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Experimental Approach for Easy Identify *Fusarium* wilt of Tomato

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ABSTRACT

Differentiating between Fusarium strains is very difficult to realize because of its diversity in species and formae specials. In order to identify a strain of Fusarium isolated from leaves of tomato hybrid (Chifa) and evaluate its virulence towards this crop, it has been inoculated in tomato hybrid (Tafna). Morphological and microscopic aspects of Fusarium colony have been defined then inoculated, through Koch's postulates, in seedlings and fruits using a spore suspension (1.5× 10^5 spores/ml). Treatment of plants has revealed that the isolate belongs to Fusarium oxysporum f. sp. lycopersici W.C. Snyder & H.N. Hansen, (1940) with high disease incidence (100 %) and complete infection obtained on tissues (roots, leaves, petioles and fruits). Statistical analysis (ANOVA) has confirmed the significant potential (P ≤ 5%) of this strain in appearance of obvious typical symptoms of disease on seedlings while the control subjects have been remained intact. Morphological and microscopic characteristics as well as inoculation in plant host are main tools to identify forma specialis of Fusarium if the host plant is already defined.

Keywords: *Inoculation, Tomato, Fusarium Oxysporum F. Sp. Lycopersici, Identification, Virulence*

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1. INTRODUCTION

Tomato is very sensitive to pathogenic fungi especially *Fusarium* spp. which are certainly the most widespread diseases in the world. Two anamorphic species belonging to this genus can attack tomato crop to wit: *Fusarium oxysporum* and *Fusarium solani* Martius (1881). The later is causal agent of foot rot of tomato (Vawdrey & Peterson 1988; Romberg & Davis 2007) and can also be pathogenic to flowers, fruits and stem of plants (Nakayama et al. 2010). However, the particular case of *F. oxysporum* is that this fungus has more than 82 listed formae speciales and classified according to host specificity (Smith 2007).

Indeed, *F. oxysporum* essential to tomato contains only two different formae specials (Blancard 2012). Root and crown rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* Jarvis & Shoemaker, (1978) and wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* W.C. Snyder & H.N. Hansen, (1940). The later has also been noticed as one of the most common diseases of fresh stored tomato fruits (Blancard 2012, Ignjatov et al. 2012).

On the other hand, within the formae speciales there are races classified according to susceptibility/ resistance of different cultivars in a given crop towards a pathogen isolates (Smith 2007). In fact, *F. oxysporum* f. sp. *lycopersici* has three physiological races (1, 2, 3) challenging the resistance of tomato to *Fusarium* wilt (Agrios 2004).

This huge diversity in species and races makes the classification of this entire species so difficult (Sayed 1976; Fisher 1982). To virtually differentiate between formae speciales of same species or even races of same forma specialis requires morphological, microscopic and molecular characteristics studies on the pathogen colony as well as the use of pathogenicity tests (Iannelli & Capparelli 1982**;** Hirano & Arie 2006; Leslie & Summerell 2006). Thereby, the characterization of a virulent *Fusarium* is realized by inoculation in the host plant (Boland & Kuykendall 1998) because this disease can be expressed perfectly on young plants as on adults (Blancard 2012).

This work focuses on the identification of *Fusarium* strain through morphological and microscopic aspects of the colony together with using Koch's postulates (in plants and fruits) in order to characterize also the forma specialis and evaluate its aggressiveness towards healthy tomato plants (Tafna) in greenhouse conditions.

2. MATERIAL AND METHODS

2.1. Fungal material

Fusarium pathotype has been obtained from fresh leaves of late season tomato (hybrid Chifa) conducted in open fields in El Meghaïer region and attacked with fungal symptoms (discoloration, wilting and drying tissues). These deformations have been expressed following a downpour. It has been reported that valuable culture is usually obtained from fresh plant diseased much more than degraded tissue (Leslie & Summerell 2006).

The fungus has been cultured by disinfection of plant tissues in sodium hypochlorite (2%), transfer of samples in agar-PDA and incubation of Petri dishes at 26 °C. Successive transplantations of the resulting colony in new PDA have been carried out under aseptic conditions.

Strain identification has been based on macroscopic and microscopic characters. Therefore, Slide culture technique has been realized to obtain the microscopic characteristics of fungus. The technique consisted of inoculate a bit of fungus mycelia at the four extremity of a small cube of PDA. The slide has been covered by a cover-slip then kept on a support to avoid direct contact with the humid base of the Petri dish. After incubating for 48 hours at 25 °C, the cover-slip has been examined under microscope using methyl blue. The criteria used for identification of species have been based on the form of macroconidia and microconidia produced in the aerial mycelium, monophialides as well as the presence or absence of chlamydospores (Siddiquee 2017).

2.2. Substrate material

Potting seedlings has been carried out on fertile mixture substrate (2/3 soil, 1/3 organic manure). Soil has been selected purely sandy (from desert) because it doesn't retain moisture and nutrients thus it is poor in pathogenic fungi. Furthermore, manure has been well disinfected by Bergerac method:

- Substrate has been placed on a metal sheet over a well-fed fire;
- It has been well wetted and stirred with a shovel for about 20 minutes, where volumes of water have been added each time to obtain a temperature of the order of 80 ° C so that can burn all the pathogens;
- Manure has finally been placed and covered with a plastic film.

A chemical amendment of NPK (15.15.15) has been added to the mixture (soil/manure) in order to ensure an adequate growth of plants, then substrate has been finally potted.

2.3. Plant material

Choosing Tafna hybrid (cultivar already tested by farmers in Wadi Righ region) is explained by its sensitivity to *Fusarium* species therefore typical symptoms of this disease will be easy and quick to be noticed.

1) Seeds

Seeds have been disinfected in sodium hypochlorite solution (2%) then in ethanol (3 minutes for each) in order to reduce the infestation associated with seeds (Leslie & Summerell 2006, McLaughlin & Martyn 1982). After that, subjects are rinsed in water and sowed in nursery tray filled with dark peat. Tray has been first covered for four days with a plastic film to promote germination.

2) Fruits

Ripe tomato fruits (Tafna) have been obtained from a greenhouse conducted at national institute of agronomic research, station of Touggourt. They have been selected for their uniformity in size and maturity (Batson & Roy 1982).

2.4. Inoculum preparation

In order to obtain *Fusarium* spores, three pure cultures of fungal isolate have been scraped with a cover-slip into Erlenmeyer containing 100 ml PDL. Flask top has been then covered by a piece of aluminum foil. Suspension has been placed over magnetic stirring in an incubator at 25 °C (Bouzoumita et al. 2017). After 5 days, resulting solution, including mycelia, macroconidia and microcnidia, has been then filtered through four layers of sterile gauze (Mouria et al. 2013) and transferred into a vial. Concentration was adjusted at 1.5×10^5 spores / ml under an optical microscope using a hemocytometer.

2.5. Pathogenicity test

Two inoculation tests have been carried out on tomato in order to identify the fungus studied.

First, inoculation of healthy seedlings (4 true leaves well spread) consists in submersion of their roots (López-Benítez et al. 2018), including the dark peat that surrounds them, in a prepared spore suspension (Hennji 1994) for 30 minutes (Çakır et al. 2014; Manzo et al. 2016). While the roots of control plants, they have been inoculated into sterile distilled water. Under greenhouse conditions, seedlings have been potted and placed as completely randomized and mono-factorial block model where prophylactic traps against *Tuta absoluta* have been installed.

Next, inoculation has been realized on detached tomatoes. Fruits have been surface-sterilized with hypochlorite solution (2%), wounded with a sterile cover-slip then inoculated with 20 µl of spore suspension (Živković 2010). Control fruits have been treated with 20 µl of sterile distilled water.

Fruits have been then placed on wire racks in closed plastic boxes with hot water added to the bottom in order to promote high relative humidity (Dillard & Cobb 1998). Boxes have been incubated at 25 °C.

Five replications have been retained for each inoculation test (seedlings and fruits) as much for the control.

2.6. Pahtogenicity detection

Evaluation of symptoms induced by *Fusarium* sp. on inoculated tomato plants has been realized after 15, 21 and 30 days from inoculation of plants based on a visual index classified on severity scale (Rodrigues & Menezes 2006):

0: No infection;

1: Slight infection which is about 25% of full scale. One or two leaves became yellow;

2: Moderate infection. Two or three leaves became yellow, 50% of leaves became wilting;

3: Extensive infection. The all plant leaves became yellow, 75% of leaves became wilting, and growth was inhibited;

4: Complete infection. The whole plant leaves became yellow, 100% of leaves became wilting, and the plant was died.

Based on this scale, disease incidence (%) has been estimated by the formula proposed by Song et al. (2006):

$$\frac{\Sigma \text{ of all ratings}}{\text{maximum rating grade} \times \text{total number of observations}} \times 100$$

On the other hand, symptoms on tomatoes have been recorded until the 16th day of inoculation (Pandey et al. 2003) using an adopted disease severity scale adopted by Batson & Roy (1982):

0: No infection;

1: Lesion ≤ 5 mm;

2: Lesion 6- 10mm;

3: Lesion 11- 15 mm;

4: Lesion > 15 mm.

2.7. Recovering of pathogen

Plants (inoculated and control) have been examined again to recover the colony of fungus. Only the infected tissues with maximum rating scale have been transferred on PDA in order to identify the fungus studied.

2.8. Statistical analysis

Analysis of variance (ANOVA) at a significance level of ($P \leq 0.05$) has been used to compare data reported for three times in all the five inoculated plants by using statistical program v. 20.0 (SPSS).

3. RESULTS AND DISCUSSION

The colony of *Fusarium* was developed quite quickly in six days on PDA. The morphological aspect was first manifested by a fluffy white mycelium shifted to pale pink (Fig. 1A) then pigmented in dark magenta (Fig. 1B). Microscopic appearance was revealed short monophialides (Fig. 1C), unicellular abundant and ellipsoidal microconidia (Fig. 1E), septate and fusiform macroconidia (Fig. 1F) and single terminal chlamydospore (Fig. 1D).

Several morphological, microscopic and molecular studies have conducted on *Fusarium* and described it on PDA. *F. oxysporum* initially manifests white abundant mycelium then becoming pale pink with a tendency towards violet. While the microscopic aspect, microconidia are unicellular oval or ellipsoid. Macroconidia are also abundant with an attenuated apical cell and a pedicellate basal cell, branched (or unbranched) in short monophialides. Chlamydospores are mostly single, have a smooth wall and formed in intercalated or terminal location (Smith 2007; Leslie & Summerell 2006, Dillard 1989, Summerell et al. 2003, Campbell et al. 2013).

Based on morphological and microscopic characteristics of fungal strain, results confirmed that the causal agent of *Fusarium* wilt isolated from fresh leaves of tomato Chifa hybrid was definitely *Fusarium oxysporum*.

Furthermore, inoculation of this strain in tomato seedlings showed a high virulence towards tomato cultivars tested (Tafna). Over three notations of symptoms, averages recorded in 1st, 2nd and 3rd notations were about 1.20 ± 0.83 (min = 0; max = 2), 3.20 ± 1.48 (min = 1; max = 5) and 5 ± 0 (min = 5; max = 5). Therefore the incidence of disease was in full swing in the last rating with complete infection of plant tissues and severity of the order of 100%. Yellowing, wilting, stunting of growth and later death of plants (Fig. 2A) as well as necrosis on one side of stem (Fig. 2B) were the main symptoms observed on inoculated subjects. Control seedlings were generally intact and healthy with no visible symptom typical to the disease studied (Fig. 2A).

Statistical analysis proved that *F. oxysporum* effectively exerted significant pathogenicity on tomato inoculated F (2. 12) 18.69, $P \leq 0.001$. Results revealed a credible null hypothesis that notation of symptoms had a significant normal distribution in the 1st and 2nd notation ($P = 0.33$ and $P = 0.77$). Likewise, significant Levene's test based on mean has assumed the null hypothesis that there is homogeneity of variances across the time ($P = 0.40$).

In fact, *F. oxysporum* f. sp. *lycopersici* induces longitudinal visible lesions on one side of stem. Necroses are first discolored then gradually dry up. Affected seedlings have a reduced growth, yellow leaves, curve and young plants may completely wilt and die (Blancard 2012). However, the same author has also reported identical symptoms on tomato affected with *F. oxysporum* f. sp. *radicis-lycopersici*, but the later produce almost browning of all the stem, rot and decomposition of roots together with brown chancre on crown that stretching upwards as a flame shape.

Based on these studies, results confirmed that the forma specialis of the pathogen agent studied was *F. oxysporum* f. sp. *lycopersici*.

Moreover, inoculation of tomatoes confirmed more this forma specialis because between the two questioned agents, only *F. oxysporum* f. sp. *lycopersici* is known to be responsible of rot fruit of tomato. Fluffy white mycelia has been manifested on fruit inoculated (Fig. 3A, B, C). The identified fungus incited lesion rating a disease index of 4 recorded in the last day of symptom notation while no symptoms have noticed in control fruit (Fig. 3D).

Successful recovering of inoculated pathogen has showed identical characteristic colonies to starting pathogen especially from leaves (Fig. 4A) roots (Fig. 4B) and petioles samples (Fig. 4C) while the control Petri dishes haven't manifested any mycelia development excepting saprophytic colonies. The most obvious part of the plant from which to recover the fungus is the part of the plant affected by the disease (Leslie & Summerell 2006). Accordingly, *F. oxysporum* f. sp. *lycopersici* has colonized plant roots, circulates with sap in vascular tissues then induce huge damage in the whole plant marking its pathogenic potency towards tomato tested.

4. CONCLUSION

In Wadi Righ region, *Fusarium* spp. can provoke frequently enormous damages during the entire agricultural companion. Presence of *Fusarium oxysporum* on wilting tomato crop conducted at El Meghaïer has been confirmed based on morphological and microscopic characteristics. Results have revealed that the forma specialis of this potent virulent agent has been certainly *Fusarium oxysporum* f. sp. *lycopersici*. This conclusion has been confirmed by pathogenicity test towards tomato (Tafna) plants and fruit.

However, pathogenicity test remains a tool influenced by plant resistance/susceptibility as well as ambient temperature. Therefore, results deserve to be complemented by testing this fungus isolate on other tomato cultivars in greenhouse or open field as well as molecular studies are highly recommended in order to precisely identify the race of fungus.

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Conflicts of Interest: The authors declare no conflict of interest.

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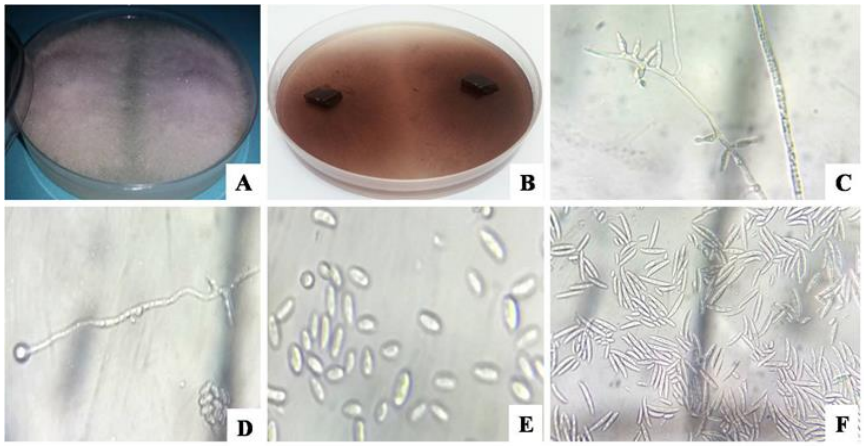

Figure 1: Macroscopic and microscopic characters of *Fusarium oxysporum* obtained with a slide culture (M × 40) **A:** Morphological aspect of a colony **B:** Pigmentation on PDA **C:** Monophialides **D:** Chalmydospore **E:** Microconidia **F:** Macroconidia

Figure 2: Pathogenicity test of *Fusarium oxysporum* in Tafna cultivar (tomato) **A:** Plants inoculated with the phytopathogenic agent **B:** Control seedlings **C:** Brown and dry necrosis visible stretched on few centimeters of stem

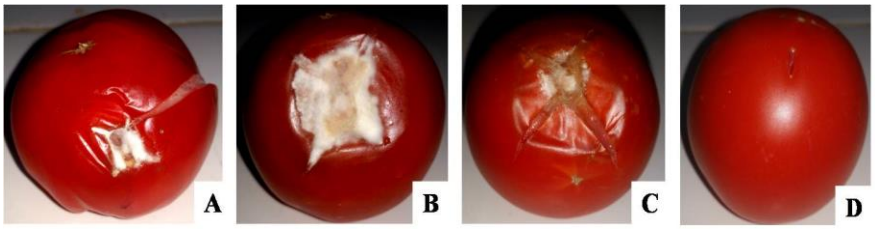

Figure 3: Result recorded after 16 days of *Fusarium oxysporum* f. sp. *lycopersici* inoculation in Tafna cultivar (tomato) **A, B and C:** inoculated fruit **D:** control fruit

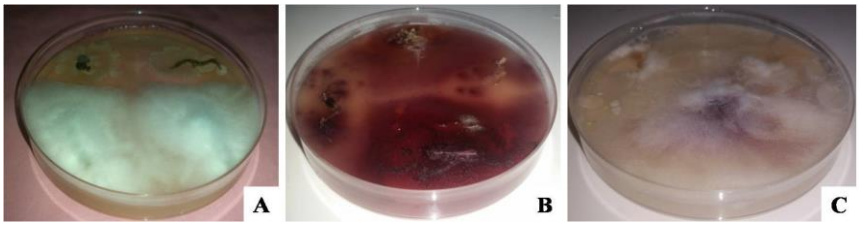

Figure 4: Recovering of *Fusarium oxysporum* f. sp. *lycopersici* from tomato samples **A:** fluffy white mycelia manifested on leaves samples **B:** Shaven colonies of roots pigmented in dark magenta **C:** Fluffy pale pink colonies from petiole samples