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In vitro Determination of *Fusarium* spp. Infection on Watermelon Seeds and their Localization

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Abstract: In this study, we used direct incubation of watermelon dissected seeds on Komada's selective medium for *Fusarium* spp. and incubation of entire seed on the same medium or on 2% agar medium. Identification of fungi was based on morphological criteria and also according to Koch's postulate. Isolates from dissected seed were identified as *F. oxysporum* f. sp. *niveum* and *F. solani* f. sp. *cucurbitae*. These fungi were found to be externally and internally seed borne in watermelon. This is the first report of localization of *Fusarium* spp. transmitted by watermelon seeds in Tunisia.

Key words: *F. oxysporum* f. sp. *niveum*, *F. solani* f. sp. *cucurbitae*, localization, seedborne fungus, *Citrullus lanatus*

INTRODUCTION

Seed health and freedom from seed borne diseases are constantly desired. Occasionally, seedling losses may originate from poor germination or pre-emergence disease. Littke (1996) identified seed borne pathogens as the prime factor in these losses. An understanding of the origin and nature of seed borne fungi may be helpful in reducing losses and improving yields (Littke, 1996).

Fusarium species are numerous and their damages vary according to the plant host. They can be responsible of vascular wilt (*Fusarium oxysporum*) or root and collar rot of a high number of vegetables (Champion, 1997).

In Tunisia, isolations from wilted watermelon plants collected from the most areas of watermelon production in the country during 1999/2000 and 2000/2001, showed the presence of the two *Fusarium* species cited above (Boughalleb and El Mahjoub, 2005; Boughalleb *et al*., 2005). These fungi are transmitted by seeds and can be preserved as conidia in the coat or as mycelia at the seeds surface (Blancard *et al*., 1991; Champion, 1997; Gargouri *et al*., 2000; Martyn and Bruton, 1989). Analysis of seed infection level is a valid investigating tool to foresee the disease development transmitted by seeds (Taylor *et al*., 2001). Few studies have been done on the localization of these pathogens on seeds (Michail *et al*., 1989; Michail *et al*., 2002). Seed chemical treatment could inhibit instantaneously fungus development in the fungicide delay of efficiency. Besides, the suitable seed treatment requires the knowledge of localization and infection level of seed as suggested by Paveley *et al*. (1997).

The objectives of this study were: (i) to isolate and identify *Fusarium* spp. from watermelon seeds, (ii) to determine the infection level for each seedlot by *Fusarium* spp., (iii) to study the effect of seed treatment on the *in vitro* infestation level.

MATERIALS AND METHODS

Seedlots: Eighteen watermelon seedlots have been provided by the Laboratoire de Contrôle des Semences et des Plants, Direction Générale de la Protection et du Contrôle de la Qualité des Produits Agricoles au Ministère de l'Agriculture des Ressources Hyrauliques, Tunisia. These seedlots were of different watermelon cultivars (L1, L3, L4, L10, L12 and L16 are of the type Charleston Gray; L6, L7, L8, L11, L13, L14, L15, L17 and L18 are of the type Crimson Sweet whereas L9 is Jubilee cultivar).

Isolation of *Fusarium* spp.: Various methods were applied for *Fusarium* isolation from watermelon seeds. This study was conducted in the plant pathology laboratory (Ecole Supérieure d'Horticulture et d'Elevage, Chott Mariem, Sousse- Tunisia). Analysis method developed by Vannacci and Gambogi (1980) and recently used by Punja *et al*. (2001) to determine the potential of fungal transmission of the melon seeds was used in our

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conditions. From each seedlot, 900 seeds were used as follow: three replicates of 100 seeds were washed with sterile distilled water and plated directly on Komada's medium, specific for *Fusarium* isolation (Komada, 1975), at the rate of 10 seeds by Petri dish. Others three replicates of 100 dissected seeds, without testa, were distributed in ten Petri dishes containing the solidified komada media; the third three replicates of 100 seeds were placed directly in Petri dish containing 2% water agar medium.

These operations were done under aseptic conditions. The plates were incubated at 25°C with an alternate of light and darkness of 12 h. The number of contaminated seeds was counted every 3 days for a period of 14 days. The infection level of each seedlot was evaluated according to the following formula:

Infection level (%) = total number of infected seeds × 100/total number of tested seeds

The use of seeds without testa permits to develop the endogenous *Fusarium* species in seed. The fungal colonies, whose appearance were similar to the *Fusarium* spp. morphology and developed around every seed, were replicated on PDA (Potato-dextrose-Agar) added with sulphate streptomycin in order to purify and to identify them.

Purification and monosporal culture of *Fusarium* isolates: Isolate purification method developed by Hansen and Smith (1932) was adopted. The isolates obtained were preserved on PDA in tubes at 5°C or in 50% glycerol at -20°C until their utilization.

Fusarium isolates identification: Cultures on PDA and LNA were incubated at 22-25°C with an alternate of light and darkness of 12 h during 4 days. Then, the diameter and coloration of the colonies and the aspect of the mycelia were recorded. The microscopic features were examined after 7 days of incubation. Identification of *Fusarium* colonies was based on the 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 Low Nutriment Agar medium (LNA) (Summerell *et al*., 2003).

Pathogenicity tests and forma specialis determination: *Fusarium* spp. isolated from the different seedlots were cultivated and used to test their pathogenicity and to determine their forma specialis. The pathogenicity of all isolates was assessed on susceptible watermelon seedlings of the Egyptian cultivar Giza supplied by G.I.L (Tunisia). Spore suspensions were prepared 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 1×10^6 conidia/mL with a hematocymeter. The seeds were surface disinfected in 5% sodium hypochlorite solution for 5 min, rinsed with running tap water and sown on vermiculite when they germinated. When the first true leaf was evident (about 2 weeks after planting), the seedlings were uprooted and the roots washed under a stream of gently flowing water. Seedlings were root-dipped into the respective inoculum for 15-20 sec, swirled several times and transplanted into 7.5 cm diameter pots (three seedlings per pot containing vermiculite) and five pots per isolate. Thus, fifteen seedlings per isolate were tested. Controls were prepared by root-dipping the seedlings into sterile distilled water. All plants were maintained in the greenhouse and fertilized when needed with a Knop fertilisation solution. The average air and soil temperatures during the experiment were 27 and 24.7°C, respectively.

Influence of the seed fungicides on the *in vitro* level infection: The regular survey of the contaminated seeds number for each petri dish, for each isolation method and for each seedlot, permit to visualize the impact of the seed treatments of seeds. Thus, we followed the number of seeds contaminated every three days for fourteen days of incubation.

Statistical analysis: An analysis of the variance-one-way (ANOVA) was done to assess differences between seedlots. The ANOVA was followed by the mean comparison and the definition of homogeneous group by Duncan's test. Analyses were performed using the SPSS Software program (SPSS Inc. Headquarters, Chicago, Illinois).

RESULTS AND DISCUSSION

***Fusarium* spp. isolation from seedlots:** One hundred and ninty colonies obtained on PDA were identified as *Fusarium* spp. on the basis of colony morphology. Their growth on PDA and LNA showed that microconidia were formed in false humid heads on long branched phialides and chlamydospores were formed intercalary on the hyphae. The colonies were identified as *F. solani* (anamorph *N. haematococca*) (Niremberg, 1976). Others colonies showed that microconidia were formed in false dry heads developed on short phialides. These colonies begun to develop three days after incubation. Some seed contamination were recovered superficially and others from seed without testa.

Table 1: Level of infection by *Fusarium solani* f sp. *cucurbitae* (*Fon*) and *F. oxysporum* f. sp. *niveum* (*Fsc*) per each watermelon seedlot and isolation method

Seedlots	Watermelon cultivars	Entire seed on Komada's medium		Dissected seed on Komada's medium		Direct incubation on 2% water agar	
		Fon (%)	*Fsc* (%)	*Fon* (%)	*Fsc* (%)	*Fon* (%)	*Fsc* (%)
Lot 1	Charleston Gray	0.00a	0.33a	0.50a	2.50a-c	0.00a	2.00b
Lot 3	Crimson	0.30a	7.90b	0.00a	0.00a	0.00a	0.00a
Lot 4	Charleston	0.00a	0.67a	0.00a	1.80a-c	0.33a	0.33a
Lot 6	Crimson Sweet	0.98a	2.95ab	0.00a	0.00a	0.33a	0.33a
Lot 7	Crimson	1.91a	0.00a	0.00a	2.73a-c	0.00a	0.00a
Lot 8	Crimson Sweet	0.00a	3.00ab	0.00a	1.12ab	1.33a	1.33ab
Lot 9	Jubilee	0.00a	4.33ab	2.13ab	4.25bc	0.00a	0.00a
Lot 10	Charleston Gray	12.00b	4.00ab	3.00b	2.58a-c	4.65b	1.66ab
Lot 11	Crimson Sweet	0.00a	0.33a	0.00a	2.00a-c	0.00a	0.00a
Lot 12	Charleston Gray	0.66a	1.00a	1.00a	0.00a	0.00a	0.00a
Lot 13	Charleston Gray	0.00a	2.26ab	0.00a	0.00a	0.00a	1.00ab
Lot 14	Charleston	0.00a	0.33a	1.17ab	4.67c	0.00a	0.00a
Lot 15	Charleston	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a
Lot 16	Charleston Gray	0.30a	5.25ab	0.70a	4.76c	0.33a	0.33a
Lot 17	Charleston Gray	0.00a	1.60ab	0.00a	0.00a	0.00a	0.00a
Lot 18	Crimson Sweet	0.0 a	5.00ab	0.66a	0.99ab	0.00a	0.00a
Lot 19	Crimson Sweet	0.33a	2.33ab	0.33a	2.65a-c	0.00a	0.00a
Lot 20	Crimson	0.00a	3.20ab	0.00a	0.35a	0.66a	0.34a

Values within a column followed by different letter(s) are significantly different at p = 0.01 according to Duncan's multiple range tests

Identification, pathogenicity and forma specialis of *Fusarium* spp.: All *Fusarium* spp. isolates collected from seeds revealed to be pathogenic to watermelon seedlings. Symptoms on watermelon seedlings appeared 14 to 21 days after inoculation as linear cortical lesions in the hypocotyl of the plants and ultimately caused seedling death for *F. solani* isolates which were then classified as *F. solani* f. sp. *cucurbitae* (*F.s. cucurbitae*) (Tousson and Snyder, 1961). Whereas, for *F. oxysporum* isolates, a vascular wilt of seedlings were observed and they were identified as *F. oxysporum* f. sp. *niveum* (*F. o. niveum*). The fungus was re-isolated confirming Koch's postulates.

The incubation of washed seeds on Komada's medium permits to isolate *Fusarium* located at the seed surface. In this case, *F. s. cucurbitae* was the most frequent for the majority of seedlots varying from 0.33% for lot 1 to 7.9% for lot 3 with exception of lot 10 where *F. o. niveum was* frequent (12%) and was localized at the seed surface. Whereas, *F. s. cucurbitae* invades the seeds surface for the others seedlots (Table 1). The incubation of seeds without testa allowed the development of *Fusarium* species internally located. This method was used by Mathur and Kongsdal (1994) to detect fungus in the embryo of some vegetables. The seedlot 3, having shown the highest level infection seeds by *F. solani,* presented an embryo free of contamination as determined by analysis on Komada's medium of seeds without testa (Table 1). The same was for L6, 13, 15 and 17. The third method of isolation (seed incubation on agar 2%) showed a low sensitivity for *Fusarium* detection. This method was practised by Abdelmonem (2000) for detection fungus transmitted by seeds for the major vegetables in Egypt. It appears from this study that *F. s. cucurbitae* is dominant (Table 1). However, McLaughlin and Martyn (1982) showed that *F. o. niveum* was isolated more frequently from seed coats. In Egypt, Michail *et al.* (1989) found that *F. o. niveum* could be externally and internally seedborne in watermelon and caused severe symptoms in pathogenicity tests. In Tunisia, the *in vitro* fungal analysis of watermelon seeds revealed their contamination by four *Fusarium* species: *F.* oxysporum, *F. solani*, *F. moniliforme* and *F. equiseti*. However, pathogenicity tests showed that only *F. oxysporum* and *F. solani* were pathogens on watermelon but their forma specialis was not determined (Gargouri *et al*., 2000).

Influence of seed treatments on the *in vitro* fungus development

Seed incubation on 2% water agar: Regular counting of the contaminated seed number plated directly on 2% water agar wasn't enough to detect infection of seeds during 8 to 10 days of incubation. At the end of a period of 14 days, some fungus hyphal growth appeared on the seedlings (Table 1).

Seed incubation on Komada's medium: Results of effect of fungicides on the *in vitro* seed infection, showed that, four days after the incubation to 25°C on Komada, we noted the highest contamination of seeds for lot 16 (7 infested seeds), L 18 (5 seeds) and L20 (3 seeds). Lot 1 (1 seed), L4 (1 seed) and L6 (1 seed) whereas for the other lots, no seed infection was noted. After seven days, the number of contaminated seeds becomes more and more important reaching the maximum in the case of L6, L8, L9, L12 and L20. Ten days of incubation permitted to detect the highest number of contaminated seeds for lot 3. For sample 10, important contaminated seeds have been

Table 2: Number of infected seed on Komada's medium for the different watermelon seedlots

Seedlots	4 days	7 days	10 days	12 days	14 days
L1	1	0	0	0	0
L3	0	1	24	0	0
L4	1	1	0	0	0
L6	1	6	2	1	2
L7	0	0	1	5	0
L8	0	5	1	2	1
L9	0	6	2	4	0
L10	0	8	12	18	10
L11	0	0	0	1	0
L12	0	2	2	0	1
L13	0	2	1	4	0
L14	0	0	0	0	1
L15	0	0	0	0	0
L16	7	6	0	4	0
L17	0	1	0	0	4
L18	5	3	1	3	3
L19	0	2	2	1	3
L20	3	2	3	2	0

detected and it was the highest after 14 days. For L10, it was strongly infested by *Fusarium* species especially located at the surface of seeds (Table 2). Previous works on the effects of seed treatment on fungus development in cucurbits confirmed these results. Among six fungicides (Captan, Thiram, Benomyl, Carbendazim, Mancozeb and Zineb) tested, Carbendazim was the most effective in inhibiting the growth of seedborne fungi of pumpkin, cucumber, watermelon and melon (Kamble *et al*., 1999). Moreover, they evidenced that some fungicides improved the germination of watermelon seeds. Randhawa *et al*. (1991) showed that the percentage of germination of watermelon seeds is improved by seed treatment with Triadimefon and Thiram, while Triadimenol and Quintozen reduced it. Furthermore, Vannacci and Gambogi (1980) demonstrated a limited development of *F. solani* f. sp. *cucurbitae* race 1 on squash seed treated with benomyl, thiram or with both products.

Determination of the *in vitro* infection levels of watermelon seedlots: Statistical analysis showed significant differences between infection levels of different seedlots (Table 1). On the basis of morphological criteria, two *Fusarium* species were identified: *F. o. niveum* and of *F. s. cucurbitae*. Their frequencies vary according to seedlots and also of isolation method. For L1, 4, 7, 9, 11 and 14, the highest infestation level was detected in the case of incubation of seeds without coat on Komada's medium. Frequency of isolation of each forma specialis varied from 0.5 to 2.13% for *F. o. niveum* and from 1.8 to 4.67% for *F. s. cucurbitae*. This leads to the conclusion that most infections recovered in internal parts of seeds. For L3, 6, 13 and 17, the seedborne fungi identified were external and *F. solani* f. sp. *cucurbitae* was more frequent. L15 was a healthy sample. No fungal species were isolated using the three *in vitro* isolation methods (Table 3).

Table 3: *In vitro* level infection of watermelon seedlots using three isolation methods of *Fusarium* spp.

Seedlots	Agar 2%	Komada (seeds with coat)	Komada (seeds without coat)
L1	2.00	0.30	3.00
L3	0.00	8.20	0.00
L4	0.67	0.67	1.80
L6	0.60	3.43	0.00
L7	0.30	1.91	2.73
L8	2.60	3.00	1.12
L9	0.00	4.33	6.38
L10	6.31	16.00	5.58
L11	0.00	0.33	2.00
L12	0.00	1.66	1.00
L13	1.00	2.26	0.00
L14	0.00	0.33	5.84
L15	0.00	0.00	0.00
L16	0.60	5.57	5.50
L17	0.00	1.60	0.00
L18	0.00	5.00	1.65
L19	0.00	2.66	2.99
L20	1.00	3.20	0.35

We noted a relatively high infestation level for lot 9 at 6.38% for dissected seeds and 4.33% for rinsed to water distilled sterile and plated in incubation on Komada's medium. Whereas, for the lot 10, the different treatments permitted to detect the highest infection level specially in the case of directly incubation of the seeds on Komada's medium (16%) and also by plating the seeds on agar 2% medium at 6.31%. Lot 10 was therefore the most infected in all cases.

CONCLUSIONS

The *in vitro* seed infection level permitted fungal analysis of a representative seedlot. For the most samples, a certain percentage of healthy seeds, some seeds are externally contaminated and a fraction is internally infected. It is important to know the fungi biology, the cultivar susceptibility as well as the environment susceptible to enhance pathogens development. Analysis of the fungi in watermelon seeds cannot guarantee that the entire sample is infected or healthy at the same level; because, this *in vitro* study as for inspections to the field, were done on representative samples taken from the lot (Rane and Latin, 1992). Thus, we suggest considering together the first two methods of isolation established in this study to be able to determine the rate of real infestation rate. Furthermore, for plant pathologists, samples must be perfectly homogeneous and representative of the whole seed lot. The sample must be sufficiently important so that the analysis can be achieved on 300 seeds as minimum. It is also necessary to consider the localization of the seedborne fungi. *Fusarium* spp. can be located internally or external in seeds. Efficient analysis methods are recommended to detect the major fungi in seedlots (Maddox, 1998).

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