Level in Watermelon Seedlots**

Six hundred seeds from each of 15 seedlots representing cvs. Charleston Gray, Crimson Sweet and Jubilee were subdivided into 300 seeds with seed coat intact (whole) and 300 with the seed coat

Table 1: Percentage seed infestation by *Fusarium solani* f. sp. *cucurbitae* (*Fsc*) and *F. oxysporum* f. sp. *niveum* (*Fon*) and total infestation levels (combined *Fon* and *Fsc*) from different watermelon seedlots isolated on Komada Medium

| | | Whole seeds (% infestation) | | | Shelled seeds (% infestation) | | |
|---|---|---|---|---|---|---|---|
| Seed company | Seedlot | *Fon* | *Fsc* | Combined *Fon* and *Fsc* | *Fon* | *Fsc* | Combined *Fon* and *Fsc* |
| Company A | Lot 1 | 0.00a | 0.33a | 0.33a | 0.50a | 2.50abc | 3.00abc |
| Company B | Lot 3 | 0.30a | 7.90b | 8.20b | 0.00a | 0.00a | 0.00a |
| Company C | Lot 6 | 0.98a | 2.95ab | 3.93ab | 0.00a | 0.00a | 0.00a |
| Company C | Lot 7 | 1.91a | 0.00a | 1.91ab | 0.00a | 2.73abc | 2.73abc |
| Company D | Lot 8 | 0.00a | 3.00ab | 3.00ab | 0.00a | 1.12ab | 1.12abc |
| Company E | Lot 9 | 0.00a | 4.33ab | 4.33ab | 2.13ab | 4.25bc | 6.38d |
| Company E | Lot 10 | 12.00b | 4.00ab | 16.00c | 3.00b | 2.58abc | 5.58cd |
| Company A | Lot 11 | 0.00a | 0.33a | 0.33a | 0.00a | 2.00abc | 2.00ab |
| Company F | Lot 12 | 0.66a | 1.00a | 1.66ab | 1.00a | 0.00a | 1.00ab |
| Company A | Lot 13 | 0.00a | 2.26ab | 2.26ab | 0.00a | 0.00a | 0.00a |
| Company A | Lot 14 | 0.00a | 0.33a | 0.33a | 1.17ab | 4.67c | 5.84bcd |
| Company F | Lot 16 | 0.30a | 5.25ab | 5.57ab | 0.70a | 4.76c | 5.50bcd |
| Company A | Lot 18 | 0.00 a | 5.00ab | 5.00ab | 0.66a | 0.99ab | 1.65abc |
| Company A | Lot 19 | 0.33a | 2.33ab | 2.66ab | 0.33a | 2.65abc | 2.99abc |
| Company F | Lot 20 | 0.00a | 3.20ab | 3.20ab | 0.00a | 0.35a | 0.35ab |
| | Pooled mean | 1.10 | 2.97 | 4.07 | 0.63 | 2.03 | 2.66 |

Values with the same letter are not significantly different at p = 0.05, Lots 1, 3, 10, 12 and 16 represent Charleston Gray type watermelon, Lots 6, 7, 8, 11, 13, 14, 18, 19 and 20 represent Crimson Sweet type watermelon, Lot 9 represents Jubilee type watermelon

Table 2: Incidence of disease caused by *F. oxysporum* and *F. solani* using different potting media

| | | Perlite and peat (1st year) | | | Perlite and peat (2nd year) | | | Sand (1st year) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Diseased plants | | | Diseased plants | | | Diseased plants | | |
| Seed Company | Seedlots | *F. oxy* | *F. sol* | Combined *F. oxly F. sol* | *F. oxy* | *F. sol* | Combined *F. oxly F. sol* | *F. oxy* | *F. sol* | Combined *F. oxly F. sol* |
| Company A | Lot 1 | 20.0d | 29.0e | 49.0ef | 16.0ef | 0.0a | 16.0a | 0.0a | 13.9bc | 13.9bc |
| Company B | Lot 3 | 8.0a | 15.0bcd | 23.0ab | 8.6bc | 17.0d | 25.7b | 3.2bc | 6.2a | 9.4ab |
| Company C | Lot 6 | 13.0ab | 20.0cd | 33.0cd | 33.5g | 5.0b | 38.5cd | 7.3e | 3.0a | 10.3ab |
| Company C | Lot 7 | 18.0cd | 10.0ab | 28.0bc | 23.5h | 12.0c | 35.5cd | 9.3f | 4.0a | 13.3bc |
| Company D | Lot 8 | 13.0ab | 12.0ab | 25.0bc | 10.5bcd | 31.5g | 42.0de | 2.3bc | 39.5f | 41.8g |
| Company E | Lot 9 | 19.0d | 43.0fg | 62,0g | 0.0a | 38.5h | 38.5cd | 2.0b | 20.0d | 22.0d |
| Company E | Lot 10 | 22.0d | 42.0fg | 64.0g | 11.5bcd | 27.3fg | 37.8cd | 2.7bc | 35.2ef | 37.9fg |
| Company A | Lot 11 | 10.0ab | 21.0d | 31.0bc | 30.0i | 3.4ab | 33.4c | 0.0a | 18.2cd | 18.2cd |
| Company F | Lot 12 | 14.0bc | 45.0g | 59.0g | 19.0fg | 27.0fg | 46.0ef | 0.0a | 11.5b | 11.5ab |
| Company A | Lot 13 | 20.0d | 13.0ab | 33.0cd | 32.0ij | 4.0ab | 36.0cd | 0.0a | 34.5e | 34.5f |
| Company A | Lot 14 | 20.0d | 41.0fg | 61.0g | 7.4b | 39.0h | 46.4ef | 0.0a | 49.0g | 49.0d |
| Company F | Lot 16 | 27.0e | 35.0ef | 62.0g | 20.0g | 31.0g | 51.0f | 5.8d | 17.9cd | 23.7de |
| Company A | Lot 18 | 11.0ab | 5.0a | 16.0a | 12.5d | 20.0de | 32.5c | 0.0a | 6.1a | 6.1a |
| Company A | Lot 19 | 21.0d | 20.0cd | 41.0de | 13.5de | 24.4ef | 37.9cd | 3.8c | 22.2d | 26.0e |
| Company F | Lot 20 | 27.0e | 29.0e | 56.0fg | 11.5bcd | 20.0de | 31.5bc | 14.0g | 21.0d | 35.0f |
| | Pooled mean | 17.5 | 25.3 | 42.9 | 16.6 | 20 | 36.6 | 3.4 | 20.2 | 23.5 |

Values with the same letter are not significantly different at p = 0.05, Lots 1, 3, 10, 12 and 16 represent Charleston. Gray type watermelon, Lots 6, 7, 8, 11, 13, 14, 18, 19 and 20 represent Crimson Sweet type watermelon, Lot 9 represents Jubilee type watermelon

removed (shelled). Isolations on Komada's medium (selective for *Fusarium* spp.) showed that 11 out of 15 seedlots were infested with *Fon*, whereas, *Fsc* was isolated from all seedlots (Table 1). *Fsc* was isolated more frequently than *Fon* on whole and shelled seeds. However, both *Fsc* and *Fon* were generally isolated more frequently on whole seeds as compared to shelled seeds. From whole seeds, the frequency of isolation of *Fsc* ranged from 0.0-7.9% compared to 0.0-12.0% of *Fon*. Based on these data, both *Fsc* and *Fon* are capable of external and internal infestation. At least some seeds from each seed company were infested with *Fsc* and/or *Fon*. There did not appear to be any relationship between seed infestation and watermelon cultivars.

**Effect of Seed Infestation Level on Disease Incidence**

Planting medium had a greater impact on disease incidence caused by *Fon* than *Fsc*. The incidence of *Fon* was greatly depressed when seeds were sowed in sand (3.4%) as compared to perlite/peat mix (17.1%) (Table 2). The incidence of *Fon* in plants sowed in perlite/peat ranged from 8.0-27.0% in the first year and 0.0-33.5% the second year. There was no association between cultivar, Seed Company and incidence of *Fon*. There were significant differences ($p = 0.05$) between seedlots. The incidence of crown rot, caused by *Fsc*, did not appear to be affected by planting medium. In fact, the pooled means for all three studies were similar ranging from 20.0-25.3%. Based on the three studies, the range of disease incidence within the 15 seedlots was 0.0-49.0%. There was no association between cultivar, Seed Company and incidence of *Fsc*. However, there were significant differences ($p = 0.05$) between seedlots. During the second survey year, we noted a considerable reduction in disease incidence for the majority of seedlots independent of cultivar type or Seed Company.

Although there were undoubtly seeds infested with both *Fon* and *Fsc*, we did not attempt to determine dual infestation. Furthermore, there were likely some plants infected by both *Fon* and *Fsc*. Based on the symptom expressed by the plant, we assigned the cause of the disease and confirmed the diagnosis with isolations in some cases. Consequently, the actual percentage of seeds and plants infected by *Fon* and *Fsc* was likely underestimated in this study. The incidence of disease, caused by *Fon* and *Fsc*, was considerably higher (Table 2) than was indicated by isolation studies (Table 1). Seed isolations predicted only 6% of the wilt and 13% of the crown rot that developed in plant grow-out studies. Clearly, isolations underestimated the amount of wilt and crown rot that developed as a result of infested seed.

## DISCUSSION

At the present time, *Fsc* is a problem on watermelon only in Tunisia production areas (Boughalleb *et al*., 2005), where in some watermelon commercial fields its incidence has been estimated at approximately 100% with a yield losses as high as 60%. *Fsc* was demonstrated to be the predominant seedborne pathogen on watermelon, so there is substantial risk that it will be spread to other areas through infested seed. Particularly troublesome is the fact that the various seedlots used in the present study represent six different commercial seed companies and three different watermelon genotypes (cvs. Charleston Gray, Crimson Sweet, Jubilee). At this time, we have no theories as to why the disease has not been detected in watermelon production areas outside of Tunisia. This is the first report that documents *Fsc* as seedborne fungus on watermelon. The fungus was both external and internal in the seed. Since *Fsc* causes a local lesion in the crown and is not known to be systemic, the route to infection of the seed is a quandry. Armengol *et al*. (2000) summarized the seedborne nature of *Fsc* in *Cucurbita* spp., although, no information is available as to the route by which *Fsc* enters cucurbit seeds. It is doubtful that seed infestation is related to fruit infection by the fungus because seed companies tend to be very cautious selecting only high quality fruit in their seed increase programs. However, Randhawa *et al*. (1991) reported that a number of commercial seed producers in India do extract the seed from rotten or partially rotten fruit.

Based on isolations, the highest percentage of recovery of both *Fsc* and *Fon* from seeds was internal which may make seed treatment less effective. Although *Fon* has been known to be seedborne from many years (Taubenhaus, 1935), the only quantitative study on infestation levels in watermelon seed and the subsequent wilt incidence was only recently published (Michail *et al*., 2002). They reported that a wilt incidence greater than 50% was possible using seeds with infestation levels (based on isolations) lesser than 10%. In the present study, a 17% wilt incidence was noted using seeds 1% infestation level. Clearly, seed isolation to predict wilt incidence in the field is inadequate. According to D. Maddox (STA Laboratorios, Inc., Longmont, CO, USA) (Personal Communication) a wet blotter

incubation technique (23-27°C and 12 h light) is used to induce fungal growth on the watermelon seed. Further research is needed to develop a more efficient system for isolating both *Fsc* and *Fon* from seed. At present, there are few requests for testing watermelon seed for *F. oxysporum* and no requests for testing watermelon seed for *F. solani* infestation. We identified *F. oxysporum* and *F. solani* by plating the fungus on a media promoting sporulation, but the development of rapid and precise detection methods for *Fon* and *Fsc* races is urgently needed by the seed industry. While Crowhurst *et al*. (1991) used RAPD markers to identify races of *Fsc*, there are no definitive PCR-based techniques to identify *Fon* (Wang *et al*., 2001; Vakalounakis and Fragkiadakis, 1999). However, Namiki *et al*. (1994) stated that DNA fingerprinting of nuclear DNA distinguished differences among forma speciales of *F. oxysporum* infecting cucurbits in Japan. More extensive studies should be undertaken to determine their utility for seed testing. Differentiating races of *Fon* will likely be difficult because the designated races 0, 1 and 2 are actually a continuum from weakly-virulent to highly-virulent (Bruton, 1998).

It is interesting to note that the incidence of *Fon* was significantly less when the seeds were planted in sand compared to the perlite/peat mix. In contrast, there was no difference in the incidence of *Fsc*. Perhaps temperature and/or soil moisture played a role. Further research will be required to determine potential interactive effects. There is little doubt that some seeds in the present study had dual infections of both *Fsc* and *Fon*. Since we did not attempt to make multiple transfers from seed isolations, the percentage of seed infestation was probably underestimated.

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