Elhazat N *et al.* (2023)
Notulae Scientia Biologicae
Volume 15, Issue 3, Article number 11361
DOI:10.15835/nsb15311361
Research Article

Comparative pathogenesis of *Fusarium* spp. obtained from diseased chickpea plants in Morocco

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Abstract

The pathogenicity of two isolates (FS1 and FS2) of the *Fusarium solani* isolated from diseased chickpea plants harvested from two different regions from Morocco, which were identified morphologically and molecular, was evaluated as the severity, incidence and index of the disease. The results show that the two isolates tested were able to induce symptoms in both parts of the plant (root and aerial), of the seven chickpea varieties ('Garbanzo', 'Farihane', 'Moubarak', 'Douyet', 'Rizki', 'Arifi' and 'Zahour'), which were associated with a reduction in all measured agronomic parameters. However, the intensity of the symptoms depends on the combination of the variety and the isolate of *Fusarium*. It appears that the FS2 isolate affected the 'Moubarak' variety much more, while the pathogenicity of the FS2 isolate was more pronounced on the 'Rizki' variety. The increase in the leaf damage index as a function of time is due to the evolution of leaf yellowing through several physiological stages (necrosis, stunting, wilting, leaf fall), before causing plant death. At the end of the experiment, the re-isolation of the two *Fusarium* isolates from the four vegetative parts of all inoculated varieties was positive.

Keywords: *Fusarium* sp.; leaf lesion; pathogenicity; root rot; varieties of chickpeas

Introduction

The cultivation of grain legumes has so far been considered marginal compared to other crops, especially cereals, despite their ability to fix atmospheric nitrogen (Bacha and Ounane, 2003) and improve soil fertility

Received: 22 Oct 2022. Received in revised form: 17 Jul 2023. Accepted: 25 Aug 2023. Published online: 14 Sep 2023.
From Volume 13, Issue 1, 2021, Notulae Scientia Biologicae journal uses article numbers in place of the traditional method of continuous pagination through the volume. The journal will continue to appear quarterly, as before, with four annual numbers.

(Martinez *et al*., 2007; Bacha and Ounane, 2003). Thanks to their ability to fix atmospheric nitrogen, legumes produce protein-rich seeds without nitrogen fertilizer (Voisin *et al*., 2015; Singh and Pratap, 2016). They contain twice as much protein as cereals (Asif *et al*., 2013).

It seems that this restriction of the extension of grain legume crops in general and chickpea in particular is due to abiotic and biotic constraints (Jha *et al*., 2014; Kaloki *et al*., 2019).

Chickpea (*Cicer arietinum* L.) is an important grain legume that is cultivated worldwide and key factor in the livelihoods of resource poor farmers (Van Der Maesen, 1987; Roy *et al*., 2010; Jendoubi *et al*., 2017; Sunkad *et al*., 2019). Today, chickpea is grown in many parts of Asia and Africa with a global production of 14.77 million tons in 2017 (FAOSTAT, 2019). Other important chickpea producing countries are Pakistan, Australia, Turkey, Iran, Myanmar, Ethiopia, Mexico (Merga and Jema Haji, 2019). It has shown impressive growth in the country during last few decades, still it faces many challenges hampering its production (Dixit *et al*., 2019). These include rainfed cultivation on poor soil inadequate application of nutrients narrow genetic base (Thudi *et al*., 2016; Srivastava *et al*., 2017) and various biotic and abiotic stresses affecting crop yield (Solh *et al*., 1994). It is important to note that consumption of chickpea foods is more common in developing countries (Jukanti *et al*., 2012).

Among fungal diseases impacting chickpea are *Fusarium* wilt, dry root rot, wet root rot, collar rot and *Ascochyta* blight, caused respectively by *Fusarium oxysporum, Rhizoctonia bataticola, Fusarium solani, Rhizoctonia solani, Sclerotium rolfsii* and *Ascochyta rabiei* (Beniwal *et al*., 1992; Ahmed and Melkamu, 2006; Navas Cortes *et al*., 2008, Srivastava *et al*., 2021; Bekele *et al*., 2021).

In Morocco, the areas reserved for the cultivation of chickpeas occupy the second place after those of broad beans. But, his production only takes 10th place (Laamari *et al*., 2016). This low yield of chickpea is attributed to abiotic constraints but in the majority of cases to attacks by pathogens (Machehouri *et al*., 2017). In this regard, Bouznad *et al*. (1990) reported that *Fusarium* wilt, a disease caused by *Fusarium sp*., soil fungi, causes significant losses in several legumes (Belabid *et al*., 2000). These plant pathogens can attack more than 100 plant species, sometimes destroying entire cereal crops (Nucci *et al*., 2007). On this point, there are more than 70 pathogens that have been reported on chickpea in different regions of the world (Morjane and Harrabi, 1995). *Fusarium* wilt appears to be one of the most devastating diseases of chickpea crops in many countries, particularly in North Africa (Reddy *et al*., 1984). *Fusarium* wilt disease can cause yield losses approaching 100% in highly infested fields (Haware and Nene, 1980; Halila and Strange, 1996; Navas Cortes *et al*., 2000; Sharma *et al*., 2016; Upasani *et al*., 2017). *Fusarium* is mainly soil borne but also persistent as a seed borne pathogen (Pande *et al*., 2007; Jimenez Diaz *et al*., 2015). The fungus can survive in the soil for more than six years (Haware *et al*., 1978; Haware 1990). *Fusarium* wilt appears in the early seedling stage as well as at maturity (Beniwal *et al*., 1992).

The present work aims to compare the pathogenicity of two species of *Fusarium solani*, by inoculation of different varieties of chickpea after their morphological and molecular characterization.

Materials and Methods

Pathogen isolation

Surveys were carried out in two different agroclimatic zones (Ouazzane and Souk larbae), and in each area, ten chickpea fields, taken at random at 10 km intervals, were surveyed during the months of May-June, during the campaigns from 2016 to 2018. Diseased plants showing symptoms of wilting, taken randomly, were taken back to the laboratory for analysis.

In the laboratory, the roots, stems and petioles of diseased plants were disinfected by rapid soaking in alcohol (90°), rinsed several times with sterile distilled water, dried on filter paper and cut into fragments of 2 mm length. The three organs are handled separately to avoid cross-contamination. For each plant, three to six fragments per organ are inserted vertically into sterile Petri dishes containing the PSA medium (Potato Sucrose Agar, 200 g potato, 20 g sucrose, 15 g Agar-agar and 1000 ml distilled water), sterilized at 120 °C for 20 min. After one week of incubation at 25 °C and in the dark, the results were evaluated according to the presence or absence of fungal colonies around the fragments of diseased plants transplanted into PSA medium.

Morphological characterization of selected Fusarium isolates

The two isolates, FS1 and FS2 obtained from the roots and leaf petioles, were first subcultured from single conidia and stored on slices of filter paper at -20 °C in the freezer.

The *Fusarium* isolates collected were cultured in 90 mm diameter Petri dishes containing sterile PSA medium. The examination was carried out according to the development of the cultures on this PSA medium. The macroscopic observations related to the aspect of the cultures, the density of the mycelium, the color, the growth and the production of the spores. While, the microscopic examination focused on the observation, under an optical microscope at ×40, ×100, ×400 and ×1000 magnifications, of the nature of the mycelium, the appearance of the conidiophore, the shape and size conidia, and the presence of conservation organs, the case of chlamydospores. The mounting liquid used for microscopic observations is cotton blue. The morphological identification was carried out based on the Tivoli guide (1988), the Nelson *et al*. (1983), Messiaen and Cassini (1968), Domsch*et al*. (1980), Nelson and Toussoun (1983) and Champion (1997).

Molecular analysis and identification

Fungal DNA extraction

The molecular identification of the two isolates was carried out after five days of culture on PSA. DNA extraction was done according to the method described by Murray and Thompson (1980) and Doyle *et al*. (1987). After freeze-drying the samples for 48 hours, a quantity of 0.1 g of the mycelium was removed and placed in 2 mL microtubes and ground for 5 min using balls in a grinding device. The mechanical destruction of the mycelial tissue is carried out under cold conditions at 4 °C in order to protect the DNA molecule against the suspected enzymatic reactions. The advantage of this step is to better expose the sample to the extraction solution, composed of 0.1 M Tris (pH 8); 5 M NaCl; 0.5M EDTA; 2% CTAB; 0.2% mercaptoethanol, and used in the next step. 1 mL buffer extraction preheated to 65 °C was quickly added to the ground material. The microtubes containing the ground mixture/extraction solution were then placed in a water bath at 65 °C for 1 hour under stirring (15 min each time). After heating, the mixture is cooled in ice for 5 minutes; and 800 µL of chloroform/isoamyl alcohol (24:1) was added. The mixture was stirred gently for 20 minutes at room temperature and centrifuged at 13000 rpm for 15 min at 4 °C. The supernatant was recovered and subsequently transferred to a sterile Eppendorf tube containing 750 µL of isopropanol cooled to -20 °C. After gentle mixing, the DNA begins to precipitate as filaments or turbidity. The precipitation lasted 1 hour at 4 °C or 30 min at -20 °C. The supernatant was drained and the pellet was recovered by centrifugation at 13.000 rpm for 15 min. Afterward, the DNA was washed twice by adding 1 ml of ethanol (75%) cooled to -20 °C at the pellet followed by centrifugation at 13000 rpm for 15 min at 4 °C. After drying the DNA under vacuum at room temperature, 200 µL of TE buffer was added to dissolve the DNA. The concentrations of the DNA extracts were measured using the Nanodrop ND-8000 spectrophotometer (Thermo Fisher Scientific, USA).

PCR amplification and sequencing

The ITS (Internal Transcript Spacer) region of ribosomal DNA, defined by (White *et al*., 1990; Gardes and Bruns, 1993) was amplified with the universal primers ITS1 and ITS4 (White *et al*., 1990). This region is non-coding and highly polymorphic.

The total volume of DNA amplification is 25 µL. The latter is composed of 5 µL of 5X buffer (reagents: MyTaq DNA polymerase Bioline kit), 1 µL of dNTP (20 mM), 1 µL of each of the primers (10 µM), 0.2 µL of Taq DNA polymerase (5 U μL^{-1}), 150 ng of template DNA and sterile bi-distilled water. Controls without DNA are carried out to test the presence of possible contaminations in the reagents and/or the buffers.

Initial denaturation lasted 1 minute at 95 °C, followed by 35 cycles of 15 seconds at 95 °C, 20 seconds at 57 °C, and 15 seconds at 72 °C, with a final extension of 3 minutes. At 72 °C in an ABI "Verity" thermal cycler. The PCR product was subjected to electrophoresis on a 1.5 % agarose gel in the presence of a 100 bp molecular weight marker.

PCR products were purified using ExoSAP-IT reagent (Affymetrix). Sequencing was performed using an ABI PRISM BigDye Terminator v.3.1 ready-to-use cycle reaction sequencing kit and an ITS1 and ITS4 primer set. Sequencing products were run on an ABI PRISM 3130XL Genetic Analyzer (AppliedBiosystems) using POP-7 polymer. The resulting ITS sequence was compared for similarity using the local base alignment search tool: (*http://www.ncbi.nlm.nih.gov/BLAST*).

Sequence submission to GenBank

The sequences from FS1 (*Fusarium solani*) and FS2 (*Fusarium solani*) have been submitted to GenBank respectively under the numbers MT111121 and MT111123.

Plant material

Healthy seeds of 7 chickpea varieties ('Garbanzo', 'Farihane', Mubarak, 'Arifi', 'Douyet', 'Rizki', and 'Zahour') were disinfected superficially by soaking for 10 min in a 10% sodium hypochlorite solution, followed by three rinses in sterile distilled water, then left to dry on sterile filter paper.

Inoculum preparation and inoculation

Preparation of fungal inoculum

The *Fusarium* isolates selected for the pathogenicity study, FS1, and FS2, showed significant sporulation on the PSA medium.

For inoculum preparation, *Fusarium* isolates are grown on PSA and incubated in the dark at 25 °C. After 7 days of incubation, the surface loaded with conidia is scraped sterile using a metal spatula. The mycelium is suspended in sterile distilled water, then stirred for 30 to 60 seconds. The resulting spore suspension is filtered through muslin cloth, to remove mycelial debris. The spore suspension is then adjusted with sterile distilled water so as to have a final concentration of 10^6 conidia/ml using a Malassez blade.

Seed inoculation

The disinfected and dried chickpea seeds are inoculated by soaking for 24 hours in a conidial suspension of 10^6 conidia ml^{-1} of the pathogens. Control seeds are soaked in sterile distilled water. Afterward, the seeds are dried a second time on sterile filter paper. Two batches of inoculated and control seeds are made up. A batch is germinated, and the seeds are placed in Petri dishes 180 mm in diameter containing filter paper soaked in sterile distilled water (5 seeds per box) and at the rate of 3 repetitions per treatment. The boxes are incubated for 7 days in the dark at 25 °C. The other batch of seeds is transplanted into polyethylene pots (13 cm x 13.5 cm) (5 grains per pot) containing a substrate consisting of sterile Mamora sand (sterilization twice in an oven at 240 °C for 4 hours Afterward, all the pots are taken back to a plastic cultivation greenhouse.

Calculation of seed germination percentage

The percentage of sprouted seeds (G) is estimated according to the following formula:

G (%) = (nt – nn) x 100 / nt

nt: Total number of seeds per box.

no: Number of ungerminated seeds.

The incidence of the disease *in vitro*, denoted I, was estimated from the number of lesions developed on the coleoptile of the germinated seeds.

I= (nt-nn) x 100 / nt

nt: Total number of seeds per box

no: Number of seeds in which the coleoptile of the seedlings developed after germination has not been altered.

Estimation of the leaf alteration index

The disease was assessed by calculating the leaf damage index, noted according to the scale established by Douira and Lahlou (1989):

Notes: Appearance of leaves	
0	Sound appearance.
1	Cotyledonal leaf: wilting or yellowing.
2	Cotyledonary leaf: fall.
3	True leaf: wilting or yellowing.
4	True leaf: necrosis.
5	True leaf: fall.

The scores related to the number of leaves constitute the Foliar Alteration Index (IAF), calculated according to the formula below. An average index is then calculated for each batch of plants.

IAF= [Σ(i×Xi)] / 6×NtF

IAF: Leaf alteration index.

I: Leaf Appearance Ratings 0 – 5.

Xi: Number of sheets with score i.

NtF: Total number of sheets.

Estimation of agronomic parameters

After 2 months of culture, the average of various measured agronomic parameters was determined: length of stems and roots, number of leaves and pods and the fresh and dry weights of aerial parts and roots before and after drying in an oven at 70 °C for 48 h, by a precision balance.

Assessment of disease severity

The roots of different plants were rinsed under running tap water and separated from their aerial parts. The visual evaluation of the severity of the disease focused on the description of the attack throughout the root system, namely at the collar, the sub-collar, and the seminal roots (Greany *et al*., 1938), according to the scale of severity class from 0 to 5 (Table 1).

Table 1. Disease severity scale (Greany *et al*., 1938)

Severity class	Degree of plant infection
0	No symptoms
1	Small scattered necrotic lesions at the level of the collar, the subcollar and the seminal roots
2	Distinct and clear necrotic lesions on the root system
3	Large necrotic lesions on the collar, subcollar and seminal roots.
4	Severe rotting of the root system and chlorosis of the plant
5	Dead plant.

Disease incidence was calculated using the following formula:

I = 100 [Nm / Nt]

Nm =Number of diseased plants

Nt =Number of plants examined

The root rot index was calculated according to the following formula:

IM = 100 Σ (Ni Si) / (5 Nt)

Ni= number of plants of severity class i

Si= severity class i

After 60 days of their inoculation with the two isolates of *Fusarium*, the plants were dug up and freed of their culture substrate by washing them abundantly with running water. For each plant, cross-sections were made every 2 cm, going from the roots to the leaves, which were subsequently disinfected by soaking in alcohol (90°), for 2 min. After drying on sterile filter paper, these fragments were inoculated into Petri dishes containing a sterile PSA medium.

The percentage of re-isolation (PR %) was calculated by applying the formula below:

PR % =Ns PX /NT×100

Ns PX: Number of segments containing fungal species X

Nt: Total number of segments used in isolation

Statistical analysis

The statistical analysis of the data was carried out using statistical software (STATISTICA). Percentages have been transformed into Arcsine √ (P: proportional on average). When the result of the variance analysis registers at least one significant difference at the 5% probability threshold, a comparison of means test is applied to these data (PPDS test).

Results

The examination of the cultures and the microscopic observations of the isolates obtained from the chickpea plants showing symptoms of wilting in the two prospected regions made it possible to describe the morphological characters of two isolates of *Fusarium solani*: FS1 and FS2 (Table 2).

Table 2. *Fusarium* isolates and their origins

Isolates	Species	Isolation parts	Isolation regions
FS1	*Fusarium solani*	Roots	Ouazzane
FS2	*Fusarium solani*	Leaf petioles	Souk larbae

On the PSA medium, *Fusarium solani* forms fluffy or cottony colonies of white to cream color with a pale underside. In addition, it older crops, the fluffy white mycelium takes on a mauve or pinkish-white color. This description is consistent with those of Chliyeh *et al*. (2017). Under the microscope, *F. solani* is characterized by a septate mycelium with the presence of unicellular or bicellular microconidia (11.5 µm) of various shapes (ovoid, fusiform, cylindrical, pear-shaped) and multicellular macroconidia (18.3 µm) fusiform and septate. The resistance spores are chlamydospores (Figure 1) in a terminal or intercalary position.

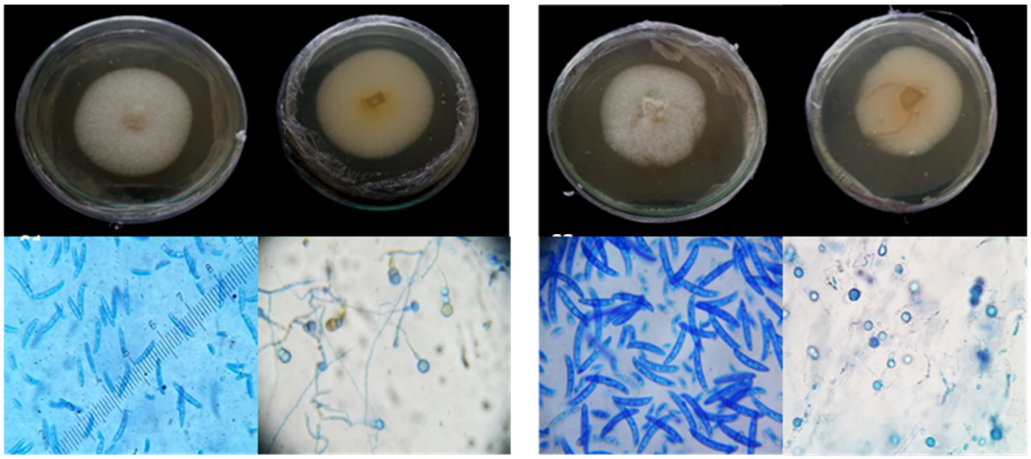

Figure 1. Appearance of cultures and morphological characters of isolates of *F. solani*: FS1, a: macroconidia and microconidia, b: chlamydospores; FS2, c: macroconidia and microconidia, d: chlamydospores (Magnification: ×400; mounting fluid: Cotton Blue).

Sequencing of the products of the two isolates FS1 and FS2 obtained by PCR amplification, using the universal fungal primers (ITS1 / ITS4), brought them closer to the species *F. solani* (100%). The two isolates FS1 and FS2 were registered in the national database and at GenBank respectively under the voucher identification number, RAB 111029 and RAB 111031, and under the accession number MT111121 and MT111123. In this regard, the identity of the FS1 isolate was evaluated and confirmed.

In vitro, the germination capacity of the seven chickpea varieties is generally high when the seeds are soaked in sterile distilled water (83% in 'Farihane', 'Mubarak', 'Douyet', 'Arifi', and 'Zahour' and 100% in 'Garbanzo' and 'Rizki'). But in the case of inoculation, the expression of the results in % of the control shows that the effect of the two isolates of *Fusarium* tested is very low on the germination of the inoculated seeds of the 7 varieties studied. In the latter case, the extent of the lesions induced on the coleoptiles after germination of the inoculated seeds is variable depending on the combination: chickpea variety X *Fusarium* isolate (Figure 2). Because, in the presence of the FS1 isolate, the percentages of reduction in the germination of the inoculated seeds compared to the control of each of the varieties 'Garbanzo', 'Farihane', Mubarak, 'Douyet', 'Rizki', 'Arifi' and 'Zahour' are respectively 6.66%; 40%, 6.66%, 40%, 40%, 8% and 10%. Whereas, with FS2 and in the same varietal order as previously, the reductions recorded compared to the control of each variety are respectively 39%, 25%, 40%, 19%, 6.66%, and 40% (Figure 3).

So, it appears from these results that the varieties 'Garbanzo' and 'Mubarak' are the least susceptible to the FS1 isolate (6.66%). And this same low sensitivity was observed in 'Rizki' in the presence of the FS2 isolate.

The incidence of the disease at the level of the coleoptile of the germinated seeds is generally high in the different varieties of chickpea tested. In this regard, it is 100% in the "Garbanzo", Mubarak, and 'Arifi' varieties, and only 86.66% in the other varieties in the presence of the FS1 isolate. But with the FS2 isolate, it is 100% in 'Douyet' and 'Rizki', and 86.66% in the other varieties. In comparison with those of the controls, these recorded incidences are very small. In ascending order of disease incidence, the varieties are ranked as follows (Figure 4):

'Garbanzo' = 'Farihane' = 'Mubarak' = 'Douyet' = 'Rizki' (10 %) < 'Zahour' (15 %) <'Arifi' (20 %).

Figure 2. Effect of isolates of *F. solani* (FS1 and FS2) tested on coleoptile development *in vitro* of different chickpea varieties

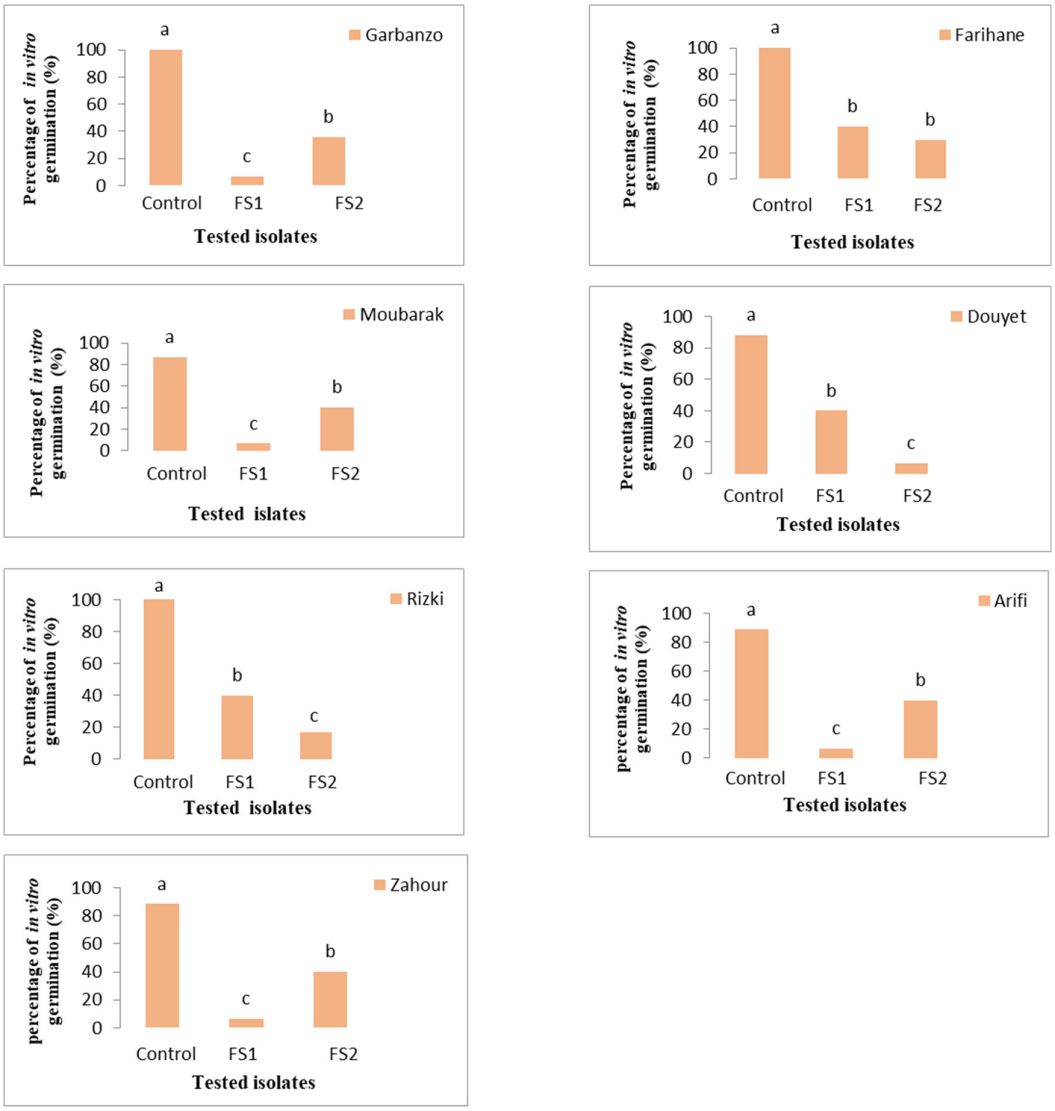

Figure 3. Percentage of *in vitro* germination of 7 chickpea varieties after 7 days of incubation against 2 isolates of *Fusarium*

Two results followed by the same letter are not significantly different at the 5% level according to the PPDS test.

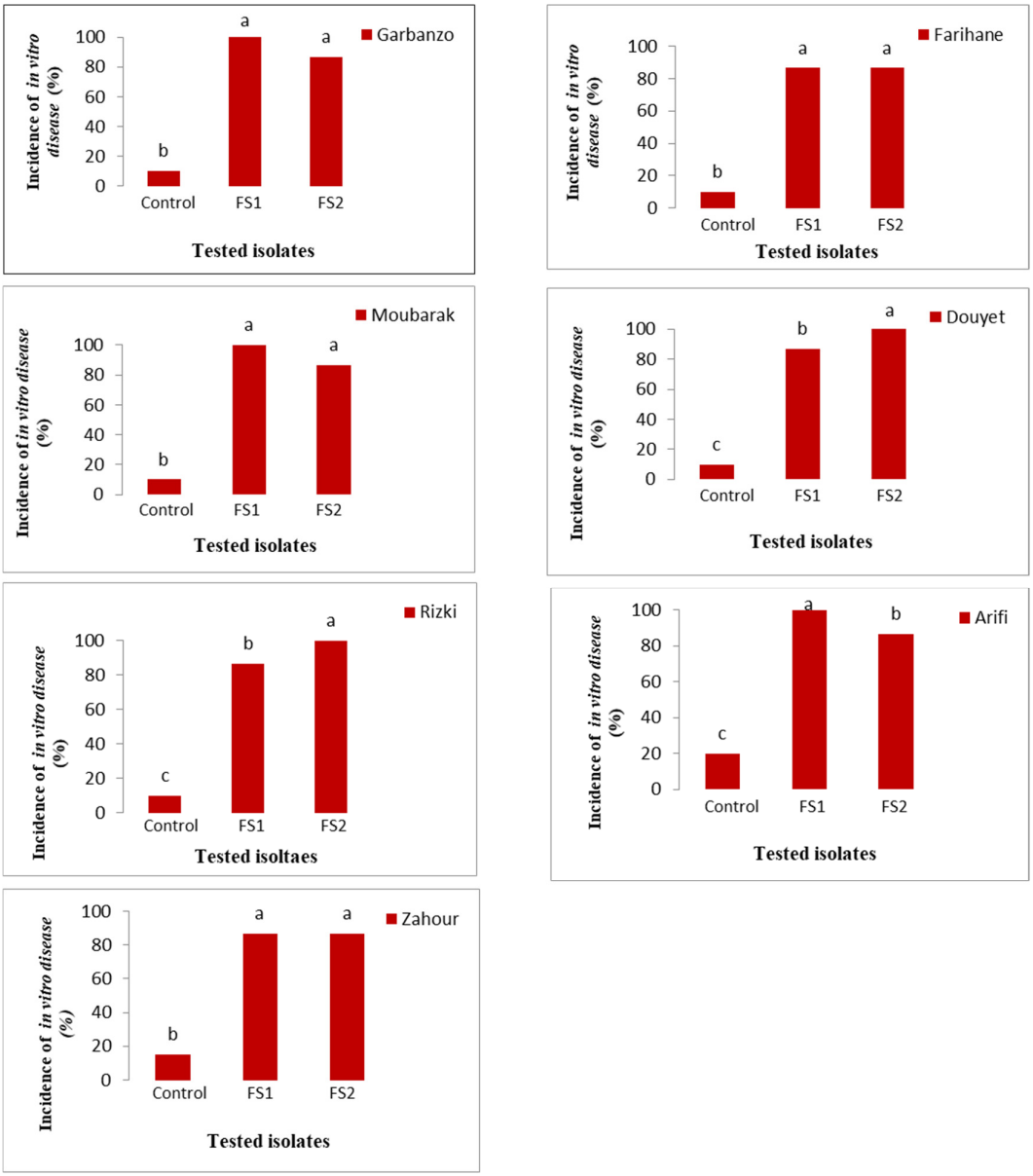

Figure 4. Incidence of the disease on different varieties of chickpea inoculated with 2 isolates of *Fusarium in vitro*

Two results followed by the same letter are not significantly different at the 5% level according to the PPDS test.

After 4 weeks of development, inoculation with the two *Fusarium* isolates resulted in variability in leaf symptoms depending on the combination (chickpea varieties x *Fusarium* isolates): stunting, wilting, leaf drop, and leaf death plants (Table 3). According to the IAF values recorded in the 6th week of culture in the presence of the FS1 isolate, it seems that the Mubarak variety is the most sensitive (IAF=0.602) and that the 'Farihane' variety is the least sensitive (IAF =0.197). However, in the presence of the FS2 isolate, the 'Mubarak' variety becomes the least sensitive (IAF=0.135) and the 'Rizki' variety is the most sensitive (IAF=0.598). In comparison with the controls at this stage, the varieties can be classified, according to the ascending order of the values, very reduced, of the following IAF (Table 3):

'Garbanzo' = 'Arifi' (0.101) < 'Douyet' = 'Rizki' (0.102) < 'Farihane' = 'Zahour' (0.103) < 'Mubarak' (0.105).

Table 3. Evolution of the leaf alteration index (IAF) as a function of time in chickpea plants

Time	Isolates	IAF						
		Varieties						
		'Garbanzo'	'Farihane'	'Mubarak'	'Douyet'	'Rizki'	'Arifi'	'Zahour'
Week 4	FS1	0.269^{ab}	0.111^{c}	0.282^{a}	0.197^{b}	0.265^{ab}	0.232^{ab}	0.214^{ab}
	FS2	0.196^{c}	0.227^{b}	0.112^{d}	0.294^{a}	0.295^{a}	0.128^{d}	0.138^{d}
	T	0.012	0.012	0.018	0.012	0.015	0.011	0.012
Week 5	FS1	0.388^{b}	0.266^{bc}	0.499^{a}	0.241^{c}	0.302^{b}	0.476^{a}	0.413^{a}
	FS2	0.417^{b}	0.411^{b}	0.152^{c}	0.500^{a}	0.536^{a}	0.164^{c}	0.162^{c}
	T	0.041	0.045	0.046	0.037	0.044	0.042	0.042
Week 6	FS1	0.596^{a}	0.197^{d}	0.602^{a}	0.251^{c}	0.296^{d}	0.562^{a}	0.405^{b}
	FS2	0.306^{b}	0.338^{b}	0.135^{d}	0.596^{a}	0.598^{a}	0.200^{c}	0.204^{c}
	T	0.101	0.103	0.105	0.102	0.102	0.101	0.103

S4: week 4; S5: week 5; S6: week 6; FS1: F. solani; FS2: F. solani, T: Control
Two results read on the same line followed by the same letter are not significantly different at the 5% level according to the PPDS test.

In the control medium, varietal differences appear at the level of the two parts of the plant: aerial and root. For the length of the stem, the highest value was recorded in 'Zahour' (36 cm), compared with the other varieties (from 31 to 32 cm). While, the best root elongation, about double of the other varieties (15 to 17.9 cm), is observed in 'Rizki' (32 cm).

However, inoculation with the FS1 isolate resulted in a reduction in stem length; which is 66% for 'Mubarak' and 'Zahour', and only 25% for 'Rizki' and 'Arifi'. For the other varieties, the decrease is 29% and 59% respectively in 'Garbanzo' and 'Farihane', and 'Douyet' (Figure 5). On the other hand, at the root level, the reduction recorded is 67% in 'Mubarak' and 'Rizki' and 21% only in 'Farihane'. For the other varieties, the reduction is 28%, 31%, 37%, and 52% respectively in 'Garbanzo', 'Douyet', 'Arifi', and 'Zahour' (Figure 5).

Similarly, in the presence of the FS2 isolate, the reduction in the length of the aerial part is 92.5% in 'Rizki' and 'Arifi', and only 20% in 'Douyet'. For the other varieties, the reduction is 28%, 31%, 52%, and 65% respectively in 'Zahour', 'Mubarak', 'Garbanzo', and 'Farihane' (Figure 5). Also, root elongation was severely affected in 'Rizki', which was more sensitive (91%), than in 'Arifi', which was relatively less sensitive (32 %). For the other varieties, the reduction is 42%, 44%, 54%, and 64% respectively in 'Zahour', 'Garbanzo', 'Farihane', and 'Douyet' (Figure 5). Therefore, in the majority of varieties tested, the FS2 isolate appears to significantly slow stem length.

For the average number of leaves on the control medium, the highest value was recorded at 'Farihane' (22 leaves per plant). On the other hand, for the other varieties, the calculated averages varied between 16 and 20 leaves per plant. For the number of flowers, the 7 varieties produced similar averages (from 7.1 to 7.6 flowers per plant), for a different number of leaves (Figure 6).

The presence of pathogen isolates affected the average number of leaves more than that of flowers (Figure 6). Because, the FS1 isolate caused a 50% reduction in rounds in 'Garbanzo', 'Douyet', and "Arifi", and only 21 to 27% in the other varieties for the average number of leaves. On the other hand, for the other parameter, the highest reduction of 59% was recorded in Mubarak. For the other varieties, the reductions varied between 32% and 40%. Along the same lines, the greatest reductions caused by the FS2 isolate on these two parameters were observed in 'Rizki' and 'Douyet' (Figure 6).

Taken together, these results indicate that the two pathogenic isolates of *Fusarium* affect the number of leaves more than that of flowers, indicating a greater effect on the activity of the apical meristem of the plant.

On a control medium, the average numbers of filled pods and those of empty pods calculated are generally very similar for the different varieties studied. In the presence of the two pathogenic isolates of *Fusarium*, the mean numbers of empty pods recorded increases that were too high compared to the control. In contrast, the highest reductions in pod filling are 77% and 72% respectively in 'Garbanzo' and 'Zahour' in combination with FS1. But with FS2, this inhibitory effect on pod filling is more marked, approaching inhibition of 96% and 82% respectively in 'Rizki' and 'Farihane' (Figure 7).

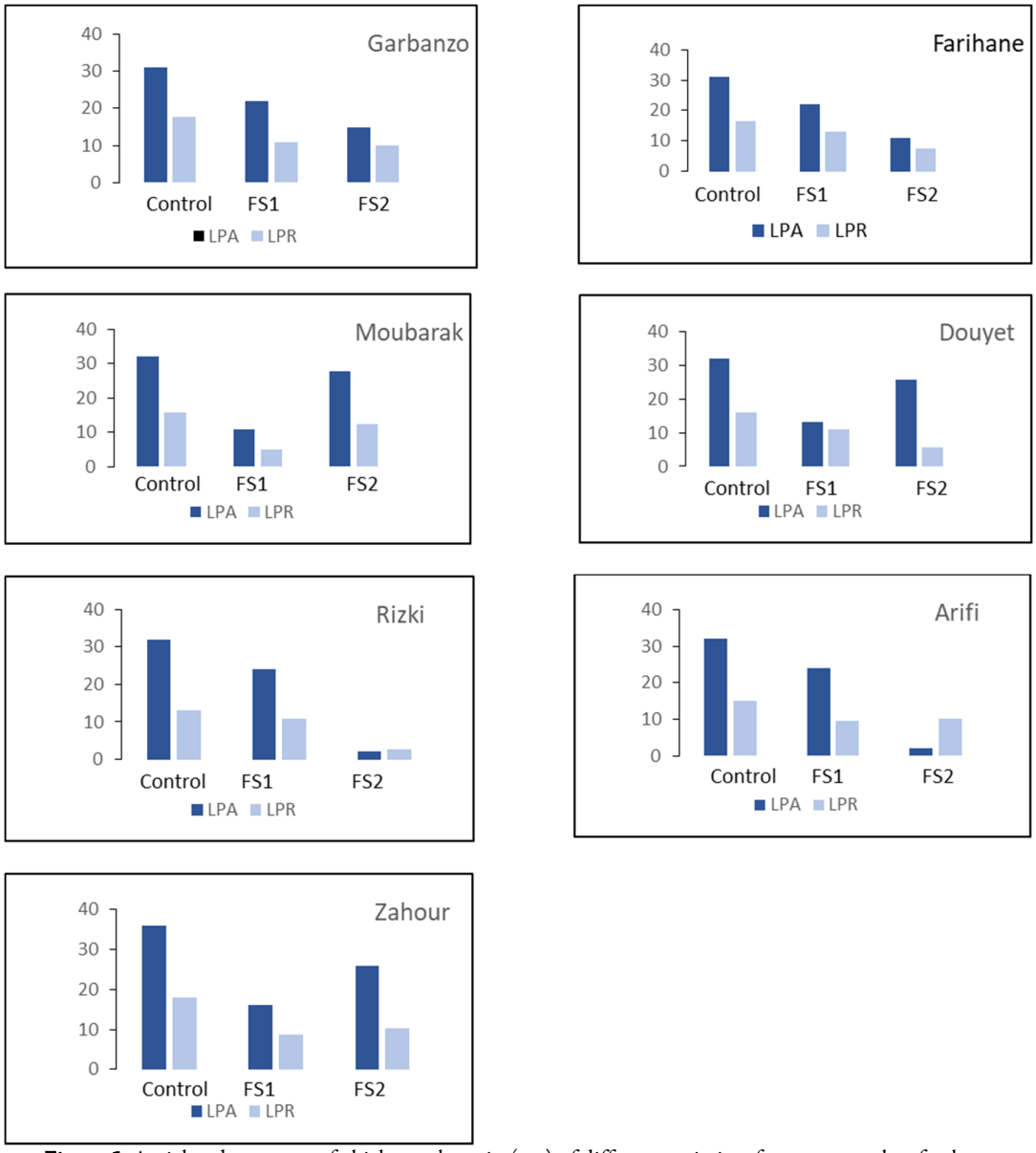

Figure 5. Aerial and root part of chickpea plants in (cm) of different varieties after two months of culture with two isolates of *Fusarium* (FS1 and FS2)

LPA: aerial part length; LPR: root part length

Two results followed by the same letter are not significantly different at the 5% level according to the PPDS

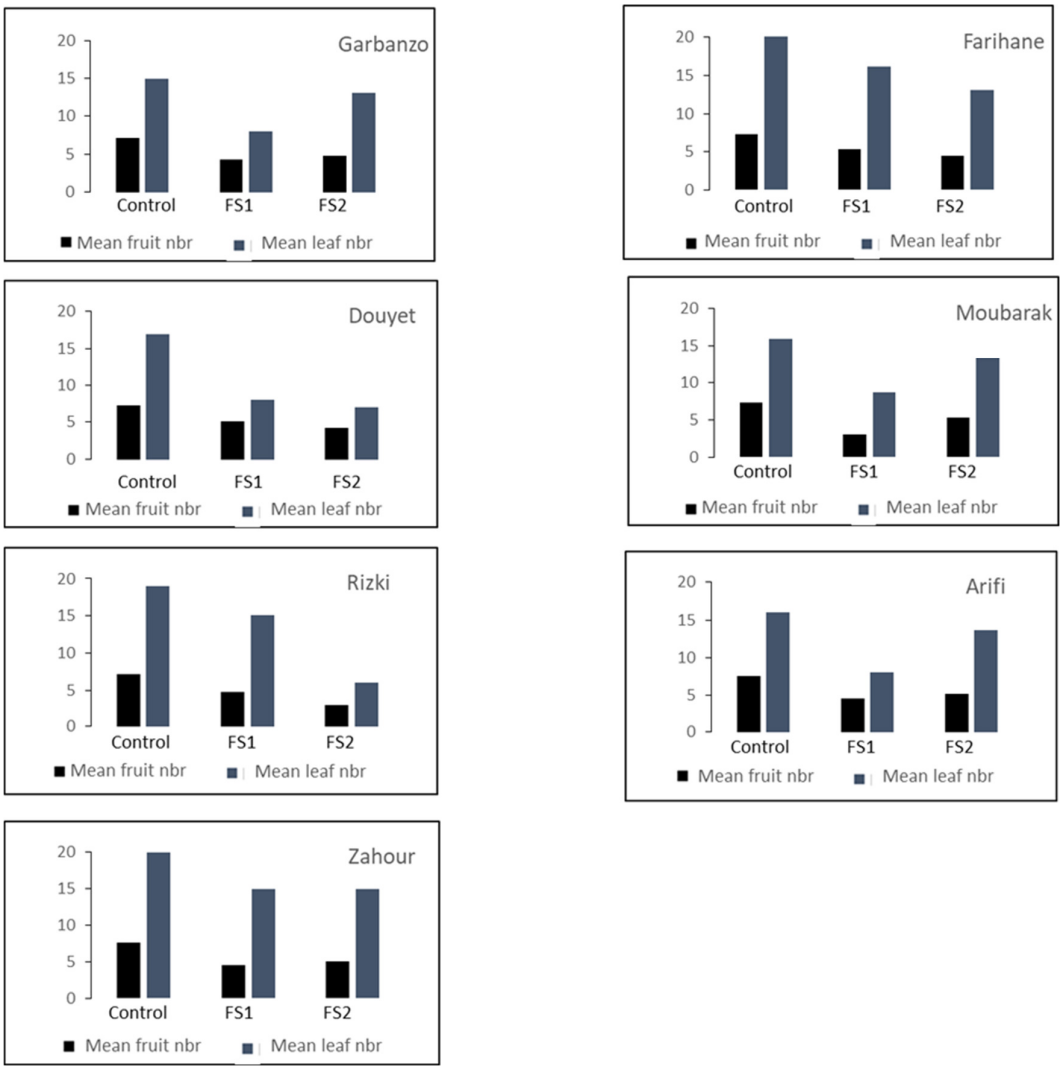

Figure 6. Effect of two *Fusarium* isolates (FS1 and FS2) on mean leaf and fruit number of chickpeas of different varieties after two months of cultivation

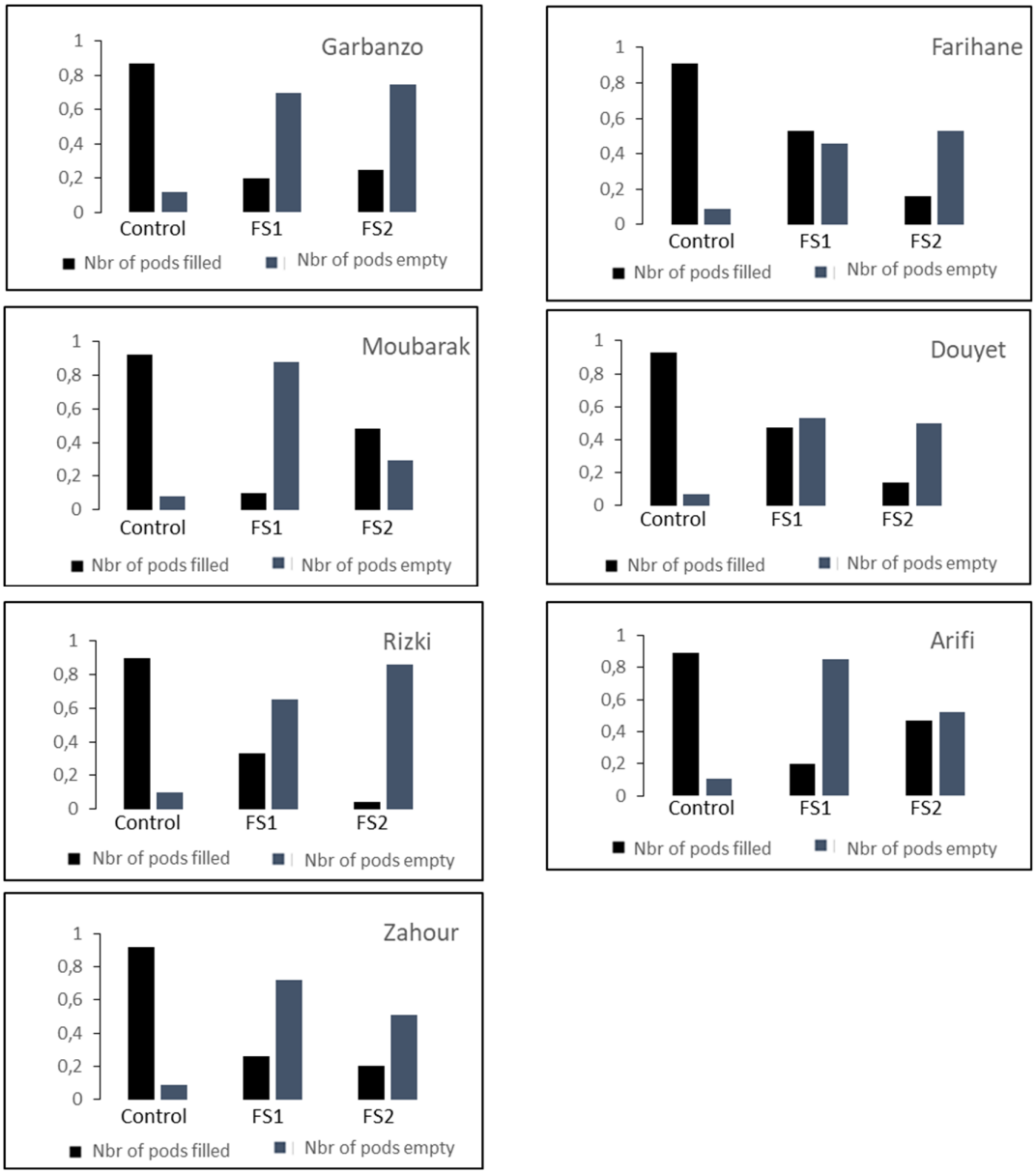

Figure 7. Comparison of average number of filled and empty pods of different pea varieties chickpeas inoculated with two isolates of Fusarium in comparison with controls

Therefore, the inhibitory action of the two pathogenic isolates FS1 and FS2 is exerted more on the filling of the pods than on their formation.

On a control medium, the comparison of aerial and root masses (fresh and dry) between the varieties shows better growth activity in ''Arifi''. On the other hand, the weakest aerial and root masses were observed respectively in 'Douyet' and 'Rizki'. For the other varieties, the masses measured are intermediate between those of these three varieties ('Arifi', 'Douyet', and 'Rizki') (Figure 8).

In the presence of the two *Fusarium* isolates, the masses of the fresh and dry matter of the aerial and root parts of the plants of each of the seven varieties showed a decrease compared to those of their corresponding controls.

For the aerial part, this reduction is 69% and 90% respectively for the fresh mass and the dry mass in Mubarak inoculated with the FS1 isolate. On the other hand, with the other isolate FS2, this reduction is 78 % and 86% respectively for the fresh mass and the dry mass in 'Rizki'. These results suggest that the 'Farihane' variety is more resistant to the FS1 isolate than the 'Mubarak' variety. While for the other pathogenic isolate (FS2), it is the 'Garbanzo' variety that seems the most resistant than the 'Rizki' variety, the most sensitive, of all the varieties studied (Figure 8).

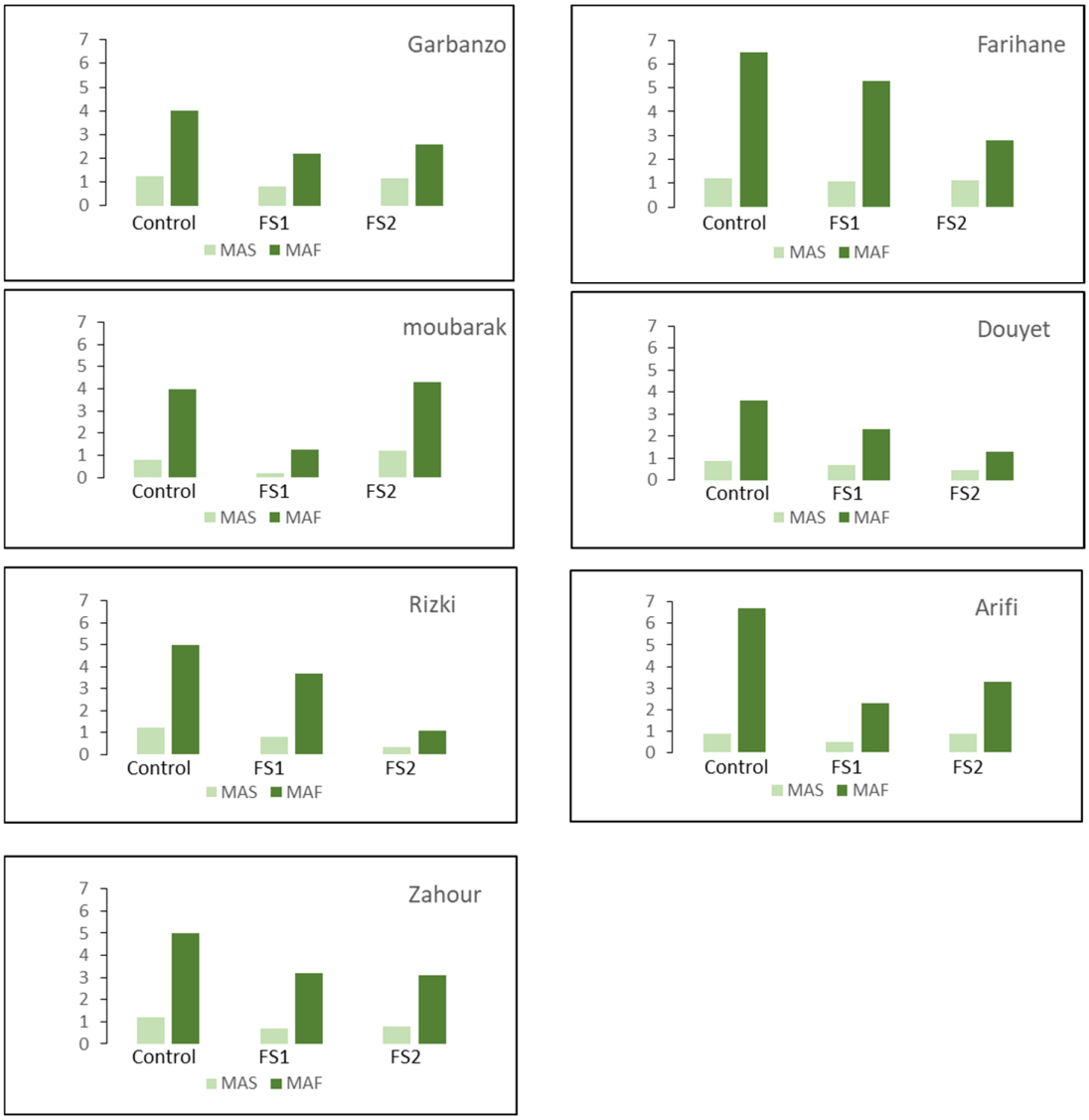

Figure 8. Effect of two *Fusarium* isolates (FS1 and FS2) on the development of fresh and dry aerial masses in (g) of chickpea plants of different varieties
MAF: fresh air mass. MAS: dry air mass

Regarding the underground part, it seems that the 'Arifi' variety is the most affected by FS1, showing a reduction of 95%. With the FS2 isolate, the low fresh root mass-produced, less than 82% compared to the control, is recorded in 'Rizki', the most sensitive. While the best root production is measured in 'Zahour', the least sensitive (Figure 9).

By comparing the behavior of the varieties studied with respect to the two isolates of *Fusarium* (FS1 and FS2), it is clear that the 'Rizki' variety is the most susceptible.

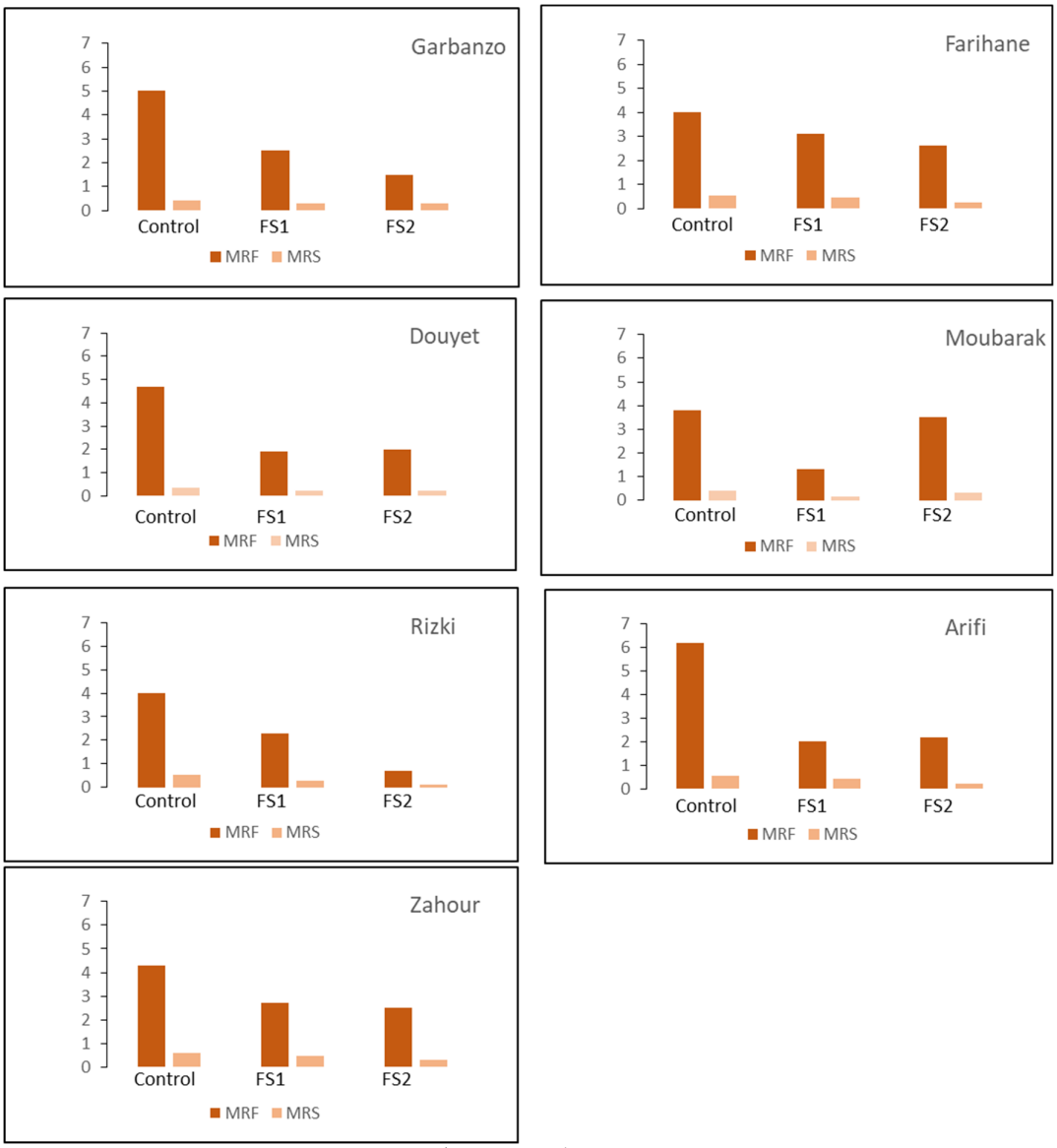

Figure 9. Effect of two isolates of *Fusarium* (FS1 and FS2) on the development of fresh and dry root masses in (g) of chickpea plants of different varieties
MRF: fresh root mass, MRS: dry root mass

After 60 days of inoculation, both isolates induced necrotic lesions on plant roots of all chickpea varieties (Figure 10). Also, the development of the vegetative part was affected (Figure 11).

According to the increasing values of the percentage of root rot severity classes calculated in the presence of FS1 (Table 4), the varieties can be classified as follows:

'Garbanzo' = 'Farihane' = 'Arifi'< 'Rizki' < 'Douyet' = 'Zahour'< 'Mubarak'

However, with the exception of 'Farihane', all other strains changed their rankings with FS2:

Mubarak = 'Farihane' = 'Douyet' = 'Zahour' < 'Arifi' < 'Garbanzo' < 'Rizki'

Figure 10. Effect two *Fusarium* isolates (FS1 and FS2) on the development of the root system on the most sensitive Chickpea varieties

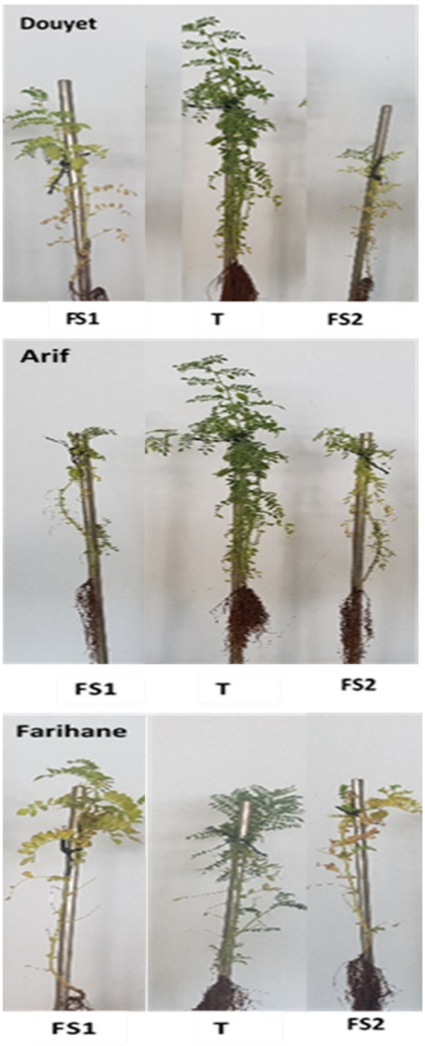

Figure 11. Aerial part of chickpea plants inoculated with two Fusarium isolates (FS1 and FS2) tested on a few chickpeas' varieties

Table 4. Percentage of root rot disease severity classes on chickpea plants

Isolates	Varieties	Severity class (%)					
		S0	S1	S2	S3	S4	S5
FS1	'Garbanzo'	0	40^{b}	26.66^{b}	26.66^{a}	6.66^{c}	0
	'Farihane'	0	40^{b}	46.66^{a}	6.66^{c}	6.66^{c}	0
	'Moubarak'	0	6.66^{c}	6.66^{c}	20^{b}	60^{y}	0
	'Douyet'	0	40^{b}	26.66^{b}	6.66^{c}	26.66^{b}	0
	'Rizki'	0	53.33^{a}	26.66^{b}	33a	10.05^{c}	0
	'Arifi'	0	40^{a}	26.66^{b}	26.66^{a}	6.66^{c}	0
	'Zahour'	0	6.66^{c}	40^{a}	33^{a}	26.66^{b}	0
FS2	'Garbanzo'	0	40^{b}	26.66^{b}	6.66^{c}	26.66^{b}	0
	'Farihane'	0	40^{b}	26.66^{b}	26.66^{b}	6.66^{c}	0
	'Moubarak'	0	6.66^{c}	46.66^{a}	40^{a}	6.66^{c}	0
	'Douyet'	0	40^{b}	26.66^{b}	26.66^{b}	6.66^{c}	0
	'Rizki'	0	6.66^{c}	6.66^{c}	20^{b}	60^{a}	0
	'Arifi'	0	53.33^{a}	26.66^{b}	33^{b}	10.05^{c}	0
	'Zahour'	0	40^{a}	26.66^{b}	26.66^{b}	6.66^{c}	0
Control	'Garbanzo'	100	0	0	0	0	0
	'Farihane'	100	0	0	0	0	0
	'Moubarak'	100	0	0	0	0	0
	'Douyet'	100	0	0	0	0	0
	'Rizki'	100	0	0	0	0	0
	'Arifi'	100	0	0	0	0	0
	'Zahour'	100	0	0	0	0	0

Two results followed by the same letter are not significantly different at the 5% level according to the PPDS test

In summary, the analysis of this parameter made it possible to identify two varieties with different behavior: the Mubarak variety most sensitive to isolate FS1 became the least sensitive with FS2 and 'Rizki' moderately sensitive to FS1 became the most sensitive to FS2.

The values of the root rot index are according to the association varieties isolates of *Fusarium*. This parameter is too high in 'Rizki' (93%) and 'Zahour' (90%), associated respectively with FS2 and FS1. But, it is about 65 ± 1% in Mubarak and 'Rizki' inoculated respectively by FS2 and FS1**.** On the other hand, in the other combinations, the values relating to the root rot index are very close to around 80 ± 04% (Figure 12).

In general, with the exception of the 'Douyet' variety, the percentages of disease incidence are very similar to those of the root rot index (± 10%); some associations are identical. But for the 'Douyet' variety, the most marked difference is observed in the presence of FS2 (Figure 13).

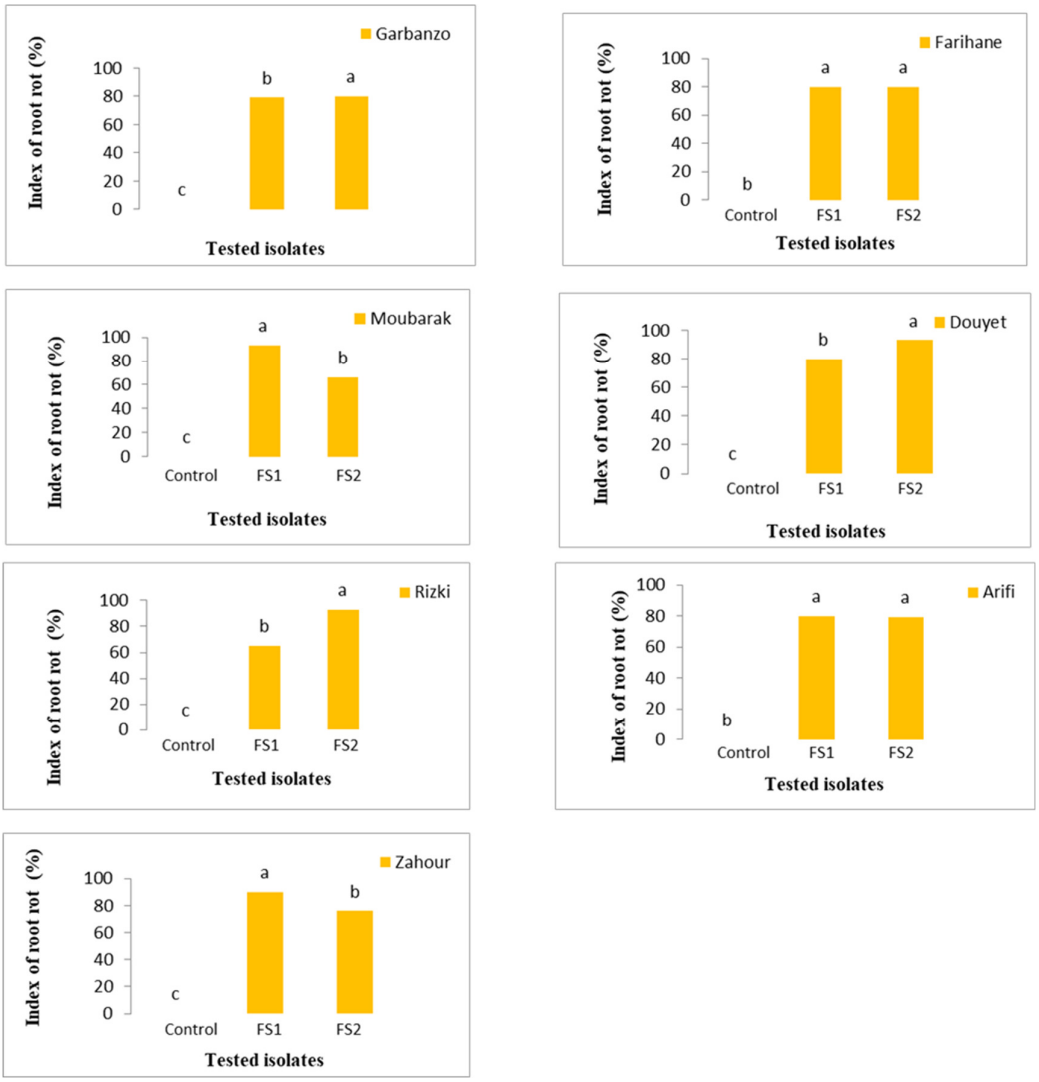

Figure 12. Root rot index (%) on different varieties of chickpeas inoculated with two isolates of *Fusarium* (FS1 and FS2)

Two results followed by the same letter are not significantly different at the 5% level according to the PPDS test

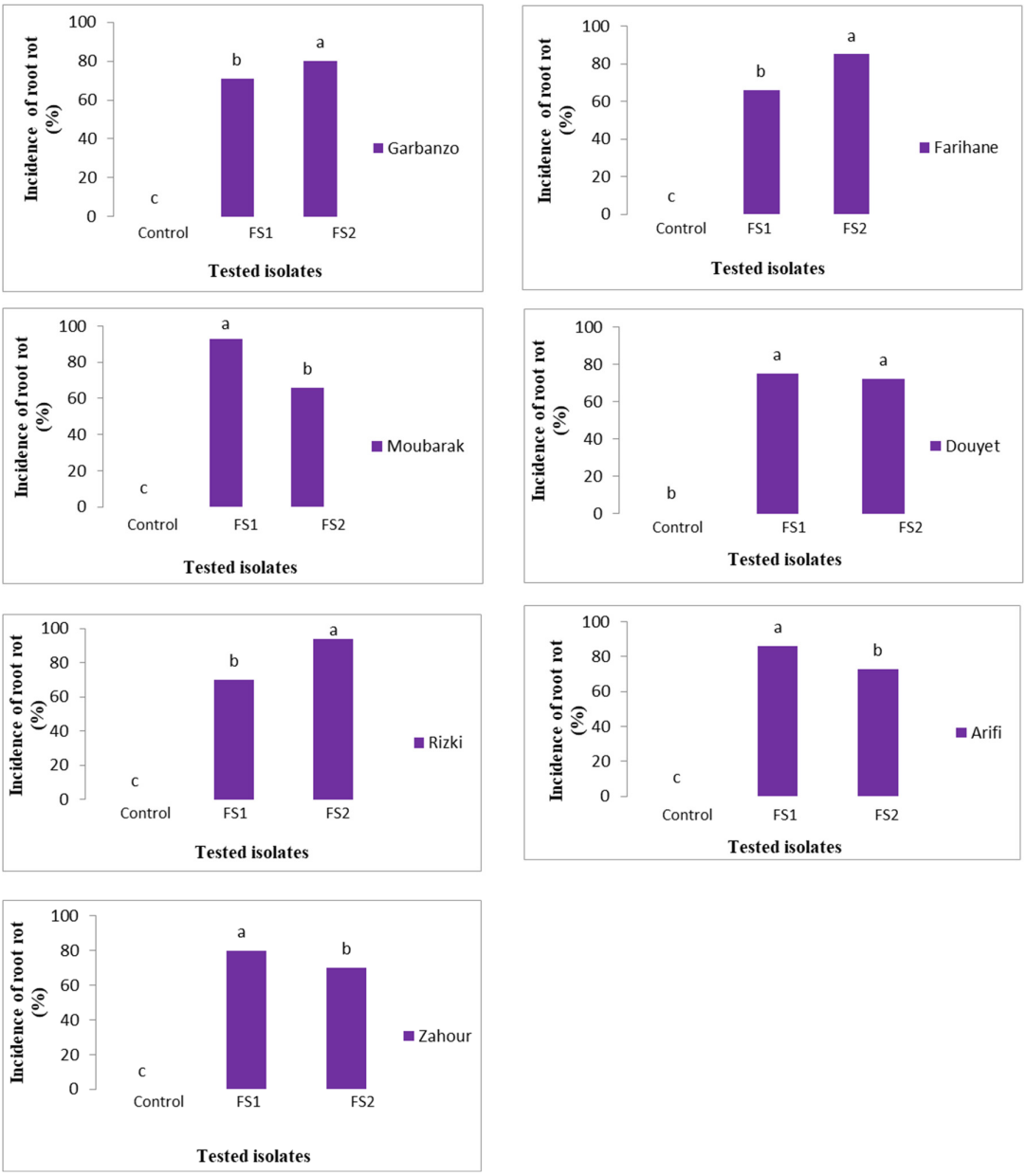

Figure 13. Incidence of root rot (%) on different varieties of chickpeas inoculated with two *Fusarium* isolates (FS1 and FS2)

Two results followed by the same letter are not significantly different at the 5% level according to the PPDS test

In comparison with the negative controls, the results of the re-isolations carried out from different organs (petioles, crowns, stems and roots) of the plants of the varieties inoculated with the two *Fusarium* isolates tested were positive (Table 5). This shows that the two pathogenic isolates of *Fusarium* were able to progress from the root to the aerial part.

In general, the percentages of re-isolation of the two isolates FS1 and FS2 from the roots and stems are between 90% and 100 %, in 'Mubarak', 'Garbanzo' and 'Zahour', and 'Rizki' inoculated respectively with FS1 and FS2. On the other hand, at the level of the collar and the petioles, the percentages of re-isolation range between 68.66%, as minimum value, and 93.33%, as a maximum value, according to the type of association: varieties X isolates of *Fusarium*.

Table 5. Percentage of re-isolation of different Fusarium isolates from different chickpea plant organs

Parts of plants	Isolates	Varieties						
		'Garbanzo'	'Farihane'	'Moubarak'	'Douyet'	'Rizki'	'Arifi'	'Zahour'
Petiole	FS1	71.66^{bc}	68.66^{c}	83.33^{ab}	81.66^{ab}	78.66^{abc}	80.00^{abc}	78.33^{abc}
	FS2	76.66^{ab}	71.66^{bc}	70.00^{c}	75.33^{ab}	86.33^{a}	83.33^{ab}	76.66^{ab}
	T	0	0	0	0	0	0	0
Collar	FS1	73.33^{a}	71.66^{c}	83.83^{a}	83.33^{a}	76.66^{a}	81.66^{a}	76.66^{a}
	FS2	71.66^{ab}	78.66^{ab}	68.66^{b}	81.66^{ab}	83.33^{a}	80.00^{ab}	78.33^{ab}
	T	0	0	0	0	0	0	0
Stem	FS1	100^{a}	88.33^{b}	100^{y}	93.33^{ab}	96.66^{b}	96.66^{ab}	100^{a}
	FS2	95.33^{ab}	85.33^{b}	92.00^{ab}	96.66^{ab}	100^{y}	93.66^{a}	96.66^{ab}
	T	0	0	0	0	0	0	0
Root	FS1	100^{y}	88.33^{c}	100^{a}	93.33^{abc}	96.66^{abc}	96.66^{abc}	100^{a}
	FS2	95.33^{ab}	92.00^{ab}	96.66^{ab}	85.33^{b}	100^{y}	93.66^{ab}	96.66^{ab}
	T	0	0	0	0	0	0	0

Two results followed by the same letter are not significantly different at the 5% level according to the PPDS test.

Discussion

The pathogenicity of two *Fusarium* isolated from diseased chickpea plants from different Moroccan regions was tested on seven chickpea varieties ('Garbanzo', 'Farihane', 'Moubarak', 'Douyet', 'Rizki', 'Arifi', and 'Zahour').

According to the in vitro test, the germination of the inoculated seeds is altered by the isolates studied, showing variability depending on the combination: variety X fungal isolate.

Also, our results showed that all measured and evaluated parameters were affected. This is due to root necrosis induced by the two pathogenic isolates; which subsequently results in blackish lesions on the roots and the collar of the plants of each variety. In addition, the symptoms of yellowing and wilting associated with this root necrosis progress from the bottom to the top of the plant, with the development of secondary roots. Incidentally, these symptoms appear late with a sudden wilting of the leaves, accompanied by an appearance of pale color compared to healthy plants. However, these symptoms can appear in certain plant species at the seedling stage. In this case, the affected leaves show flaccidity followed by a dull green color and drying leading to the early death of the plant. This typical wilting reported by other works (Boureghda, 2009; Halila *et al*., 2014; Jendoubi *et al*., 2017), was observed in some plants of the present work.

These results are in agreement with those obtained by several authors (Navas-Cortes *et al*., 1998; Jimenez-Gasco and Jimenez-Diaz, 2003; Dubey and Singh, 2004; Jimenez-Gasco *et al*., 2004; Honnareddy and Dubey, 2006; Bekkar, 2007; Pande*et al*., 2007; Raju*et al*., 2008; Shah *et al*., 2009). The same symptoms have also been observed in several plots of the world (Westerlund *et al*., 1974), notably in California (Koike *et al*., 2009) in India (Trapero-Casas and Jimenez-Diaz, 1985), in Spain, (El- Aoufir, 2001; Trapero-Casas and Jimenez-Diaz, 1985) in Tunisia (Halila *et al*., 2010), and in Algeria (Zemouli-Benfreha *et al*., 2014).

Indeed, Jeon *et al*. (2013), found that *F. oxysporum* and *F. solani* were able to cause severe wilts and root rots in *Platycodon grandiflorus*. The same root rot caused by *F. solani* in this study is similar to that observed in okra (Rahim *et al*., 1992; Fayaz *et al*., 2014). According to Messiaen and Lafon (1991), *Fusarium* species are the most widespread soil-borne pathogenic microorganisms of crops such as tomato (Mc Govern, 2015), radish (Lee *et al*., 2021), peas (Michielse and Rep, 2009), lettuce (Mbofung and Pryor, 2010), leading to considerable crop losses. Generally speaking, these losses may be a consequence of destructive vascular wilts (Bodah, 2017)

and/or browning of the vessels and yellowing of the leaves resulting in complete drying of the plants (Jendoubi *et al*., 2017).

From a physiological point of view, all these symptoms observed are only the result of the interaction of several inhibitory mechanisms, which are exerted, upon infection, by the pathogenic agent on its host. Along the same lines, Gupta *et al*. (1986) showed that the infection begins with the germination of chlamydospores. Afterwards, the germ tube enters through the epidermis of the root system inducing root rot, which is due to the secretion of pectolite enzymes (Aboul-Soud *et al*., 2004; Halila *et al*., 2009; Cunnington *et al*., 2007). Generally, after migrating to the upper parts of the plant (Aboul-Soud *et al*., 2004), the pathogen mycelium branches to xylem vessels to produce microconidia (Cunnington *et al*., 2007; Halila *et al*., 2009; Aboul-Soud *et al*., 2004). Afterward, these microconidia detach and will be transported into the vascular system via the flow of transpiration (Aboul-Soud *et al*., 2004). Once at the level of the aerial organs, the microconidia germinate producing mycelia, which will penetrate the walls of the adjacent vessels and thus become systemic in the tissues of the host (Cunnington *et al*., 2007). Therefore, the inhibition of water supply observed in infected plants is due to the closure or clogging of conductive vessels following the germination of microconidia (Halila *et al*., 2009; Cunnington *et al*., 2007; Aboul-Soud *et al*., 2004). Also, Sharma and Muehlbauer (2007), reported that *Fusarium* blocks or reduces the passage of water and nutrients to the leaves, resulting in vascular wilt. So, it is this restriction in water supply that will trigger the closure of the stomata; which will be followed by the withering of the leaves ending in the complete death of the plant.

Fusarium oxysporum f.sp *ciceris* may survive in soil and on crop residues as chlamydospores for up to six years in the absence of host plant and spread by means of both soil and infected seeds (Haware *et al*., 1996).

Also, it is important to note that this reduction, of different agronomic parameters caused by the two isolates of *Fusarium* in the seven varieties studied, was observed in the presence of other phytopathogenic isolates of the same genus on other crops by Nirina *et al*. (2014) (tomato, cucumber, and bean) and by Sghir *et al*., 2016 (tomato and eggplant). Thus, a genotype showing high resistance in one year may become moderately resistant in another year solely due to variation in race distribution (Sharma *et al*., 2019) and other weather parameters like high soil temperature (> 25 °C) and less soil moisture (Rafiq *et al*., 2020).

The analysis of the results relating to the growth and the vegetative development of the plants of seven varieties of chickpea, made it possible to identify two varieties with different behavior opposite the two *Fusarium* isolates tested: Mubarak is relatively the most sensitive to the FS1 isolate and 'Rizki' is the most susceptible to the FS2 isolate. It also seems that the inhibitory action of these two pathogenic isolates is exerted more on:

- The number of leaves than that of flowers, indicating a more marked effect on the activity of the apical meristem of the plant;
- The filling of the pods only on their formation by affecting the surface and the photosynthetic activity of the leaves.

Conclusions

In conclusion, all the isolates turn out to be aggressive, with a degree of aggressiveness which differs from one variety to another, thus resulting in considerable losses. Therefore, it is essential to develop means of preventive and curative control suitable for this crop in order to minimize the damage and subsequently improve the yield and quality of the chickpea crop.

Authors' Contributions

All authors contributed to manuscript preparation: laboratory works, manuscript writing, identification of the fungus, translation of the manuscript and results analysing.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

Acknowledgements

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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