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RESEARCH PAPER

Integrated management of the *Fusarium* vascular wilt disease of *Cucurbita pepo* in Iraq

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الأدارة المتكاملة لمرض الذبول الوعائي الفيوزارمي في *Cucurbita pepo* في العراق

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Abstract. Fusarium vascular wilt disease is one of the most harmful diseases that affect a broad range of plant species including zucchini (*Cucurbita pepo*). The objective of the research was to investigate the presence of the phytopathogenic fungus Fusarium oxysporum the causal agent of the disease in zucchini fields. Forty five isolates of F. oxysporum were isolated from four locations in province of Dyala during 2014-2015. Isolate Foq9 was the most virulent in the in vitro pathogenicity test. In vitro significant suppression efficiency were observed of the fungicides Topsin M 70 WP (Tm) and Tecto 500 SC (Tc) and two botanical extract solution extracted from Garlic (Gr) and Ginger (Gn) against the pathogen on the potato sucrose agar (PSA). In greenhouse experiments all of the agents decreased the percentage of disease incidence and severity significantly, while the tetra-inoculum (Tm+Tc+Gr+Gn) was superior which exhibited 0% disease incidence and severity compared to the negative control which was 95% and 79% respectively. Also the combined application of the botanical extracts (Gr+Gn) reduced disease incidence and severity significantly. All of the treatment increased plant growth criteria represented by dry weight of the plant compared to the control.

Keywords: *Fusarium oxysporum*; Garlic extract; Ginger extract; PCR; Tecto; Topsen M.

المستخلص: يعد مرض الذبول الوعائي الفيوزارمي احد اهم الامراض الضارة التي تصيب طيف واسع من النباتات ومنها القرع *Cucurbita pepo* . هدف البحث للتحري عن وجود الفطر الممرض *Fusarium oxysporum* المسبب للمرض في حقول القرع. تم عزل ٤٥ عزلة من الفطر *F. oxysporum* من ٤ مناطق مختلفة في محافظة ديالى خلال الفترة ٢٠١٤–٢٠١٥. تم اختبار امراضية العزلات الفطرية مختبرياً وكانت العزلة Foq٩ الأكثر امراضية. واظهرت نتائج التشخيص الجزيئي ان ٢٩ عزلة من العزلات الفطرية تعود للفطر *Fusarium spp*. . مختبرياً حققت معاملات المبيد الفطري الكيميائي *Topsin M* ٧٠ *WP* (*Tm* والمبيد *Tecto* ٥٠٠ *SC* (*Tc* و محاليل المستخلصات النباتية الطبيعية المستخلصة من الثوم (*Gn*) والزنجبيل (Gr) زيادة معنوية في معدل تثبيط الفطر الممرض على الوسط الزرعي أكاروز البطاطا (*PSA*). وتحت ظروف البيت الزجاجي حققت جميع العوامل المستخدمة خفضاً معنوياً في النسبة المئوية للمرض وشدته، وقد تفوقت معاملة اللقاح الرباعي (*Tm+Tc+Gr+Gn*) على باقي المعاملات إذ بلغت نسبة المرض وشدته في معاملتها ٠٪ قياساً الى معاملة المقارنة التي بلغت ٩٥٪ و ٧٩٪ على التتابع ،كما حققت معاملة تداخل المستخلصات النباتية (*Gr+Gn*) خفضاً معنوياً في نسبة المرض وشدته. كما ان جميع المعاملات حققت زيادة معنوية في معايير نمو النباتات متمثلةً بالوزن الجاف للنبات قياساً الى معاملة المقارنة.

الكلمات المفتاحية: تفاعلات البلمرة المتسلسلة، فيوزاريوم اوكسيسبوريوم، مبيد توبسن م، مبيد تيكتو، مستخلص الثوم، مستخلص الزنجبيل.

Introduction

Zucchini (Cucurbita pepo L. var. giromontina) of the family Cucurbitaceae is an important vegetative crop in Iraq and worldwide (Hassan, 1991), the total cultivated area in Iraq was 39836 ha with production of 167576 tons (CSS, 2012). This crop infected by many harmful plant pathogens and the fungus *Fusarium oxysporum,* the causal agent of the Fusarium wilt disease is one of them. Serious economic losses were observed last decade in the zucchini production area due to this pathogen. In Poland, this pathogen was observed for the first time in 2008 in the tunnel and field cultivation of infected Zucchini fields (Jamiołkowska et al., 2011). In South Korea, the pathogen was isolated for the first time from infected zucchini plants in 2013 (Choi et al., 2015). Symptoms appears as stunning, wilting of the foliage system, drying older leaves, brown discoloration in the vascular system of the plant and eventually plant death especially during the period of flowering and fruit setting (Lee and Park, 2001). Fusarium wilt disease controlled usually through crop rotation, soil fumigation or solarization, using resistance cultivars, biological control and grafting on the rootstocks of resistant host, but eventually this pathogen is not easy to control using single method of plant protection (Agel and Martyn, 2013). Application of chemical fungicides is the fastest effective means of disease control (Nicholson, 2007), some systematic fungicide such as Benomyl, Carbendazim and Thiophanate methyl proved positive efficiency against broad range of the soilborne pathogenic fungi including *Fusarium* spp. (Rajput at al., 2006; Amini and Sidovich, 2010). Botanicals (natural plant extracts) used more recently as eco-friendly means of plant disease control alternative to the chemicals, natural extraction of some plant species such as neem (*Azadirachta indica*), Ginger (*Zingiber officinale*) and garlic (*Allium sativum*) fungicidal activity against number of the plant pathogens including species belonging to the

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Table 1. Pathogenicity effect of Fusarium spp. isolates to zucchini seeds

Location	Isolate	Germination (%)	Location	Isolate	Germination (%)
	Control	100.0	Muqdadia	Fom10	35.0
Khalis	Fok1	60.0	Muqdadia	Fom11	42.5
Khalis	Fok2	22.5	Muqdadia	Fom12	60.0
Khalis	Fok3	40.0	Khanaqin	Foq1	22.5
Khalis	Fok4	37.5	Khanaqin	Foq2	17.5
Khalis	Fok5	42.5	Khanaqin	Foq3	15.0
Khalis	Fok6	65.0	Khanaqin	Foq4	42.5
Khalis	Fok7	22.5	Khanaqin	Foq5	45.0
Khalis	Fok8	20.0	Khanaqin	Foq6	47.5
Khalis	Fok9	47.5	Khanaqin	Foq7	35.0
Khalis	Fok10	65.0	Khanaqin	Foq8	32.5
Khalis	Fok11	27.5	Khanaqin	Foq9	5.0
Khalis	Fok12	22.5	Khanaqin	Foq10	25.0
Khalis	Fok13	40.0	Khanaqin	Foq11	52.5
Muqdadia	Fom1	57.5	Khanaqin	Foq12	47.5
Muqdadia	Fom2	40.0	Khanaqin	Foq13	60.0
Muqdadia	Fom3	40.0	Khanaqin	Foq14	42.5
Muqdadia	Fom4	15.0	Khanaqin	Foq15	22.5
Muqdadia	Fom5	32.5	Khanaqin	Fos1	40.0
Muqdadia	Fom6	20.0	Mindly	Fod2	30.0
Muqdadia	Fom7	30.0	Mindly	Fod3	30.0
Muqdadia	Fom8	35.0	Mindly	Fod4	47.5
Muqdadia	Fom9	22.5	Mindly	Fod5	42.5
LSD (0.05) = 2.4					

Each number is the mean of 4 replicates.

bacteria and fungi through direct inhibition activity or via enhancing plant growth and plant resistance (Sanjay and Tiku, 2009; Al.Samarrai at al., 2012). Integrated management of the plant diseases demonstrated high efficiency in controlling several plant pathogens globally, throughout using multiple strategies and tactics to maintain the pathogen under the economic injury (El Khoury and Makkouk, 2010). This study aimed to estimate the disease control ability of the both fungicides Topsin M 70 WP and Tecto 500 Sc and two botanical extracts solutions were garlic (*A. sativum*.) and ginger (*Zingiber officinale*) each agent individually and in combination against *F. oxysporum* in vitro and under greenhouse conditions.

Materials and Methods

Isolation and Identification of the Pathogen

Thirty six samples were collected from different symptomatic zucchini plants. Samples were taken from four areas in the Iraqi province of Diyala (north east of the capital Baghdad) during the growing season in 2014-2015. Segments (0.5 cm) from the root and stem of the samples were washed under running tap water for 25 min and surface disinfected with 0.5% sodium hypochloride solution for 3 min. Four segments were then placed in Petri dishes (9 cm) containing sterilized PSA mixed with 200 mg\l of the antibiotic (Amoxicillin) and incubated at 25 °C for 7 days. Each fungal growth around the segments were transferred individually on the separate PSA plate and incubated for 7 days, each isolate purified using single spore method as described in Hussein (2014). Isolates were identified morphologically based on the cultural characteristics of colony, diameter,

Table 2. Molecular identification of the *Fusarium* spp. isolates

Isolate	Result of amplification	Isolate	Result of amplification
Fok1	-	Fom11	+
Fok2	+	Fom12	-
Fok3	+	Foq1	+
Fok4	-	Foq2	-
Fok5	+	Foq3	+
Fok6	+	Foq4	+
Fok7	-	Foq5	+
Fok8	+	Foq6	-
Fok9	+	Foq7	+
Fok10	-	Foq8	-
Fok11	+	Foq9	+
Fok12	-	Foq10	+
Fok13	+	Foq11	-
Fom1	-	Foq12	+
Fom2	+	Foq13	-
Fom3	+	Foq14	+
Fom4	+	Foq15	+
Fom5	-	Fos1	-
Fom6	+	Fod2	+
Fom7	+	Fod3	+
Fom8	+	Fod4	-
Fom9	+	Fod5	+
Fom10	-		

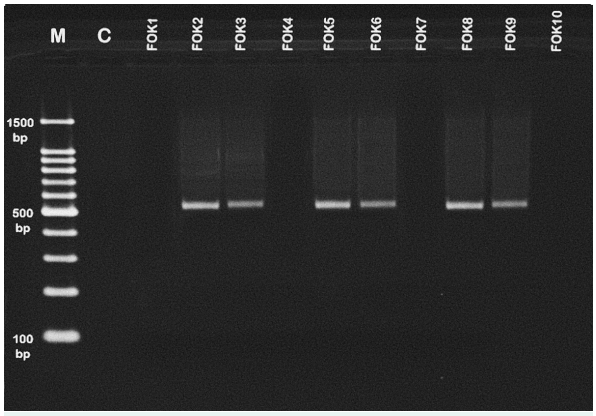

Figure 1. PCR amplification of DNA samples of Fusarium spp. using universal primers (ITS-Fu-f and ITS-Fu-r): M= DNA ladder, C= Negative control

radial growth and microscopic characteristics of conidiophores, microconidia, macroconidia and chlamydospore (Booth, 1971; Domsch et al., 2007). Identification was confirmed in the Plant Protection Department/ College of Agriculture/ University of Baghdad. The percentage of appearance and frequency was calculated using the following formulae (Hussain and Juber, 2014):

Appearance (%) = $Spec_{Occ}$ / $Sample_{T}$ x 100

In which $Spec_{Occ}$ is the samples of species occurrence) and $Sample_{T}$ the total No.of samples ,and

Frequency (%) = N_{Occ} / N_{T} x 100

Where N_{Occ} is the number of segments that showed infection and N_{T} is the total number of segments used in the experiment.

Pathogenicity test

In-vitro experiments were conducted to estimate pathogenicity ability of the 45 initially isolates of *F. oxysporum* on the seed of the zucchini cultivar Amjad. Spore suspensions of the pathogen were prepared and amended to 108 CFU/mL using hemocytometer. Ten mL of the spore suspension were added to the Petri dish containing 6 layers of the sterile filter paper (9 cm) and 10 mL-sterile distilled water, 10 seeds of the zucchini were added to each plate and incubated at 25°C for 14 days, four replicates amended for each treatment. The percentage of the seed germination was calculated according to the formula below:

Ge (%) = $Seed_{G}$/ $Seed_{T}$ x 100

Where $Seed_{G}$ is the number of seeds that germinated and $Seed_{T}$ the number of seed used in the trial (Elouaer and Hannachi, 2012).

Molecular identification assay

DNA extraction

DNA of the 45 initially isolated of *F. oxysporum* were extracted for the PCR detection, mycelium of each isolate collected individually with a sterile spatula from potato dextrose agar medium (PDA) after 7 days of incubation

Table 3. Mean inhibition effect of the fungicides and botanical extracts in vitro. (n = 4)

Treatment	Inhibition (%)
Control (Foq9)	0.00
Foq9 + Tm	100.00
Foq9 + Tc	100.00
Foq9 + Gr	88.06
Foq9 + Gn	80.89
LSD (0.05) = 0.53	

Foq9 = isolate of F. oxysporum, Tm= Topsin M 70 WP, Tc= Tecto 500 SC, Gr= Garlic extract, Gn= Ginger extract.

Table 4. Effect of the fungicides and botanical extracts in controlling *Fusarium* wilt disease under greenhouse conditions.

No.	Treatment	Germination (%)	Incidence (%)	Severity (%)	Dry weight (mg/plant)
1	Control	100.0	0.0	0.0	479
2	Foq9	30.0	95.0	79.0	86
3	Tm	100.0	0.0	0.0	479
4	Tc	100.0	0.0	0.0	484
5	Gr	100.0	0.0	0.0	571
6	Gn	100.0	0.0	0.0	589
7	Fos9+Tm	87.5	67.5	41.8	444
8	Fos9+Tc	80.0	70.0	45.0	395
9	Fos9+Gr	75.0	65.0	50.5	292
10	Fos9+Gn	77.5	67.5	56.0	301
11	Fos9+Tm+Tc	92.5	37.5	33.0	487
12	Fos9+Tm+Gr	92.5	50.0	45.5	498
13	Fos9+Tm+Gn	95.0	52.5	41.3	474
14	Fos9+Tc+Gr	100.0	50.0	45.3	502
15	Fos9+Tc+Gn	100.0	55.0	40.8	480
16	Fos9+Gr+Gn	100.0	47.5	30.3	559
17	Fos9+Tm+Tc+Gr	95.0	35.0	23.5	545
18	Fos9+Tm+Tc+Gn	100.0	30.0	21.8	586
19	Fos9+Tm+Gr+Gn	100.0	25.0	19.5	595
20	Fos9+Tc+Gr+Gn	97.5	32.5	23.5	602
21	Fos9+Tm+Tc+Gr+Gn	100.0	0.0	0.0	710
	LSD (0.05)	0.84	2.0	14.76	71

Each number reflects 4 replicates.

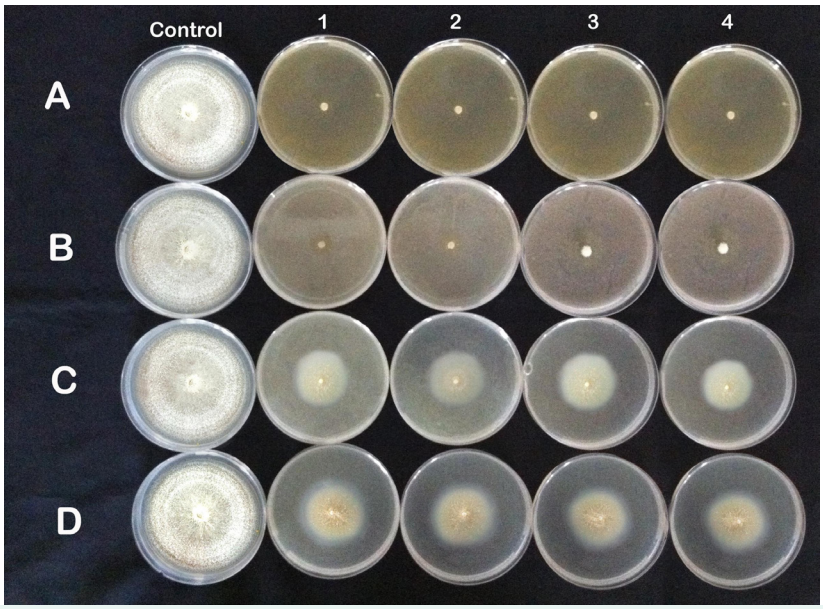

Figure 2. Inhibition effect of the fungicides and botanical extracts against *F. oxysporum in vitro* A. Topsin M 70 WP, B. Tecto 500 SC, C. Garlic extract, D. Ginger extract. Columns are replicates.

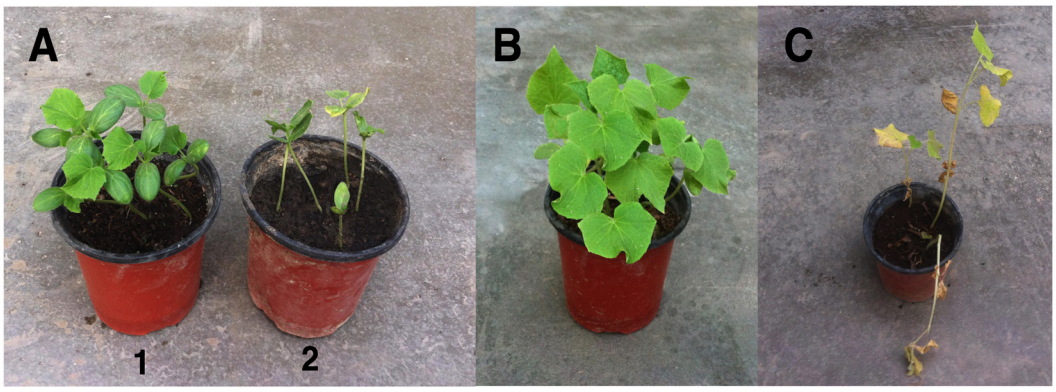

Figure 3. Control Fusarium wilt disease under greenhouse conditions A1.Treatment of (Fos9+Tm+Tc+Gr+Gn), A2. Negative control, B. Treatment of (Fos9+Gr+Gn), C. Negative control

at 25±2°C, lyophilized and grinded, fungal DNA extraction kit were used of Chelex 100 kit (Bio-Rad Laboratories, Inc., USA). DNAs were maintained at -20°C until used.

Polymerase chain reaction assay (PCR)

Universal primer pair ITS-Fu-f and ITS-Fu-r which is specific for the species of *Fusarium* were used with the following nucleotide sequences: 5`-CAACTCCCAAACCCCTGTGA-3`and 5`-GCGACGATTACCAGTAACGA-3` (Abd-Elsalam et al., 2003). The master mix of REDTaq ReadyMix PCR Reaction Mix (Sigma-Aldrish, USA) were used in the PCR reaction , each 20µL of the PCR reaction consist of 10µL REDTaq ReadyMix, 8.6µL PCR H_2O, 0.4µL forward primer, 0.4µL reverse primer at the concentration of 5 pmol and 1.0µL template DNA at the concentration of 50 ng/µl. Thermocycler (Applied Biosystems 2720 Thermal Cycler, USA) as programmed at the optimized amplification parameter according to the method of Hussein (2016) except the annealing step which set at 58°C for 1 min, negative control were amended as PCR mixture free of DNA in each run. PCR products were size fractionated on 1.5% agarose gel (Bio Basic, Canada) using Loading Quick 100 bp DNA ladder (Toyobo, Japan) added as 6 µL per lane, gels were visualized using Micro DOC system with U.V. Transilluminator (Cleaver, UK).

Botanical extracts preparation

Natural extracts of garlic and ginger were prepared by blending 100 gm of dried garlic cloves and 100 gm of fresh ginger rhizome with 250 mL sterile distilled water for 5 min and completed to 1000 mL with sterile distilled water. The suspensions were filtered through sterile cheesecloth into sterile conical flask (Mohana and Raveesha, 2007).

Inhibition activity assay

Inhibition activity of the fungicides and botanical extracts were estimated using poisoning food technique on the PSA medium, the fungicide Topsin M 70 WP (Tm), active ingredient is Thiophanate Methyl (Produced by Nippon Soda Co. Limited, Jappan) was prepared according to the manufacturer instructions at a concentration of 1.5 g/L, the fungicide Tecto 500 SC (Tc), active ingredient is Thiabendazole (Produced by Syngenta, Switzerland) at the concentration of 2 mL/L, one milliliter of each fungicide (Tm and Tc) and 1 mL of each botanical extracts (Gr and Gn) individually added to the petri plate, the plates were prepared by 15-20 mL of autoclaved PSA, each plate inaculcated with 0.5 cm disc of the fungal isolate Foq9 (7 days old), the control was the fungal isolate Fos9 alone. Four replicates were prepared for each treatment, the plates were incubated at 25°C until the radial growth of the pathogen reached the edge of plate in the control. The inhibition zones were calculated using the following formula:

Inhibition (%)

$=R - r / R \times 100$ (Mohana and Raveesha,2007)

where R = Radial growth of mycelium in control,

r = Mycelial growth in treatment.

Assessment efficacy of the fungicide and botanical extracts under greenhouse conditions

Experiment was conducted under greenhouse conditions to evaluate the disease controlling ability of the fungicides and botanical extracts individually and in combination. Plastic pots of 13 cm diameter were filled with autoclaved mixture of soil and compost (1:1). The treatment were Tm, Tc, Gr, Gn, Foq9+Tm, Foq9+Tc, Foq9+Gr, Foq9+Gn, Foq9+Tm+Tc, Foq9+Tm+Gr, Foq9+Tm+Gn, Foq9+Tc+Gr, Foq9+Tc+Gn, Foq9+Gr+Gn, Foq9+Tm+Tc+Gr, Foq9+Tm+Tc+Gn, Foq9+Tm+Gr+Gn, Foq9+Tc+Gr+Gn, Foq9+Tm+Tc+Gr+Gn, Foq9 (negative control) and positive control (without any treatment). Fungal inoculum prepared by adding 5 discs (0.5 cm) of the fungus 7 days old to each 100 gm of the autoclaved Pennisetum glaucum in sterile conical flask with 10 mL sterile distilled water and incubated 7 days at 25 ±2°C. The fungal inoculum were added

to the pots as 10 g/pot and watered, after 3 days each pot received 10 seeds of zucchini cultivar of Amjad, the fungicide Tm and Tc, and the botanical extracts Gr and Gn were soil drenched as 10 mL/pot on the same day of seeds transplanting and at the same concentration of the previous experiment. Percentage of the seed germination was calculated after 15 days of seeds transplanting. Percentage of the disease incidence and severity were calculated after 60 days of seeds transplanting according to the following formula:

Disease Incidence (%)
=(No.of infected plants)/
(Total No.of plants assessed)×100
(Masood et al.,2010)

The disease severity index (DSI) was estimated using the score chart of (0-7) rates which was described by Schneider (1984) and the percentage of the disease severity was calculated using the following formula:

$$DSI\ (\%) = (\Sigma\ (f \times v))/(N \times X)\times 100$$
(Grau and Radke,1982)

Results and Discussion

Isolation and Identification of the Pathogen

The results indicated that the soilborne fungus *F. oxysporum* was predominant, which exhibited 78.57 % percentage of appearance with frequency of 62.50 %. The high density of this pathogen in the soil of these areas may be due to inadequate agricultural practices such as repeated crop cultivate annually without crop rotation, weak cleanup process from infected plant debris. Furthermore, chlamydospores of the fungus can survive in the soil for several years even in the absence of a host (Stover, 1962). Saprophytic fungi such as *Aspergillus* spp., *Rhizopus* spp. and *Pen'cillium* spp. appeared with percentage ranged between 15.50- 24.40 % and frequency of 19.40-25.00 %.

Pathogenicity test

The forty five isolates of *Fusarium* spp., which identified morphologically as *F. oxysporum* showed variation in their pathogenicity *in vitro*, while the percentage of seed germination ranged between 5.0 – 90.0 % (Table 1), the isolate Foq9 exhibited 5.0 % percentage of seed germination, followed by the isolate Fom4 and Foq 2 were they exhibited 15.0 %, 17.5 % germinated respectively. This variation between the isolates may be due to enzyme secreting (Irzykowska et al., 2012) ability.

Molecular identification assay

Results of the DNA amplification of the 45 fungal isolates showed that 29 isolates were amplified positively with the universal primer of *Fusarium* spp. (Table 2), as they generated bands at 570 bp (Fig. 1). The rest of the isolates didn't amplify with theses primers, and this is may be because of technical errors.

Inhibition activity assay

According to Table 3, all of the agents used in this experiment significantly reduced the fungal radial growth of the *F. oxysporum*, so far both of the fungicides Topsin M 70 WP and Tecto 500 SC exhibited 100.0% percentage of inhibition, while the botanical extracts of garlic and ginger exhibited 88.06%, 80.89% respectively, compared to control which filled the plates after 7 days of incubation (Fig. 2). Sahar et al. (2013) found that in vitro experiment Topsin M inhibited the growth of *F. oxysporum* significantly as 76.66 %.

Assessment efficacy of the fungicides and botanical extracts under greenhouse conditions

The results showed that all of the agents used individually and in combination increased the percentage of the seed germination up to 75-100 % compared to the negative control (pathogen alone) which was 30 % (Table 4). All of the treatments reported significant disease controlling ability, while the percentage of the disease incidence and severity ranged between 0-70 %, 0-56 % respectively compared to the negative control which was 95 % and 79 % respectively, among these the treatment of tetra-inoculum (Tm+Tc+Gr+Gn) was superior which exhibited 0 % percentage of disease incidence and severity, followed by the tri-inoculum (Tm+Gr+Gn) which was 25.0 %, 19.5 % respectively. Further, the treatment of botanical extracts in combined application (Gr+Gn) reduced the disease incidence and severity to 47.5 % and 30.3 % respectively, which reflect a positive efficiency of the natural extracts in plant diseases control as alternative to chemicals or in combination with them to preserve the environment from harmful effect of the chemicals (Fig. 3).

The tetra-inoculum (Tm+Tc+Gr+Gn) reported highest average of dry weight of plants which was 710 mg/plant compared with negative and positive control which was 86 and 479 mg/plant respectively, followed by the treatment of tri-inoculum (Tc+Gr+Gn) which was 602 mg/plant. The combined application of Gr+Gn exhibited significant increase in the dry weight of plant which was 559 mg/plant. Tariq and Magee (1990) found that volatile components of garlic extracts inhibited germination of the microconidia, macroconidia and mycelium of the *F. oxysporum* f. sp. *lycopersici in vitro*. Al.Rahmah et al. (2013) has been found that ginger extracts has strong fungistatic and fungicidal activities against *F. oxysporum* and *Pythium aphanidermatum* with minimal inhibitory concentration. Furthermore, Sahar et al. (2013) proved that foliar application of Topsin-M with concentration of 0.1 % was significantly reduced the disease incidence of Fusarium wilt disease on eggplant.

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