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MOLECULAR IDENTIFICATION AND INTEGRATED MANAGEMENT OF THE *Fusarium* f. sp. *cucumerinum* THE CAUSAL AGENT OF *Fusarium* WILT DISEASE OF *Cucumis sativus* L. IN IRAQ

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Fusarium oxysporum f. sp. *cucumerinum*

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Trichoderma viride

Bacillus pumilus

Salicylic acid

Cucumber

PCR

ABSTRACT

This study was aimed to isolate and identify the phytopathogenic fungus *Fusarium oxysporum* f. sp. *cucumerinum* (Foc) the causal agent of the *Fusarium* vascular wilt disease of cucumber in the middle and south of Iraq. The results of study indicated the presence of the pathogen with percentage of appearance 71.43% with frequency 46.43%. Molecular identification carried out using polymerase chain reaction technique indicated the presence 18 isolates of Foc pathogenic group I and 6 isolate of Foc pathogenic group II. Among these, the isolate Foc-b3 was most virulent under *in vitro* pathogenicity test on the cucumber seed and it exhibited 0% seed germination compared with control. An attempt of integrated management of *F. oxysporum* f. sp. *cucumerinum* under *in vitro* conditions was also carried out in present study and a significant inhibition were reported on the application of various bioagents such as *Chaetomium cupreum* (Cc), *Trichoderma viride* (Tv) and *Bacillus pumilus* (Bp) on the potato dextrose agar (PDA). Further, under greenhouse conditions, combined application (tetra inoculums) of Cc+Tv+Bp+ Salicylic acid (Sa) was significantly reduced the percentage of the disease incidence and severity up to 0% as compared with the control where it was reported 97.50%, 75.72% respectively. Effect of this combination was also reported on the dry weight of the plants and significant improvement (0.975 gm/plant) was reported as compared to control (0.098 gm/plant).

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1 Introduction

Cucumber (*Cucumis sativus* L.) is an important vegetable crop throughout the world; it has similar importance in Iraq also (Saeed et al., 2015). Crop grown in various part of the country, and the area of cultivation reached 92.887.00 ha with total production of 405.610.00 ton in 2013 (FAO, 2015). In Iraq, crop production is affected by various physical and biological factors, among these serious economic losses was caused by *Fusarium* vascular wilt disease which is caused by the *Fusarium oxysporum* (Schlechtend.:Fr.) f. sp. *cucumerinum* (Owen) Snyder & Hansen (Armstrong & Armstrong, 1978). Foc is a host specific pathogen (Armstrong et al., 1978) and widely distributed in many cucumber grown areas such as South Africa, Japan, Thailand, England, France, Germany and Australasia (Booth, 1971; Plantwise, 2015). This disease was recorded first time on the cucumber in 1951 (Zitter et al., 1996) while In Iraq, it was first time recorded in the province of Wasit in 2012 (Risan, 2012). Symptoms appear typically on the plant as foliar wilting, necrotic lesions of the stem base followed by vascular discoloration on the roots and stem (Vakalounakis, 1988; Vakalounakis et al., 2004). Three physiological races of the Foc viz 1, 2 and 3 have been identified (Armstrong et al., 1978). Information regarding molecular identification and characterization are in scarcity especially in context of Iraq.

Disease management is a major problem for cultivars; various chemical, physical, biological and cultural practices are carried out for the management of this disease. Among these, biological control offers an eco-friendly means of plant diseases management. Strategies of using beneficial microorganisms for controlling plant pathogens help in reducing the negative impacts of the chemicals on the environment and human health and it also cost effective (Higa, 1991; Parr & Hornick, 1994). Various species such as *Bacillus*, *Pseudomonas*, *Chaetomium*, *Trichoderma* and *Penicillium* have been used widely in plant disease controlling (Vessey, 2003). *Trichoderma* species are widely used as biocontrol agents against plant pathogens since 1920s (Harman, 2006). Species belong to *Chaetomium* are normally found in the soil and organic compost, and has potential of cellulose and other organic materials degradation, species such as *C. cupreum* and *C. globosum* also has been proved to be good antagonistic agents against various plant pathogens (Soytong et al. 2001). Role of phytohormones such as Salicylic acid, Jasmonic acid, Absciscic acid and ethylene in inducing systemic resistance in plants against the broad range of the pathogens through activating plant enzymes such as peroxidase (POD) and polyphenol oxidase (PPO) has been well documented (War et al., 2011). This study was amid to investigate the occurrence of *F. oxysporum* f. sp. *cucumerinum* (Foc) in the middle and south of Iraq, isolated pathogens were identified by molecular

identification technique. Further, effort of disease management by using eco-friendly means by using biocontrol agents *C. cupreum*, *T. viride*, *B. pumilus* and plant growth regulator Salicylic acid was also attempt.

2 Materials and methods

2.1 Sampling, isolation and identification

Samples were collected from various cucumber grown areas of Baghdad, Babil and Karbala provinces of Iraq during the growing season in 2014-2015. Infected samples were collected from rhizosphere and basal segment of the stem. Collected samples were washed by 1% Hypochlorite sodium for 1 min and cut off in to 0.5 cm, disinfected sample were rinsed twice in sterile distilled water and dried. Four dried samples were incubated in laminar flow bench on Petri plat containing sterilized PDA (CDH Bioscience Co., India) and incubated at 25±2 °C for 7 days. Fungus grown around the infected segments were transferred individually on the separate PDA and incubated for 7 days at same temperature (Hussein & Juber, 2015). Fungal isolates were identified according to the morphological characteristics described by (Booth, 1971; Booth 1977; Ellis, 1971; Domsch et al., 2007). Percentage of the appearance and frequency of the fungi calculated according to method of Hussein & Juber (2014).

2.2 Identification of *F. oxysporum* f. sp. *cucumerinum* using specific polymerase chain reaction (PCR) assays

2.2.1 DNA extraction

DNA of the initially isolated 31 fungal isolates which basically identified as *F. oxysporum* based on the cultural and microscopic morphology characteristics were extracted using the fungal DNA extraction kit (EZ-10 Spin Column Fungal Genomic DNA Mini-Preps Kit, Bio Basic Inc., Canada) according to the manufacture instructions. For this all the isolates were purified by using single spore technique according to the method given by Hussein & Juber (2015). Mycelium with spores were harvested by sterilized glass rod from each isolate individually and transferred into 1.5 ml clean eppendorf tubes and kept in -20 °C until DNA extraction process were established.

2.2.2 Primers preparation

Four specific primers used to identify *F. oxysporum* f. sp. *cucumerinum* pathogenic group I, II, III and IV (Bioneer Co., South Korea) (Table 1). Primers were diluted individually by adding sterile distilled water according to the company instructions to obtain the concentration of 100 pmol/μl and stored at -20 °C until uses.

Table 1 Specific primers and amplification conditions used in the PCR process.

primer of pathogenic group	Sequence (5'→3')	Annealing temp./ cycles
I	F: AGGTGACCGTCGTAGCAAG R: AGGTGACCGTTGAGAGGA	54 °C/ 25
II	F: GGAGGGTGTGGAGATGC R: GCAGGGTGTACTCCAGGCAC	61 °C/ 35
III	F: GATGACCGCCAATGTGTACT R: GATGACCGCCGACCTTGGCCG	62 °C/ 25
IV	F: GGAGGGTGTACAATCACAAT R: GGAGGGTGTAAAGCATTC	55 °C/ 25

F= Forward, R= Reverse (Najafiniya & Sharma ,2011)

2.2.3 Polymerase chain reaction (PCR) assays

Extracted fungal DNA were amplified using standard master mix (AccuPower PreMix series, Bioneer Co., South Korea) in the PCR experiments, reaction mixture consist of 250 µM each dNTP, 1.5 mM MgCl₂, 30 mM KCL, 10 Mm Tris-HCL (pH 9.0), 1 U Taq DNA polymerase, 1.5 µl of each primer, 3µl DNA template in concentration of 50 ng/µl and 14 µl sterile distilled water in eppendorf tube 0.2 ml, negative control were performed in all the experiments which was DNA template replaced with sterile distilled water, the quality and concentration of the DNA were tested using spectrophotometer (NanoSpec Cube, Nanolytic, Germany) at the wavelength 260/280 nm (Maniatis et al., 1982). Samples were enrolled into the Thermocycler (Model 480, Perkin-Elmer Co., USA), amplification program have been organized for each primer in similar conditions except annealing temperature step which was specific to each primer particular (Table 1) according to the method of Najafiniya & Sharma (2011), each program started with 1 initial denaturation cycle at 95°C for 4 min, followed by 25 cycles except with primer of pathogenic group II where it was 35 cycles, each cycle consist of denaturation step at 95°C for 1 min, annealing step for 1 min at 54 °C, 61 °C, 62 °C, 55 °C for the primer of pathogenic group I, II, III, IV respectively, and an extension step at 72°C for 2 min, followed by 1 cycle final extension step at 72°C for 7 min.

2.2.4 Gel electrophoresis

Amplification products were separated on the 1.5% agarose gel (Bio Basic Inc., Canada) by adding 1 gm agarose to 100 ml TBE buffer (Tris-Borate-EDTA, 10x solution, Bio Basic Inc., Canada) which is consist of 0.089 M Tris Base, 0.189 M Boric Acid, 0.112 M EDTA after diluted to 1x by adding 10 ml TBE buffer to 90 ml sterile distilled water and poured into agarose electrophoresis kit (Model AE-6100, ATTO Co., Japan), TBE buffer x1 concentration 1:10 (TBE: sterile distilled water) added to on the solid agarose gel in the agarose EP kit, 10 µl of PCR products for each isolate individually and 2 µl of green DNA dye (Bio Basic Inc., Canada) mixed and loaded into the holes of the agarose gel, 1 µl of the 1 kb DNA ladder marker (Thermo Scientific, USA) mixed gently with 1

µl 6x DNA loading dye (Thermo Scientific, USA) and 4 µl deionized water were loaded into the hole of the agarose gel as well as negative control. The gels were visualized under U.V. light using U.V. Transilluminator and Jell documentation system (Cleaver, UK).

2.3 Pathogenicity test

In-vitro pathogenicity test was conducted for the 31 isolated and purified fungal isolates of *F. oxysporum*, using filter paper technique. For this spore suspension were prepared by pouring 10 ml of the sterile distilled water into fungal mycelium containing petri plate; mycelia were scraped by sterile glass rod and pour the liquid from the plate into the cheese cloth placed folded over a sterile conical flask. The spore suspension was prepared at 5x10⁴ spore concentration by using sterile water and hemocytometer. Seeds of cucumber cultivar Green Paris (Vilmorin/ France) uncoated with fungicide were surface sterilized and 10 seeds were transferred into each petri plate (9 cm diameter) containing 5 layers of filter paper (9 cm diameter), each plate were watered with 1ml spore suspension and 3 ml sterile distilled water. All the plates were incubated at 25 °C for 14 days and watered as needed, each treatment has 4 replicates. Percentage of the seed germination was calculated according to the formula below:

$$\text{The Percentage of germination} = \frac{\text{No.of seeds germinated}}{\text{Total No.of seeds}} \times 100$$

2.4 In vitro antagonism assay

Antagonistic activity was conducted on the PDA medium by using dual culture technique with biocontrol fungi *C. cupreum*, *T. viride* and *B. pumilus* against the fungal isolate Foc-b3 according to the method of Lamsal et al. (2012). Among these, *C. cupreum* and *B. pumilus* was obtained from previous study while the fungus *T. viride* which obtained from Ministry of Science and Technology, Department of Agricultural Research, Iraq. A disc of 0.5 cm of PDA containing 7 days old pathogen was inculcated on the dual PDA plate (9 cm diameter) about 2 cm apart from the plate edge and similar dice size of the bioagents Tv and Cc and 20 µl of the bacteria Bp

was inoculated individually on the opposite side of the plate about 2 cm apart from the plate edge, separate plates of PDA were amended as a control containing a disc of the pathogen only, all the plates were incubated at 28 °C for 10 days. Inhibition effect was calculated by measuring the distance of the fungal growth radius of the pathogen toward the bioagents compared with the control according to the following formula:

$$\text{Inhibition (\%)} = \frac{R-r}{R} \times 100$$

(Lamsal et al., 2012)

Where: R = is the radius of the pathogen in control plate, r = is the radius of the pathogen in dual culture plate.

2.5 Management of *Fusarium* wilt disease of cucumber under greenhouse conditions

Greenhouse study was conducted to evaluate the disease suppression and plant growth ability of selected bioagents Cc, Tv, Bp and plant growth elicitor Sa (Merck Millipore Co., Germany) either individually or in combination. Inoculum of the fungal isolates of Cc, Tv and Foc-b3 were prepared by adding 5 disc (0.5 cm) of 7 days old fungal culture to the conical flask (500 ml) containing 200 gm autoclaved millet seeds (*Pennisetum glaucum*) and incubated at 25 °C for 14 days. Inoculum of the Bp was prepared on sterilized nutrient broth medium and incubated at 30 °C for 7 days with constant shaking, spore density amended to 10⁸ CFU. ml⁻¹ by hemocytometer, while Sa solution was prepared at the concentration of 150 µg.ml⁻¹. Mixture of the autoclaved soil and compost (2:1) were filled into the plastic pots (13 cm diameter), inoculum of the pathogen was added as 1% (w/w) to the pots and watered, three days after inoculation, pots were planted with 10 seeds of the cucumber, in the same time 40 ml/pot of the Bp inoculum was added as soil drench, inoculum of Cc and Tv added as 1% (w/w), the Sa solution was soil drenched as 40 ml/pot after 10 days of seeds planting. Pots are arranged according to the completely randomized design (CRD) with 4 replicates in the greenhouse and watered as needed, percentage of seed germination calculated after 10 days. Percentage of disease incidence was calculated after 8 weeks of seed planting according to the formulas described by

Masood et al. (2010). Disease severity was estimated by using the score-rating chart below which is described by Schneider (1984) with simple modification: 0= No symptoms, 1= mild vascular discoloration in roots, 2= extensive vascular discoloration in roots, 3= mild discoloration in crown, 4= extensive discoloration in crown, 5= mild disintegration of crown and roots tissue, 6= extensive disintegration of crown and roots tissue, and 7= wilted or plant dead. The Percentage of disease severity index (DSI) was calculated by using the formula described by Grau & Radke (1982). The dry weights of plants were also recorded during the study.

3 Results and Discussion

3.1 Isolation and identification

Results of the isolation study along with association number of fungi were indicated in (table 2). Among various isolated pathogenic fungi, *F. oxysporum* was predominant, and it exhibited 71.43%, 46.43% percentage of appearance and frequency respectively. Morphological characteristic of 7 days old Foc was white colony with a purple tinge, reverse pale purple pigment on the bottom of the plate, floccose texture, diameter ranged 4.5 - 5cm. The fungus formed small microconidia with oval shaped, ellipsoid, cylindrical, straight to curved with 1- 2 septate born on the simple phialides arising laterally on the hyphae or from short sparsely branched conidiophores, thin-walled macroconidia born on the branched conidiophores appeared as fusoid shaped with a definite foot cell with 3-5 septate, whilst terminal or intercalary chlamydospores were formed rarely with rough walled arranged solitary or in chains (Booth, 1971 ; Compbell et al., 2013).

3.2 Identification of *F. oxysporum* f. sp. *cucumerinum* using PCR assay

The PCR analyses of *F. oxysporum* were demonstrated that 24 isolates were belong to special form of *F. oxysporum* f. sp. *cucumerinum* (table 3), among these 18 isolates were observed to amplify fragments with primer of the pathogenic group I and, their bands shown at 1.320 kb (Fig. 1).

Table 2 Fungi associated with the samples isolated from the collected root and shoot samples.

Fungus name	Appearance (%)	Frequency (%)
<i>Aspergillus niger</i> Van Tieghem	42.86	15.63
<i>Cladosporium cladosporioides</i> (Fresen.) G.A. de Vries	28.57	18.30
<i>Fusarium oxysporum</i> Schlesht	71.43	46.43
<i>Fusarium solani</i> (Mart.) Sacc.	21.43	10.71
<i>Pencillium</i> sp.	35.71	20.54
<i>Phoma</i> sp.	14.29	4.91
<i>Rhizctonia solani</i> (Kuhn)	7.14	2.23
<i>Rhizopus</i> sp.	28.57	13.39
<i>Ulocladium atrum</i> Preuss	14.29	3.13

Table 3 Detection pathogenic group I, II, III and IV of *F. oxysporum* f. sp. *cucumerinum* using specific primers.

Isolate	Primer				Isolate	Primer			
	I	II	III	IV		I	II	III	IV
Control	-	-	-	-	Foc-b9	+	-	-	-
Foc-g1	+	-	-	-	Foc-b10	-	+	-	-
Foc-g2	+	-	-	-	Foc-b11	+	-	-	-
Foc-g3	-	+	-	-	Foc-b12	+	-	-	-
Foc-g4	+	-	-	-	Foc-b13	-	-	-	-
Foc-g5	+	-	-	-	Foc-k1	-	-	-	-
Foc-g6	+	-	-	-	Foc-k2	+	-	-	-
Foc-g7	+	-	-	-	Foc-k3	-	-	-	-
Foc-b1	+	-	-	-	Foc-k4	-	-	-	-
Foc-b2	-	-	-	-	Foc-k5	-	+	-	-
Foc-b3	+	-	-	-	Foc-k6	+	-	-	-
Foc-b4	+	-	-	-	Foc-k7	-	-	-	-
Foc-b5	+	-	-	-	Foc-k8	+	-	-	-
Foc-b6	-	+	-	-	Foc-k9	-	-	-	-
Foc-b7	-	+	-	-	Foc-k10	-	+	-	-
Foc-b8	+	-	-	-	Foc-k11	+	-	-	-

Foc-1g - Foc-7g = Baghdad, Foc-1b - Foc-13b = Babil, Foc-1k - Foc-11k = Karbala

Figure 1 Gel electrophoresis of the amplification product from PCR using genomic DNA of *F. oxysporum* f. sp. *cucumerinum* isolates using specific primers.

A. Primer of pathogenic group I, B. Primer of pathogenic group II, M. DNA ladder marker, C. Negative control.

Table 4 Pathogenicity effect of *F. oxysporum* isolates to cucumber seeds.

Isolate	Germination (%)	Isolate	Germination (%)
Control	100.0	Foc-b9	30.0
Foc-g1	40.0	Foc-b10	22.5
Foc-g2	35.0	Foc-b11	30.0
Foc-g3	32.5	Foc-b12	22.5
Foc-g4	37.5	Foc-b13	60.0
Foc-g5	20.0	Foc-k1	65.0
Foc-g6	35.0	Foc-k2	22.5
Foc-g7	22.5	Foc-k3	45.0
Foc-b1	20.0	Foc-k4	47.5
Foc-b2	47.5	Foc-k5	35.0
Foc-b3	5.0	Foc-k6	32.5
Foc-b4	27.5	Foc-k7	57.5
Foc-b5	15.0	Foc-k8	25.0
Foc-b6	17.5	Foc-k9	52.5
Foc-b7	42.5	Foc-k10	15.0
Foc-b8	40.0	Foc-k11	40.0
SEM Value = 1.7			
LSD (0.05) = 3.4			

Each number reflects 4 replicates.

Six isolates amplified with primer of the pathogenic group II and, their bands were shown at 0.770, while 7 isolates did not amplified with any of the four primers, it may be due to be these isolates belonging to other form or due to technical error.

3.3 Pathogenicity test

The 31 isolates of *F. oxysporum* showed variation in their pathogenicity, while the percentage of the seed germination was reported between 5 - 65% as compared to the control which shows 100% germination (table 4). Among all the tested isolates, Foc-b3 inhibit maximum seed germination and exhibited 5% seed germination. Further, isolates belong to the province Babil showed higher pathogenicity than other two provinces; this variation in the pathogenic ability between the isolates may be refer to the genetic variation between races of the fungus or may be due to the geographical distribution, properties of the soil and environmental conditions.

3.4 *In vitro* antagonism assay

All the bioagents were effectively inhibited the mycelial growth of the pathogen on the PDA. *In vitro* comparison with the control (fungus alone) which filled the petri plate after 10 days of incubation (table 5), the bioagent Tv was superior which exhibited 82.69% percentage of inhibition this inhibition was followed by Cc and Bp where they exhibited 68.85%, 66.92% inhibition respectively (Fig. 2). These results are in agreement with Fitsum et al. (2014) who obtained high percentage of inhibition 80.39% in dual culture plate of *T. viride* against *Colletotrichum lindemuthianum* *in vitro*.

Table 5 Inhibition effect of the bioagents against *F. oxysporum* *in vitro*.

Treatment	Inhibition (%)
Control	0.0
Foc-b3 + TV	82.69
Foc-b3 + Cc	68.85
Foc-b3 + Bp	66.92
LSD (0.05) = 0.65	

Foc-b3= *F. oxysporum* f. sp. *cucumerinum*, Tv= *T. viride*, Cc= *C. cupreum*, Bp= *B. pumilus*
Each number reflects 4 replicates.

3.5 Management the *Fusarium* wilt disease of cucumber under greenhouse conditions

The results indicated that all of the treatments used in this experiment were positively increased the percentage of the seed germination, while the percentage ranged between 75-100% compared with the negative control (Pathogen alone) which shows only 52.5% seed germination (table 6). Disease management ability of various treatments under greenhouse conditions varies and superiority was shown by the tetra inoculum containing Tv+ Cc+ Bp+ Sa, treatment significantly reduced the percentage of disease incidence and severity up to 0% as compared with the negative control which shows 97.50% & 72.75% respectively (Figure 3).

This combination was followed by the triinoculum of Tv+ Bp+ Cc which exhibited 32.50%, 26.50% disease incidence and severity respectively. All of the treatments increased the dry weigh of the plants significantly, among these tetra inoculum shows superiority over the other treatments and exhibited 0.975 gm/plant compared with the negative control which was 0.098 gm/plant. These results are agreed with the results of other studies, such as Soyong & Soyong (1997) where they found that the tomato plants treated with *C. cupreum* in the form of mycofungicide completely prevented the damages caused by *F. oxysporum* in Thailand. Similar types of results were obtained by John et al. (2010) those who reported that *T. viride* is an effective bioagent against the plant pathogens *F. oxysporum* and *Pythium arrhenomanes* in soybean trial. Further, they reported that *T. viride* not only controlled the pathogens but also simultaneously increased the plant growth and modified the rhizosphere of soybean plant.

The bioagents *C. cupreum* and *T. viride* suppressed the pathogen through several mechanisms including mycoparasitism, antibiosis and competition for space and nutrient resources. The mycoparasitism activity of the *T. viride* inducing its growth toward the pathogens, it creates coiling on the target fungi and induced inactivation of the pathogen's enzymes by producing several antifungal extracellular enzymes such as protease.

Figure 2 Inhibition effect of the bioagents against *F. oxysporum* using dual culture method.

A.) Tv+ Foc-b3 , B.) Cc+Foc-b3 , C.) Bp+Foc-b3

Further it was also reported that enzymes secreted from *T. viride* suppressed the enzymes produced by pathogens i.e. chitinase and glucanase. These biocontrol agents, also playing a big role in rhizosphere modification, biofertilization and stimulation of plant defense mechanisms (Harman, 2006). The soil bacteria *B. pumilus* also inhabiting in the rhizosphere microbial growth and acting as one of the PGPR group (Hussein & Juber, 2015). This bacteria involved in promoting plant growth directly or indirectly through production of several regulatory chemicals in the rhizosphere area. The

PGPR generally enhance the plant growth directly through amending plant hormone or facilitate by resources such as phosphorus and nitrogen acquisition, and indirectly by the forms of biological control agents through suppression negative impact of the phytopathogens on plant growth development (Ahemed & kibret, 2014). The phytohormone Salicylic acid is also performing an essential role in plant growth regulation and plant's reaction to the biotic and abiotic stresses by involving locally and systemically induced resistance (Halim et al. 2006).

Table 6 Effect of the bioagents on the *Fusarium* wilt disease of the cucumber under greenhouse conditions.

No.	Treatment	Germination (%)	Incidence (%)	Severity (%)	Dry weight (gm/plant)
1	Control	100.0	0.0	0.00	0.396
2	Foc-3b + Tv	80.0	60.0	51.25	0.301
3	Foc-3b + Cc	77.5	67.5	56.50	0.230
4	Foc-3b + Bp	82.5	67.5	50.75	0.290
5	Foc-3b + Sa	75.0	70.0	62.00	0.129
6	Foc-3b + Tv + Cc	100.0	50.0	30.00	0.378
7	Foc-3b + Tv + Bp	100.0	52.5	41.50	0.404
8	Foc-3b + Tv + Sa	87.5	55.0	43.00	0.398
9	Foc-3b + Tv + Cc + Bp	100.0	32.5	26.50	0.562
10	Foc-3b + Tv + Cc + Sa	97.5	35.0	28.25	0.497
11	Foc-3b + Tv + Cc + Bp + Sa	100.0	0.0	0.00	0.975
12	Foc-3b + Cc + Bp	95.0	45.0	33.00	0.317
13	Foc-3b + Cc + Sa	95.0	47.5	41.25	0.249
14	Foc-3b + Bp + Sa	92.5	65.0	56.25	0.312
15	Tv	100.0	0.0	00.00	0.550
16	Cc	100.0	0.0	00.00	0.502
17	Bp	100.0	0.0	00.00	0.587
18	Sa	100.0	0.0	00.00	0.418
19	Foc-3b	52.5	97.5	72.75	0.098
	SEM Value	7.3	9.4	10.53	0.114
	LCD (0.05)	14.7	18.9	21.09	0.229

Each number reflects 4 replicates.

Figure 3 *Fusarium* wilt disease control under greenhouse condition.

A= Tetrainoculum of (Tv+ Cc+ Bp+ Sa) , B= Negative control (Foc-b3), C= 1. Tetrainoculum of (Tv+ Cc+ Bp+ Sa) , 2. Control , 3. Negative control. D= 1. Negative control , 2. Control, 3. Tetrainoculum of (Tv+ Cc+ Bp+ Sa).

Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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