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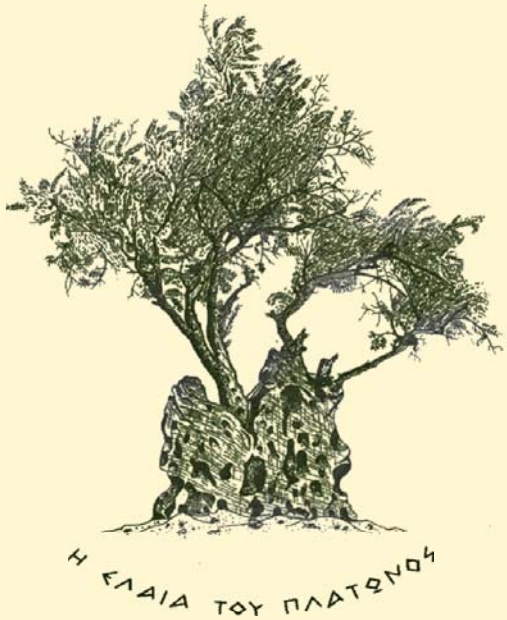

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Tomato bacterial endophytes in controlling Verticillium wilt and their advantage on biostimulation

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Summary A total of 688 bacterial endophytes were isolated from both greenhouse and field-grown tomatoes in the Tehran and Alborz provinces, Iran in order to obtain effective bacterial endophytes against the fungus *Verticillium dahliae*. 128 bacterial isolates, with respect to their different phenotypic characteristics were further analysed. All bacteria with positive hypersensitivity reaction on tobacco and geranium leaves and potato soft rot were eliminated, and totally 39 isolates were selected for in vitro antagonism and greenhouse tests. The potential biocontrol isolates were evaluated using seed treatment and soil drench methods on two tomato cultivars. The results indicated that seven bacterial isolates had a high potential for the control of the fungus and reduced the severity disease to 95-98%. This reduction was coincided with an increase in some growth factors like plant dry weight, root dry weight, plant height, root length, root fresh weight and plant fresh weight ranged between 92-98%. The seven antagonists' preliminary identification was confirmed using 16SrRNA gene sequencing analysis. The BLAST analysis was performed, and the bacteria were also identified as *Bacillus pumilus* (two isolates)*, Bacillus subtilis, Bacillus safensis, Enterobacter ludwigi, Serratia marcesens* and *Pseudomonas beatica*. Biocontrol mechanisms examination indicated that protease production was positive for all isolates and differentiated isolates *E. ludwigii* and *P. beatica* as higher producers with protease levels up to 65%. The three bacteriocins producing isolates inhibited the phytopathogenic mycelium up to 70% in dual culture assay. Also, five of the isolates produced siderophores and *P. baetica, S. marcesens* and *E. ludwigii* produced remarkable amount of auxin hormone.

Additional keywords: biocontrol, endophytic bacteria, tomato, Verticillium wilt

Introduction

Tomato (*Lycopersicon esculentum* Mill.) is considered as one of the most important commercial vegetable crops in Iran and all over the world. Iran ranks as the sixth largest producer of tomato in the world (http://www.fao.org/faostat/en/#rankings/countries_by_commodity). However, one of the main problems in tomato production is fungal diseases causing damping-off, wilts and blights. Verticillium wilt, in particular, caused by the soil-borne fungus *Verticillium dahliae,* is considered as an economically important disease that limits the production of a broad range of agricultural crops, including tomato (Gayoso *et al*., 2007). The tomato yield losses due to this disease are estimated by 50% (Jabnoun *et al*., 2009). Several methods have been proposed and implemented for managing the Verticillium wilt.

Host plant resistance is generally the most favorable control method for plant disease management, but resistant cultivars to Verticillium wilt are not available for many crops and therefore the management of the disease is notoriously difficult (Rekanovic *et al*., 2007; Song and Thomma, 2016). Synthetic agrochemicals are applied as conventional and common method to control plant diseases. However, the use of chemical pesticides adversely affects the environment and human health (Prabhat *et al*., 2013). The use of biological control agents has increasingly become popular in plant protection as a more environmentally friendly alternative to chemical pesticides (Martin and Bull, 2002; Naraghi *et al*., 2006; Naraghi *et al*.,

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2008; Heydari and Pessarakli, 2010). This can include the soil inoculants and foliar sprays of beneficial microorganisms, while the increased knowledge about microbial ecology in the phytosphere has stimulated new thoughts for biocontrol approaches (Rabiey *et al*., 2019).

Application of bacterial endophytes to tomato plants and their effects on disease control along with plant growth promotion are considered as a novel method in tomato Verticillium wilt management (Latha *et al*., 2019). Endophytic bacteria usually colonize the whole tomato plant, obtain the essential nutrients from plant tissues and protect the host plants against biotic and abiotic stresses (Botta *et al*., 2013). According to Amaresan *et al.* (2012), the most identified endogenous bacteria with promoting plant growth capability in tomato belong to *Bacillus* and *Serratia* genera. They have the ability to control phytopathogens such as *Fusarium oxysporum, Colletotrichum capsici, Sclerotinia rolfsii* and *Pythium* sp. and are also able to produce siderophore, indole acetic acid and solubilize phosphorus. Several other reports recommend bacterial endophytes as an appropriate alternative to chemical fungicides. Some of these bacteria like *Bacilli* and *Streptomyces* isolates have been commercialized (Kwak and Weller, 2013; Lagzin *et al*., 2013). The latter can induce systemic resistance in their host plants to different diseases and abiotic stresses through the production of diverse antimicrobial compounds, enzymes and siderophores (Botta *et al.,* 2013; Kandel *et al.,* 2017). In addition, they promote plant growth and yield in many agricultural and horticultural crops (Rosenblueth and Martínez-Romero, 2006). Therefore, endophytic bacteria are considered as biocontrol agents which protect their host under unfavorable conditions better than soil, rhizoplane and phylloplane microbes and they can be transmitted through gene transfer between generations in plants, an attribute that also differentiates them from rhizosphere bacteria (Nejad and Johnson, 2000).

Since, host resistance against Verticillium wilts is not available for many plant species, and combating with this disease is notoriously difficult (Song and Thomma, 2016), host-induced gene silencing (HIGS) has been identified as a promising strategy for improving the plant resistance against these pathogens (Song and Thomma, 2016). Specifically, HIGS by employing RNA silencing mechanisms to silence the targets of invading pathogens has been successfully applied in crop disease prevention (Qi *et al*., 2019).

The objective of this study was to isolate and identify effective bacterial endophytes from tomato plants in order to control the tomato Verticillium wilt and promote the growth parameters.

Materials and methods

Evaluation of biocontrol action of endophytic bacteria against *V. dahliae* in tomato plants

Plant sampling. Tomato plants sampling was carried out from 20 greenhouses and 10 farms free of fungal disease during spring and summer of 2017 in Tehran and Alborz provinces. Ten healthy plants were selected from each greenhouse and field.

Isolation of endophytic bacteria. The root, stem, leaf and fruit samples of each greenhouse and field were mixed for isolation of endophytic bacteria. The samples were rinsed with tap water for 10 min., then sterilized with 75% ethanol for 40 sec. and in 5% sodium hypochlorite for 5 min., and disinfected in 70% ethanol for 30 sec. Afterwards, the samples were rinsed with sterile distilled water at least three times and the specimens' surface was scraped with sterile scalpel and immersed in distilled water for 30 min. All samples were crushed in sterile mortar, and a loopful from each suspension was cultured on nutrient agar medium (NA, Merck, Germany). All plates were incubated for 3-4 days at 37°C. The distinguished colonies were selected and purified. The representative strains were maintained in nutrient broth medium at -20°C (NB, Difco, USA) containing 15% (w/v) glycerol (Atugala and Deshappriya, 2015).

Grouping of the endophytic bacteria on the basis of phenotypic characteristics. Thirty seven (37) representative strains were characterized initially, with respect to certain phenotypic tests including Gram staining, mobility, aerobic or anaerobic growth, fluorescent pigment production on King's B medium, levan, potato soft rot, oxidase, arginine dihydrolyase as well as gelatin and starch hydrolyses and also nitrate reduction test (Schaad *et al*., 2001). Hypersensitivity reaction (HR) was also examined on tobacco and geranium leaves.

Pathogenic isolate. Verticillium dahliae strain VD-Co-P-G-22, isolated from wilted tomato, was obtained from the Iranian Research Institute of Plant Protection, Tehran, Iran and used in all experiments of this study.

In vitro screening of endophytic bacteria antagonistic activity against V. dahliae. The antifungal activity of 128 endophytic bacteria was evaluated in vitro by dual culture assay on PDA medium with three replications. Sterile distilled water was used as a control. An agar disc with *Verticillium dahliae* mycelium was placed 5 cm away from the bacterial cultures, and the plates were incubated at 25°C. The percent of inhibition was calculated using the following formula: Percent Growth Inhibition= [(R-r) ×100]/R, where R is the fungal growth radius in the control, and r is the fungal growth radius in the antagonist presence. (Munif *et al*., 2012).

Effect of endophytic bacteria on seed germination and seedling growth under laboratory and greenhouse conditions. For bacterization of seeds surface of two tomato cultivars, "Falat" and "Super amber", the seeds were immersed for 40 min. in the individual bacterial suspension with a concentration of 1×108 cfu/ml. The treated seeds were then placed on filter paper for seed germination. The germinated seeds were sown in sterile soil and growth rates were monitored daily for one month. All tests were conducted in a completely randomized design with three replications (Vichova and Kozova, 2004; Jabnoun et al., 2009).

Evaluation of biocontrol capability of selected endophytes under greenhouse conditions. Seeds of the cv. Falat were disinfected with sodium hypochlorite and ethanol, thoroughly washed with sterilized distilled water and planted in a plastic tray (Atugala and Deshappriya, 2015). Three weeks later, the roots were individually placed in bacterial suspension of 37 endophytic bacteria isolates (3×108 cfu/ml). Phosphate buffer (PBS) was used as a negative control. The treated seedlings were transferred to some pots containing infested soil with *V. dahliae* (Nejad *et al*., 2000). After three weeks, tomato roots and stems were crushed and the extracts were streaked on nutrient agar (NA) medium for determining the bacterial colonization. The disease severity was measured after 90 days of cultivation using the following scale: 0 = no symptoms, 1= infection symptoms on the root, 2 = infection symptoms on the root and crown, 3 = infection symptoms on the stem base, and 4 = infection in the stem middle and end (Jabnoun *et al*., 2009). The experimental design was a completely randomized design with three replicates. ANOVA for a factorial design (factors: endophytic bacteria, *V. dahliae*) was conducted, using the SAS program. The treatments means was compared by Duncan's multiple range test.

Measuring plant growth parameters. Different growth parameters including shoot and root fresh and dry weight, and growth lengths were measured. Ten tomato plants at the age of 1-month, treated with 37 bacterial isolates were assigned to measure the parameters by random (Atugala and Deshappriya, 2015).

Identification of the most efficient bacteria. A combination of phenotypic characteristics and 16S rDNA sequences was applied in order to identify the endophytic bacteria (Kumar *et al*., 2016).

For DNA extraction and PCR reaction, the selected bacterial isolates were cultured in NA medium and after 24 hours, a single colony was selected and mixed in the sterile microtubes containing 500 µl of NaOH and 10 µl of SDS. The samples were placed in boiling water for 5-10 min. after vortexing for 3 min. (gram-negative bacteria for 5

min. and gram-positive for 10 min.). This was followed by ice-cold shock treatment for 3 min. Then, the samples were centrifuged at 13000 rpm at 25°C. 100 µl of the upper phase containing DNA was transferred into the 1.5 ml Eppendorf tubes and was preserved at -20°C (Elboutahiri *et al.*, 2009). The primers pairs that were applied were P1 and P6 with the sequences as followings: (5'ATC CAGAGTTTGATCCTGGTCAGAACGAACGCT -3') and (5'-CGGGATCCTACGGCTACCTTGT-TACGACTTCACCCC-3'), respectively (Kumar *et al.*, 2016).

For the PCR product electrophoresis and sequencing, the PCR mixture was prepared in a 25µl final volume containing 2 µl of extracted DNA (10 ng/µl), 1 µl of $MgCl_2$ (50 mM), 0.6 µl of dNTPs (10 mM), 1 µl of each primer (10 ng/µl), 0.1 µl of Taq polymerase, 3 µl of PCR buffer (10X) and 16.3 µl of SDW. The thermal cycling conditions for initial denaturation were for 2 min. at 94°C, followed by 25 cycles for 30 S at 94°C (denaturation), for 30 S at 52°C (annealing), for 2 min at 72°C (extension), and a final extension step for 20 min at 72°C. Mixtures without DNA templates (water) were used as negative control. 4 µl of each PCR product was mixed with a 1 µl staining marker (GeneRuller 100bp) and run in 1% agarose in 1x TAE buffer. 1 µl of 100 bp DNA ladder SMO323 (Thermo Scientific) was applied. The gel was photographed using Gel Document Uvitec/DM 500. The PCR products with sharp bands were sent to the two Microsynth (Switzerland) and Bioneer (South Korea) companies for sequencing.

Detection of antifungal metabolites produced by representative endophytic bacteria

Detection of antifungal metabolites was examined in five bacterial isolates, *E. ludwigii* NZ101, *P. baetica* NZ108, *B. subtilis* NZ104 and NZ103, *B. pumilus* NZ106, namely through the production of protease, hydrogen cyanide (HCN), inhibitory extracellular metabolites, volatile antifungal metabolites, indole-3-acetic acid (IAA), siderophore and bacteriocins.

Protease production. The bacterial isolates were spot inoculated on SCA medium (Simmons' citrate agar) and the Perti plates were kept for 48 hours at 25°C. The colorless halo formation around the bacterial colonies indicated proteolytic proteins activity (Gull and Hafeez, 2012).

HCN production. Bacteria were heavily inoculated on SCA growth medium (Simmons' citrate agar) and a filter paper strip of 1×1 cm containing 5 ml of copper ethyl acetoacetate, 5 mg of 4,4' methylene bis (N,N'-dimethyl) aniline and 2 ml of chloroform was placed inside the lids upside-down. All Petri plates were incubated for 48 hours at 28°C. Any change in the filter paper color (to blue color) was considered as a positive result of hydrogen cyanide production (Gull and Hafeez, 2012).

Production of inhibitory extracellular metabolites. Bacterial isolates were prepared at a concentration of 1×10^7 cells/ml, cultured on PDA medium and were incubated at 28 °C for three consecutive days. After that, DW washed the bacteria from the surface of the culture medium, and a sterilized cotton swab soaked in chloroform was placed on lids. After 30 min, a 15-day fungal growth was cultured in the middle of each plate. The inhibitory zones were measured after incubation of plates at 28°C for 10 days using the formula proposed by Raut and Hamed (2016).

Production of volatile antifungal metabolites. All bacterial suspensions were prepared at a concentration of 1×10^7 cells/ml, cultured on nutrient agar + 2% glucose (NGA) and were incubated for 24 hours at 25°C. Meantime a 15day fungal culture was placed in the middle of the PDA medium and the two plates were sealed together with Parafilm M®. The control sets did not contain the bacteria. The inhibitory zones were measured after incubation of plates at 28°C for 10 days as mentioned above.

Production of IAA. The bacterial isolates were grown in tubes containing 100 ml of KB medium for 16 h at 30°C. Tubes containing 100 ml of KB medium were inoculated with bacteria and incubated for at least 16 hours at a temperature of 30°C. The 0.6-0.7 absorbance value was determined at 600 nm.

The suspension was centrifuged at 8,000 rpm for 10 min and the supernatants were mixed with Salkowski reagent in a 2:1 ratio and ethanol, chloroform and concentrated sulfuric acid were mixed in a ratio of 2: 2: 2. The tubes were placed in a dark box for 30 minutes. The pink color developed due to the production of IAA was measured by spectrophotometry at 530 nm. The concentration of IAA in each culture was calculated with respect to the standard curve (Merck, Germany) (Gull and Hafeez, 2012).

Production of siderophore. The KB and PDA culture media containing FeCl3 in the final concentrations of 25, 20, 15, 10 µg/L and control without iron, were prepared, respectively, and a 15-day Verticillium culture was inoculated and incubated at 28°C for 5 days in darkness (Raut and Hamed, 2016; Gull and Hafeez, 2012).

Bacteriocin production and their antibacterial activity. Antagonistic isolates were cultured on a NB medium for 24 hours at 25°C. The supernatants that were obtained by centrifugation for 20 min. at 8000 rpm were adjusted to pH 6.5 with 1M NaOH. Trypsin (1 mg ml^{-1}) was added to each tube and isolates were incubated for 12 hours at 37°C. Two wells with the depth of 5 mm were made on each NA medium, and each one of the bacterial isolates was added to each well, up to the time of completely absorbing by medium. Finally, the Petri plates surface were sprayed with *Escherichia coli* indicator (isolate code: PTCC1330) and incubated for 1 week. Any inhibitory effect was considered as a result of bacteriocins (Tagg and McGiven, 1971).

Results

Biocontrol action of endophytic bacteria against *V. dahliae* in tomato plants

Altogether, 688 endophytic bacterial isolates were obtained from the root, stem, leaf, and fruit of tomato plants. 128 isolates were selected in initial screening based on phenotypic traits. Finally, 37 isolates which did not cause hypersensitivity reactions and soft rot in potato slices were selected for further studies.

All 37 representative isolates were examined for their inhibitory effects against *V. dahliae* using the agar diffusion method on PDA medium. The results indicated that there was a significant difference in the fungal growth inhibitory effect. The maximum inhibitory effect was produced by *P. beatica* with 3.80 mm, *E. ludwigii* with 3.70 mm and *B. subtilis* with 3.60 mm zone diameters, respectively.

The results from the tests on the effect of endophytic bacteria on germination of tomato seeds and seedling growth (viability and vigor) under laboratory and greenhouse conditions indicated that the seeds viability in cv. Falat and super amber cultivars was 99% and 50%, respectively. As a result, all experiments were carried out on tomato cv. Falat.

By analyzing the data attained from the greenhouse test, it was demonstrated that there was a significant difference ($P < 0.0001$) between tomato plants treated with endophytic bacteria and *V. dahliae* in comparison with positive (inoculated with pathogen) and negative controls (non-inoculated) (Fig. 1). Moreover, five distinct groups of a, b, c, d and e were obtained as indicated in Table 1. In group e, the non-inoculated controls along with seven isolates were recognized as high potential biological control agents reducing the disease severity to 95-

Figure 1. Seed treatment and reduction of disease symptoms and growth promoting effect of the endophytic bacterium *Pseudomonas beatica* NZ-101 (middle) compared to healthy control (right) and *Verticillium dahliae* infected tomato plant (left).

Table 1. Disease incidence and control disease against *Verticillium dahliae*, and means of fresh and dry weight of seedlings, seedling length, root fresh and dry weight, and root length of tomato seedlings, cv. Falat, inoculated with endophytic bacteria.

Treatment	Disease incidence	Disease Control (%)	Seedling wet weight (g)	Seedling dry weight (g)	Seedling height (cm)	Root wet weight (g)	Root dry weight (g)	Root length (cm)
Inoculated Control	94.5 ± 0.28 a	4.50	19.15 ± 0.005 h	4.67 ± 0.005 i	13.83 ± 0.005ef	6.11 ± 0.005i	1.98 ± 0.005 m	4.83 ± 0.005 j
RA^{3}_{1}	64.50 ± 0.28 b	45	26.40 ± 0.005 de	9.65 ± 0.005 e	14.16 ± 0.005ef	11.48 ± 0.005 ef	5.27 ± 0.005 g	7.66 ± 0.005ef
RH^{1}_{1}	45.50 ± 0.28 c	65	49.71 ± 0.005 c	18.20 ± 0.005 d	24.00 ± 0.005 c	31.66 ± 0.005 bc	12.62 ± 0.005 d	16.50 ± 0.005 d
NZ 104	**2.50 ± 0.28 e**	**98**	**70.07 ± 0.005 a**	**30.73 ± 0.57 a**	**35.66 ± 0.57 a**	**42.31 ± 0.005 a**	**17.23 ± 0.57a**	**25.50 ± 0.57 a**
RE^{1}_{1}	20.20 ± 0.11 d	82.5	49.71 ± 0.58 c	18.20 ± 0.57 d	24.00 ± 0.57c	31.66 ± 0.58bc	12.62 ± 0.58 d	16.50 ± 0.57 d
RA^{2}_{1}	50.50 ± 0.28 c	46.5	24.62 ± 0.57 defg	7.89 ± 0.83 gh	9.66 ± 0.56 gh	9.84 ± 0.01 h	3.20 ± 0.57 l	5.66 ± 0.56 ij
MA^{1}_{2}	49.50 ± 0.28 c	52.5	26.40 ± 0.57 de	9.65 ± 0.58 e	14.16 ± 0.58ef	11.48 ± 0.57ef	4.94 ± 0.01 gh	7.66 ± 0.01 ef
SB^{1}_{6}	89.5 ± 0.28 a	8.5	26.40 ± 0.57 de	9.65 ± 0.58 e	14.16 ± 0.58 ef	11.48 ± 0.58 ef	5.27 ± 0.58 g	7.83 ± 0.56 e
MA^{3}_{3}	55.50 ± 0.28 c	45.5	25.50 ± 0.57 def	9.65 ± 0.58 e	13.00 ± 0.57 gef	10.22 ± 0.01 ghf	4.20 ± 0.57 ij	7.83 ± 0.56 e
RC^{1}_{3}	50.50 ± 0.28 c	52.50	49.71 ± 0.005 c	18.20 ± 0.58d	24.00 ± 0.57 c	31.66 ± 0.58 bc	12.62 ± 0.01 d	16.50 ± 0.57 d
NZ 101	**2.50 ± 0.28 e**	**98**	**70.07 ± 0.005 a**	**30.73 ± 0.57 a**	**35.66 ± 0.57 a**	**42.31 ± 0.005 a**	**17.23 ± 0.57a**	**25.50 ± 0.57 a**
NZ 102	**4.50 ± 0.28 e**	**95**	**69.74 ± 0.01 a**	**29.13 ± 0.57 b**	**33.83 ± 0.01 a**	**41.21 ± 0.58 a**	**16.95 ± 0.01 a**	**23.66 ± 0.01b**
RB^{13}_{3}	70.50 ± 0.28 b	35.5	26.40 ± 0.57 de	9.65 ± 0.57 e	14.16 ± 0.01 ef	11.48 ± 0.57ef	5.27 ± 0.57g	7.66 ± 0.01ef
SC^{7}_{6}	92.50 ± 0.28 a	6.5	21.73 ± 0.01 gh	6.99 ± 0.57 h	10.00 ± 0.57 gh	10.11 ± 0.005 gh	3.25 ± 0.57 i	6.00 ± 0.57 hi
BC^{1}_{3}	55.50 ± 0.28 c	48.50	49.71 ± 0.005 c	18.20 ± 0.57 d	24.00 ± 0.57 c	31.66 ± 0.58 bc	12.62 ± 0.01 d	16.50 ± 0.57 d
RD^{7}_{6}	25.50 ± 0.28 d	77.50	27.40 ± 0.57 d	8.65 ± 0.02 gf	14.66 ± 0.001 e	12.48 ± 0.57 e	4.27 ± 0.005 ij	6.66 ± 0.001 gh
NZ 103	**3.50 ± 0.28 e**	**96**	**69.74 ± 0.01 a**	**29.13 ± 0.57 b**	**33.83 ± 0.01 a**	**41.21 ± 0.58 a**	**16.95 ± 0.01 a**	**23.66 ± 0.01b**
NZ 106	**4.50 ± 0.28 e**	**95**	**69.74 ± 0.01 a**	**29.13 ± 0.57 b**	**33.83 ± 0.01 a**	**41.21 ± 0.58 a**	**16.95 ± 0.01 a**	**23.66 ± 0.01b**
BB^{1}_{6}	30.20 ± 0.11 d	65.80	25.40 ± 0.11def	8.65 ± 0.11gf	12.00 ± 0.57 gehf	11.28 ± 0.01 gef	3.90 ± 0.57 kj	5.00 ± 0.57 j
BA^{1}_{3}	32.50 ± 0.28 d	64.50	47.71 ± 0.005 c	17.47 ± 0.01 d	18.50 ± 0.57 d	29.83 ± 0.01 d	11.07 ± 0.04 f	16.33 ± 0.01 d

SB^{1}_{6}	94.50 ± 0.28 a	4.50	23.79 ± 0.01 efg	9.05 ± 0.02 ef	11.66 ± 0.01 gehf	9.15 ± 0.02 h	4.04 ± 0.01 kij	5.66 ± 0.01 ji
SD^{1}_{6}	65.50 ± 0.28 b	35.50	24.03 ± 0.01 defg	7.22 ± 0.01 h	12.00 ± 0.57 gehf	10.15 ± 0.02 gh	4.38 ± 0.01 ij	7.00 ± 0.57 gef
MA^{1}_{4}	59.50 ± 0.28c	38.50	26.06 ± 0.03ed	8.68 ± 0.01 gef	13.16 ± 0.01 gef	11.48 ± 0.01 ef	5.48 ± 0.01 g	7.16 ± 0.01gef
MA^{1}_{2}	25.50 ± 0.28 d	72.50	24.20 ± 0.01defg	8.20 ± 0.11 gf	9.16 ± 0.01 h	10.22 ± 0.01 ghf	3.34 ± 0.02 i	7.00 ± 0.57 gef
BA^{1}_{6}	30.50 ± 0.28d	68.50	25.40 ± 0.11 def	8.59 ± 0.005 gf	14.16 ± 0.01 ef	11.15 ± 0.02 gf	4.54 ± 0.02 ih	6.83 ± 0.01 ghf
MB^{5}_{6}	55.50 ± 0.28 c	40.50	19.15 ± 0.02 h	4.67 ± 0.01 i	9.00 ± 0.57h	6.11 ± 0.005 i	1.98 ± 0.005 m	3.83 ± 0.01 k
BC^{2}_{4}	52.50 ± 0.28c	45.50	25.79 ± 0.005 de	9.05 ± 0.02 ef	14.00 ± 0.57ef	11.48 ± 0.005 ef	5.04 ± 0.02 gh	7.33 ± 0.01gef
NZ 108	**2.50 ± 0.28 e**	**98**	**70.07 ± 0.005 a**	**30.73 ± 0.57 a**	**35.66 ± 0.57 a**	**42.31 ± 0.005 a**	**17.23 ± 0.57a**	**25.50 ± 0.57 a**
BA^{4}_{6}	25.50 ± 0.28 d	72.5	22.20 ± 0.11 fgh	7.20 ± 0.11 h	10.66 ± 0.01 ghf	9.85 ± 0.02 h	3.62 ± 0.01 kl	6.66 ± 0.01gh
SA^{1}_{6}	94.50 ± 0.28 a	4.50	6.39 ± 0.005 i	1.66 ± 0.01 j	3.00 ± 0.57 i	2.06 ± 0.01 j	0.67±0.01 n	1.33 ± 0.01 l
MA^{2}_{6}	45.50 ± 0.28 c	54.5	47.71 ± 0.005 c	17.47 ± 0.01 d	18.50 ± 0.18 d	29.83 ± 0.01 d	11.07 ± 0.01 f	16.33 ± 0.01 d
ME^{3}_{6}	46.50 ± 0.28 c	52.5	49.71 ± 0.005 c	18.20 ± 0.02 d	24.00 ± 0.57 c	31.66 ± 0.01bc	12.62 ± 0.01 d	16.50 ± 0.01d
SA^{2}_{6}	94.50 ± 0.28a	4.50	6.39 ± 0.005 i	1.60 ± 0.02 j	3.00 ± 0.57 i	2.06 ± 0.01 j	0.67 ± 0.01 n	1.33 ± 0.01 l
MA^{5}_{6}	48.50 ± 0.28 c	54.50	49.71 ± 0.005 c	18.20 ± 0.11 d	24.00 ± 0.57 c	31.66 ± 0.01 bc	12.62 ± 0.01 d	16.50 ± 0.57 d
MD^{3}_{6}	65.50 ± 0.28b	35.50	48.71 ± 0.005 c	17.47 ± 0.01 dc	22.16 ± 0.01 c	30.50 ± 0.15 dc	11.74 ± 0.18 ec	16.16 ± 0.01dc
MA^{5}_{6}	56.50 ± 0.28 c	42.50	26.40 ± 0.23 de	9.65 ± 0.02 e	14.16 ± 0.01 ef	11.48 ± 0.01 ef	5.27 ± 0.01 g	7.66 ± 0.01 ef
MD^{5}_{6}	55.50 ± 0.28 c	42.5	49.71 ± 0.005 c	18.20 ± 0.11 d	24.00 ± 0.57 c	31.66 ± 0.01 bc	12.62 ± 0.01 d	16.50 ± 0.57 d
NZ 107	**3.50 ± 0.28 e**	**96**	**69.74 ± 0.01 a**	**29.13 ± 0.57 b**	**33.83 ± 0.01 a**	**41.21 ± 0.58 a**	**16.95 ± 0.01 a**	**23.66 ± 0.01b**
SD^{6}_{6}	65.50 ± 0.28 b	42.5	48.71 ± 0.005 c	17.47 ± 0.01 d	22.16 ± 0.01 c	30.50 ± 0.15 cd	11.74 ± 0.18 e	16.16 ± 0.01 a

RA^{3}_{1}= *Pseudomonas* sp., RH^{1}_{1} = *Bacillus* sp., **NZ 104= *Bacillus subtilis*,** RE^{1}_{1}=*Pseudomonas* sp., RA^{2}_{1}=*Bacillus* sp., MA^{1}_{2}= *Bacillus* sp., SB^{1}_{6}= *Enterobacter* sp., MA^{3}_{3}= *Bacillus* sp., RC^{1}_{3}= *Bacillus* sp., **NZ 101 = *Pseudomonas beatica*, NZ 102= *Serratia marcesens*,** RB^{13}_{3}= *Enterobacter* sp., SC^{7}_{6}= *Serratia* sp., BC^{1}_{3}= *Bacillus* sp., RD^{7}_{6}= *Pseudomonas* sp**., NZ 103= *Bacillus pumilus*, NZ 106= *Bacillus pumilus*,** BB^{1}_{6}=*Bacillus* sp., BA^{1}_{3}= *Bacillus* sp., SB^{1}_{6}= *Enterobacter* sp., SD^{1}_{6}= *Serratia* sp., MA^{1}_{4}= *Bacillus* sp., MA^{1}_{2}= *Enterobacter* sp., BA^{1}_{6}= *Pseudomonas* sp., MB^{5}_{6}= *Bacillus* sp., BC^{2}_{4}= *Pseudomonas* sp., **NZ 108= *Enterobacter ludwigii*,** BA^{4}_{6}= *Pseudomonas* sp., SA^{1}_{6}= *Serratia* sp., MA^{2}_{6}= *Bacillus* sp., ME^{3}_{6}=*Enterobacter* sp., SA^{2}_{6}= *Bacillus* sp., MA^{5}_{6}= *Bacillus* sp., MD^{3}_{6}= *Enterobacter* sp., MA^{5}_{6}= *Bacillus* sp., MD^{5}_{6}= *Pseudomonas* sp., **NZ 107= *Bacillus safensis*,** SD^{6}_{6}= *Bacillus* sp.

Means followed the same letters are not significantly different ($P < 0.0001$). Separation of means using Duncan's Multiple Range Test.

98%. In group d with seven representative isolates, the disease severity has a reduction between 70-90%. The group c containing fifteen isolates had intermediate control effects (45-70%). The two lowest groups were as followings: the group b with five isolates and 25-45% disease reduction effects and the group a, with four bacterial strains that could lower the disease by 5-20%.

The statistical analyses on the dry weight, root dry weight, seedlings length, root length, root fresh weight and fresh weight of the seedlings treated with representative isolates, showed a significant difference amongst the treatments. The seven isolates of *P. baetica* NZ101, *B. subtilis* NZ104 and *E. ludwigii* NZ108, *S. marcesens* NZ102, *B. pumilus* NZ103, *B. safensis* NZ107 and *B. pumilus* NZ106 have increased the growth factors by 92-98%. Other isolates had a modest effect in comparison with the control (Table 1).

BLAST analyses demonstrated that the isolates NZ101, NZ102, NZ104, NZ106, NZ108 belong to the species *P. baetica, S. marcesens, B. subtilis, B. safensis* and *E. ludwigii*, respectively. Both NZ103 and NZ106 belong to the species *B. pumilus*.

Detection of antifungal metabolites produced by isolates of endophytic bacteria

Six isolates including *P. baetica* NZ101, *S. marcesens* NZ102, *B. pumilus* NZ103 and NZ106, *B. subtilis* NZ104 and *B. safensis* NZ107 could significantly inhibit the *V. dahliae* growth by production mechanism of the protease enzyme at P <0.0001 (Fig. 2). *E. ludwigi* NZ108 failed to produce this enzyme completely, whereas *P. baetica* NZ101 showed the highest protease activity by 65% (Table 2).

S. marcesens NZ102 and *B. pumilus* strains NZ103 and NZ106 produced hydrogen cyanide in the medium containing glycine (Table 2).

Regarding the production of agar-permeable extracellular inhibitor metabolites, a significant decrease (P <0.0001) in *V. dahliae* mycelial growth was observed. The highest growth inhibition with 2.76 mm in diameter was associated to *P. baetica* NZ101, *E. ludwigi* NZ108 and *B. subtilis* NZ104 (Table 2).

A significant reduction by 68% (P <0.0001) was observed in the *V. dahliae* mycelial growth compared to the control, due to volatile antifungal metabolites production by the isolates *P. baetica* NZ101, *E. ludwigi* NZ108 and *B. subtilis* NZ104 (Table 2).

Three representative isolates, *P. baetica* NZ101, *S. marcesens* NZ102 and *E. ludwigii* NZ108, produced the auxin hormone IAA, in concentrations calculated with respect to the standard curve (Merck, Germany) (Table 2).

Siderophore production by almost all bacteria had a significant effect on the fungal mycelium growth of *V. dahliae* at concentrations of 5, 10, 25 and 50 ug/l (P <0.0001). The results indicated that the isolates *E. ludwigii* NZ101, *P. baetica* NZ108, *B. subtilis* NZ104 and NZ103, *B. pumilus* NZ106 reduced or inhibited the *V. dahliae* mycelial growth, at the media emended with 10 and 5 µg/l of FeCl3 (Table 2).

The mean value of the inhibition zone caused by bacteriocin producing strains indicated that *P. baetica* NZ101, *E. ludwigii* NZ108, NZ104 and *B. subtilis* NZ104 could decrease *V. dahliae* growth up to 70% compared to the control (Table 2).

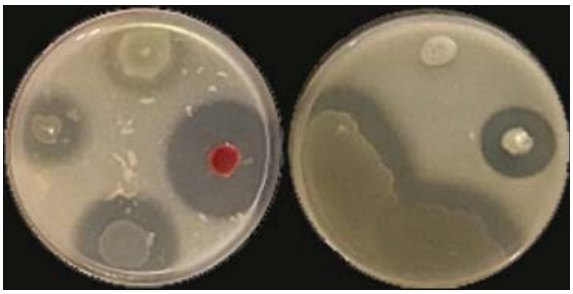

Figure 2. Protease enzyme production by different bacteria on SMA medium. Clockwise from top, at the left plate: *Pseudomonas baetica, Serratia marcesens, Bacillus pumilus* and *Bacillus subtilis*, and at the right plate: *Bacillus safensis, Bacillus pumilus* and *Enterocater ludwigii*.

Table 2. Effect of protease, bacteriocin, extracellular and volatile antifungal metabolites, and siderophore on inhibitory growth of *Verticilliun dahliae* in tomato plants, cv. Falat, inoculated with endophytic bacteria. Production of IAA and hydrogen cyanid by the endophytic bacteria.

Treatment	Protease (mm)	Bacteriocin (mm)	Extracellular metabolites (mm)	Volatile antifungal metabolites (mm)	Siderophore (10-µl ferric chloride)	Concentration of IAA (µg/ ml)	Optical density at 530 nm	Hydrogen cyanid
P. baetica NZ101	1.33 ± 0.01 a	1.23 ± 0.01 e	2.76 ± 0.01 c	1.50 ± 0.05 e	1.33 ± 0.01 a	25	0.059	–
S. marcesens NZ102	0.23 ± 0.01 e	1.89 ± 0.05 c	3.69 ± 0.03 b	3.69 ± 0.03 b	0.80 ± 0.005 c	25	0.059	+
B. pumilus NZ103	0.63 ± 0.01 c	2.89 ± 0.001 d	3.29 ± 0.02 b	2.73 ± 0.001 c	1.20 ± 0.01 ab	3.12	0.004	+
B. subtilis NZ104	0.83 ± 0.01 b	1.23 ± 0.01 e	2.76 ± 0.01 c	1.65 ± 0.05 e	1.20 ± 0.01 ab	12.5	0.025	-
B. pumilus NZ106	0.23 ± 0.01 e	2.06 ± 0.01 b	3.29 ± 0.02 b	2.50 ± 0.01 d	1.20 ± 0.01 ab	3.12	0.004	+
B. safensis NZ107	0.63 ± 0.01 c	2.16 ± 0.01 ab	2.76 ± 0.01 c	1.65 ± 0.05 e	0.80 ± 0.005 c	12.5	0.025	-
E. ludwigii NZ108	0.00 ± 0.00 f	1.23 ± 0.01 e	2.76 ± 0.01 c	1.65 ± 0.05 e	1.33 ± 0.01 a	25	0.059	-

Means followed the same letters are not significantly different ($P < 0.0001$). Separation of means using Duncan's Multiple Range Test.

Discussion

One of the most applicable methods for tomato wilt disease management caused by *V. dahliae* is the use of biological control agents, such as fungal and bacterial antagonists, both *in vitro* and *in vivo* conditions. Furhermore, disease control along with plant growth promotion traits has been considered as a novel method in tomato Verticillium wilt disease management all over the world (Martin and Bull, 2002; Munif *et al*., 2012; Thomas and Upreti, 2015). In this study, 688 endophytes bacteria were isolated from roots, stems, leaves and fruit of tomato plants and their antagonistic activities against *V. dahliae* were evaluated. Among them, 39 bacterial isolates with negative hypersensitivity reaction (HR) on tobacco and geranium and failure to decay potato slices were selected for greenhouse investigations.

Seven endophytic bacterial isolates, *B. pumilus* (NZ103 and NZ106)*, B. subtilis* NZ104*, S. marcesens* NZ102*, E. ludwigii* NZ108, *B. safensis* and *P. baetica* NZ108*,* were found to be highly potent in controlling *V. dahliae* and reducing the severity of the symptoms by 98%. In addition, the endophytes could accelerate the growth of tomato plants, based on several growth characteristics, in greenhouse conditions. These findings are in agreement with the results of Martin and Bull (2002) and Naraghi *et al*. (2008), supporting the fungal and bacterial antagonists and bacterial endophytes application against Verticillium wilts in other crops. Munif *et al*. (2012) identified 32 genera of endophytic bacteria in tomatoes enable to control fungal pathogens with dominant genera *Bacillus* and *Pseudomonas* against *Fusarium* and *Rhizoctonia*.

Tomato seeds infested with *V. dahliae* and treated with individual endophytic bacteria of the seven isolates demonstrated that some bacterial metabolites play a significant role in disease symptoms reduction in comparison with the controls i.e. with respect to the mechanisms investigated, it was established that *P. baetica* NZ101 could produce the highest protease and auxin IAA level, whereas, the isolates of *S. marcesens* NZ102 and *E. ludwigii* NZ108 could only produce auxin IAA in order to modulate plant growth. These results are consistent with the Gull and Hafeez (2012) findings, indicating that 8 isolates of *Pseudomonas fluorescens* could increase plant growth and also reduce root decay disease by *R. solani* in wheat plants. The results are also consistent with the research work presenting that bacteria isolated from canola roots and shoots not only improved the seed germination, seedling length and rapeseed and tomato growth, but also lowered the disease symptoms of *V. dahliae* and *F. oxysporum* (Nejad and Johnson, 2000). In respect to hydrogen cyanide production, three isolates of *S. marcesens* NZ102 and *B. pumilus* NZ103 and 106 were able to produce hydrogen cyanide in glycine containing medium. The results were in agreement with the results of Gall and Hafeez (2012).

The production of secondary metabolites in producing various antibiotics, enzymes and biofilm is well documented (Morikawa, 2006). The endophytic bacteria *E. ludwigii* NZ108, *P. baetica* NZ108*, B. subtilis* NZ104 and *B. pumilus* (NZ103 and NZ106) produced siderophore on PDA medium containing 10-µl ferric chloride iron to reduce or inhibit *V. dahiae* growth. The three first species produced bacteriocin that could reduce *V. dahiae* growth by 70% as well as volatile antifungal metabolites and diffusible inhibitors in agar media to decrease the normal mycelial growth by 68%. Henis and Inbar (1986) report that extracellular and volatile antifungal compounds production by *B. subtilis* strains were effective in reducing plant diseases. According to Ongena *et al.* (2008), *B. subtilis* can induce resistance to pathogens by producing secondary metabolites.

In conclusion, this study isolated, identified and evaluated the endophytic bacteria diversity in different tomato plant organs from tomato plants sampled in Iran in relation to their effect on Verticillium wilt disease control and plant growth. Four endo-

phytic bacterial isolates, *P. baetica* NZ101, *B. subtilis* NZ104, *E. ludwigii* NZ108 and *B. safensis* NZ107 inhibited fungal growth and indirectly stimulated plant growth by producing different types of antimicrobial metabolites. Since no similar research has been performed in Iran before, the results are notable for future application of these most promising bacterial endophytes in biocontrol of the destructive Verticillium wilt in greenhouse tomato.

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Ενδοφυτικά βακτήρια της τομάτας στην αντιμετώπιση της αδρομύκωσης και το πλεονέκτημά τους στη βιοδιέγερση

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Στη μελέτη αυτή απομονώθηκαν 688 ενδοφυτικά βακτηριακά στελέχη από φυτά θερμοκηπιακής και υπαίθριας καλλιέργειας τομάτας, τα οποία συλλέχθηκαν από τις επαρχίες της Τεχεράνης και του Alborz, Ιράν και ελέγχθηκαν ως πιθανοί ανταγωνιστικοί παράγοντες έναντι του μύκητα *Verticillium dahliae*. Ακολούθησε περαιτέρω επιλογή 128 αντιπροσωπευτικών απομονώσεων βάσει της διαφορετικότητας των φαινοτυπικών χαρακτηριστικών τους. Στη συνέχεια μετά από δοκιμή υπερευαισθησίας αποκλείστηκαν τα στελέχη που εμφάνισαν θετική αντίδραση σε φύλλα καπνού, γερανίου και στην υγρή σήψη της πατάτας. Βάσει αυτών επιλέχθηκαν συνολικά 39 απομονώσεις για περαιτέρω δοκιμές ανταγωνισμού *in vitro* και σε συνθήκες θερμοκηπίου. Από αυτά, επτά ενδοφυτικά ανταγωνιστά στελέχη με τα πλέον επωφελή χαρακτηριστικά ταυτοποιήθηκαν βάσει της ανάλυσης του γονιδίου 16S rRNA ως *Bacillus pumilus* (δύο στελέχη), *Bacillus subtilis, Bacillus safensis, Enterobacter ludwigi, Serratia*

marcesens και *Pseudomonas beatica*. Αξιολογήθηκε η βιολογική δράση των απομονώσεων με τη χρήση μεθόδων επεξεργασίας σπόρων και διαβροχής εδάφους σε δύο ποικιλίες τομάτας. Τα αποτελέσματα έδειξαν ότι και οι επτά βακτηριακές απομονώσεις είχαν υψηλό δυναμικό για τον έλεγχο του μύκητα και μείωσαν τη δριμύτητα της ασθένειας κατά 95-98%. Αυτή η ανταγωνιστική δράση συνδυάστηκε με την αύξηση ορισμένων παραμέτρων ανάπτυξης όπως το ξηρό βάρος φυτού, το ξηρό βάρος ρίζας, το ύψος φυτού, το μήκος ρίζας, το νωπό βάρος ρίζας και το νωπό βάρος φυτού, κατά 92-98%. Η διερεύνηση των μηχανισμών βιολογικής δράσης τους έδειξε παραγωγή πρωτεάσης από όλες τις απομονώσεις, με υψηλότερα επίπεδα, έως 65%, από τα στελέχη *E. ludwigii* και *P. beatica*. Τρεις από τις απομονώσεις που παράγουν βακτηριοσίνες ανέστειλαν την ανάπτυξη του μυκηλίου του φυτοπαθογόνου μύκητα έως και 70% σε δοκιμή διπλής καλλιέργειας. Επίσης, πέντε από τις απομονώσεις παράγουν σιδηροφόρα, ενώ τα ενδοφυτικά βακτήρια *P. baetica, S. marcesens* και *E. ludwigii* παρήγαγαν αξιοσημείωτη ποσότητα αυξίνης.

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Infections of bean plant and field soil are linked to region, root rot pathogen and agro-ecosystem

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Summary Understanding pathogen-agrosystem interaction is particularly essential when applying a control method to minimize pathogen prevalence prior to plant infection. To meet this requirement, frequency of major root rot pathogens isolated from bean root and seed, and their soil populations were examined in farmers' fields. Multivariate analyses evidenced more frequent isolations of *Fusarium solani* and *Rhizoctonia solani* from root and seed compared to *Macrophomina phaseolina* and *Fusarium oxysporum*. Two Fusarium species had denser soil populations than *R. solani* and *M. phaseolina*. More frequent isolations of pathogens were detected in root and seed collected from Abhar and Khodabandeh compared to Kheirabad region. Agronomic and soil variables corresponded less closely to root infections compared to soil infestation and seed infections. Bean market class, herbicide application, and planting depth were linked to root, seed and soil infestations. Such information provides a basis for increased confidence in choosing appropriate control strategies for a pathogen and region in sustainable agriculture.

Additional keywords: Fungus, Legume, *Phaseolus vulgaris,* principal component analysis, soil-borne

Introduction

Common or dry bean (*Phaseolus vulgaris* L.) is considered as an essential legume food crop in Iran where more than 116,000 ha of bean crops are planted annually (Anonymous, 2014). Under various soil and environmental conditions, soil-borne pathogens may infect beans in any possible combination and cause root-rot-disease complexes (Abawi and Pastor Corrales, 1990; Naseri and Hemmati, 2017).

Our earlier findings based on assessing commercial bean fields demonstrated that the most prevalent of root rot pathogens are: *Fusarium solani* (Mart.) Saec. f. sp. *phaseoli* (Burk.) Snyd. and Hans, *Rhizoctonia solani* Kuhn, *Macrophomina phaseolina* (Tassi) Goid., and *F. oxysporum* Schlechtend:Fr. Moreover, reductions in Zanjan's bean production due to root rots could be estimated as high as 65% (Naseri, 2008). Later and shallower planting, irrigating at 6-9 days intervals, herbicide and manure use, higher soil organic matter and rhizobial nodulation, manual seeding, proper plant density, and sprinkler irrigation lowered root rot diseases and crop damage by these pathogens (Naseri and Tabande, 2017). Furthermore, growing beans after maize and seed treatment with fungicide lowered Fusarium root rot caused by *F. solani* and improved bean productivity (Naseri and Marefat, 2011; Naseri, 2014a; Naseri *et al*., 2016). Growing beans after potato and tomato decreased Rhizoctonia root rot caused by *R. solani* (Naseri and Moradi, 2015). High soil pH and sand use, and growing beans following legumes or cereals use intensified charcoal root rot caused by *M. phaseolina* (Naseri, 2014b). Cultivation in sandy soil with neutral pH reduced Fusarium wilt caused by *F. oxysporum* (Naseri, 2014c).

Although the above-mentioned studies focused on measuring disease symptoms in interaction with agro-ecological conditions, associations of agro-ecosystem with pathogens populations in bean root and

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seed, and field soil is little understood. Naseri and Mousavi (2015) examined interrelationships among development of root rots, bean yield and populations of pathogens isolated from root, seed and soil, which revealed that the intensity and spread of bean root rots are linked to high soil populations of *R. solani* and *F. solani* (Naseri and Mousavi, 2015). Hence, a better epidemiological understanding of agro-ecological indicators of pathogens populations in the plant and soil affecting this disease complex deserves more consideration. Moreover, based on our multi-point assessments over the bean growing season (Naseri and Mousavi, 2015), fungal species and experimental sites affected isolations of *F. solani*, *R. solani*, *M. phaseolina* and *F. oxysporum* from seed, soil and root. Based on this research, it would be interesting to compare these soil-borne pathogens for their potential to infect root, seed and soil in the bean-root-rot pathosystem. Furthermore, information on association of the region and cropping system with infections of the root, seed and soil by root rot pathogens would assist in identifying the importance of inoculum sources for each pathogen and region under commercial production conditions to improve experimental designs for disease control and sustainable agronomy purposes. Thus, the objectives of this investigation were to: (1) evaluate the effect of pathogen on frequencies of *F. solani*, *R. solani*, *M. phaseolina* and *F. oxysporum* isolated from root, seed and soil samples, (2) determine the influence of region on infections of root, seed and soil by the pathogens, and (3) compare the correspondences of pathogens infecting either root, seed or soil with 14 agronomic and soil descriptors.

Materials and Methods

Experimental site assessment

The present study area was estimated at 6149 ha in a North-Western part of Iran, Zanjan, with a cold semi-arid climate, hot and dry summer season from June to September. Zanjan province has 315.4 mm and 11.7°C mean annual rainfall and temperature, respectively. To assess soil populations of major root rot pathogens, 13 producers' fields were randomly selected from the main bean growing region as representative experimental sites. Agronomic and soil characteristics of the fields are represented in Table 1.

The methodology of collecting plant, seed and soil samples, fungal isolation, identification and pathogenicity confirmation have been reported earlier (Naseri and Mousavi, 2015). Briefly, monosporic isolates of *Fusarium* spp. isolated from root, seed and soil samples were transferred to carnation leaf agar and identified to species level according to key characteristics provided by Nelson *et al.* (1983). Isolates of *F. solani* f. sp. *phaseoli* were specified according to the greenhouse studies followed by specific molecular detection (Khodagholi *et al.,* 2013).

The following samples were collected at pod maturity stage of bean growth from either of three observations per field: (1) two to four symptomatic plants (wilt and yellowing along with cankered and discolored roots); (2) seeds of 50 bean plants; (3) four samples (*ca* 1 kg in total) from the soil surrounding bean roots.

Laboratory assessment

Concisely, four pieces per symptomatic root were surface sterilized (for 2 min in 1% NaOCl) and then cultured on potato dextrose agar (PDA, Difco, Detroit, MI, USA). After identification, isolation frequencies of *F. solani*, *R. solani*, *M. phaseolina* and *F. oxysporum* were defined as the total number of isolations of each fungal pathogen from all root segments (four 1 mm^3 pieces per root) per root sample expressed as percentage (Naseri, 2008).

A subsample of 12 seeds (48 seeds in total per field) from each seed sample, were surface sterilized, rinsed in sterile distilled water, and then placed onto PDA amended with 30 mg l^{-1} streptomycin (streptomycin sulfate, Sigma, St Louis, MO). After purification and identification, the isolation

Table 1. Agronomic and soil properties of commercial bean fields studied.

Factors	Fields												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Bean market class[a]	P	W	R	R	R	P	R	R	R	P	R	R	W
Field size (ha)	2	2	1.2	1	1	0.5	1	1.5	1	0.3	3	0.3	2
Herbicide[b]	T	P	T	T	0	B	T	T	R	T	T	T	P
P-Fertilizer[c]	1	1	0	0	1	1	0	0	0	1	0	0	1
Planting date (day)	26	30	30	30	30	27	35	30	30	30	10	16	20
Planting depth (cm)	5	4	4	5	15	5	10	10	7	6	10	5	8
Previous crop[d]	W	A	W	W	B	P	P	A	A	W	W	P	W
Region[e]	A	A	A	A	A	K	K	K	K	K	O	O	O
Soil EC (ds/m)	1.3	3.6	3.5	1.3	2.9	1.7	2.2	1.1	2.1	3.3	1.8	1.5	1.5
Soil pH	7.7	7.4	7.6	7.7	7.5	7.5	7.5	7.4	7.7	7.7	7.8	7.5	7.4
Clay (%)	24	38	32	44	30	36	31	44	32	38	44	32	40
Sand (%)	54	32	36	26	36	28	38	18	36	28	14	36	28
Silt (%)	22	30	32	30	34	36	31	38	32	34	42	32	32
Urea[c]	1	1	1	0	1	0	1	0	1	1	1	0	0

[a] P = Pinto, R = Red, and W = White beans.
[b] B = bentazon, P = paraquat, R = roundup, and T = trifluralin.
[c] 0 = Not applied and 1 = applied.
[d] A = Alfalfa, B = bean, P = potato and W = wheat.
[e] A = Abhar, K = Kheirabad and O = Khodabandeh.

frequencies of each major root rot pathogen was determined as the total number of isolations from 12 seeds expressed as a percentage (Naseri and Mousavi, 2015).

For soil samples, 1 ml aliquots of the 10^{-4} dilution of a soil subsample in Ringer's solution were plated on soil-extract-streptomycin-agar (James, 1958). The number of colony forming units (CFU) per gram for the four root rot pathogens was counted. Thus, 52 soil samples collected from 13 fields were examined (Naseri and Mousavi, 2015).

Statistical analysis

In an attempt to improve data homogeneity, data for the soil populations of root rot pathogens at pod maturity stage were log-transformed before statistical analysis. In order to simplify interpretations of significant associations within the large data set, principal component analysis (PCA) was used to compare root rot pathogens infecting root, seed and soil between bean growing regions (R software, Development Core Team, 2006). Thus, a total of 12 infection variables were described for the three infection courts (root, seed and soil) and four fungal pathogens. The non-metric variables, pathogen species and region, were regarded as factors in the first and second PCAs to determine the significant differences in the soil populations and frequencies of isolations from roots and seeds between the pathogens and regions. The PCA method enabled joint analysis of the regional information at which variables were of a heterogeneous nature. This also allowed us to reduce the number of variables accounting for the total variance in the dataset. A new variable, which was defined by PCAs, combined the 12 infection variables into a linear regression. The significance of differences was examined using Monte Carlo permutation tests. The third PCA compared the associations of agronomic and soil variables with root, seed and soil infestations.

Results

Pathogen effect

All the four major bean-root-rot patho-

gens, *F. solani*, *R. solani*, *M. phaseolina* and *F. oxysporum*, were isolated from root, seed and soil samples collected from the 13 farmers' fields studied as experimental sites. In the two-dimensional plot of the first PCA (PCA1) based on frequencies of the pathogens isolated from roots and seeds, and soil populations examined at 13 experimental sites, the first principal component (PC1) accounted for 57.6% of the total data variance. The horizontal axis, PC1, was termed as plant infection indicator due to receiving high positive loadings from frequencies of the four pathogens isolated from roots (0.86) and seeds (0.89; Fig. 1). PC1 accounted for the largest proportion of the total variance and may, therefore, be considered as the major descriptor of infections caused by bean-root-rot pathogens under production field conditions.

The vertical axis, PC2, captured 30.2% of the data variance. This PC was termed the soil infestation indicator because of having a high positive loading (0.89) for the density of root-rot-pathogen populations in field soils (Fig. 1). This significant contribution of pathogens populations in the soil to the second PC of PCA1 demonstrated the considerable role of prevalence of the bean-root-rot pathogens studied in field soil to characterize fungal infections associated with this complex soil-borne disease in bean crops.

The multivariate analysis, PCA1, demonstrated that the infection of root, seed and soil significantly differed among the four major root rot pathogens (Monte Carlo permutation test; $P = 0.0019$; Fig. 1). The horizontal axis, plant infection indicator, distinguished *F. solani* and *R. solani* from *M. phaseolina* and *F. oxysporum*, indicating more frequent isolations of *F. solani* and *R. solani* from root and seed samples in comparison with *M. phaseolina* and *F. oxysporum*. In addition, the highest and lowest plant infections were detected for *F. solani* and *F. oxysporum*, respectively. Based on the vertical axis, soil infestation indicator, *F. solani* and *F. oxysporum* were distinguished from *R. solani* and *M. phaseolina*. This indicated denser soil populations of the two Fusarium species than *R. solani* and *M. phaseolina* in the bean-root-rot pathosystems studied. *F. solani* and *R. solani* had the densest and sparest populations in the soils sampled from bean production fields.

Region effect

All the four major bean-root-rot pathogens, *F. solani*, *R. solani*, *M. phaseolina* and *F. oxysporum*, were isolated from root, seed

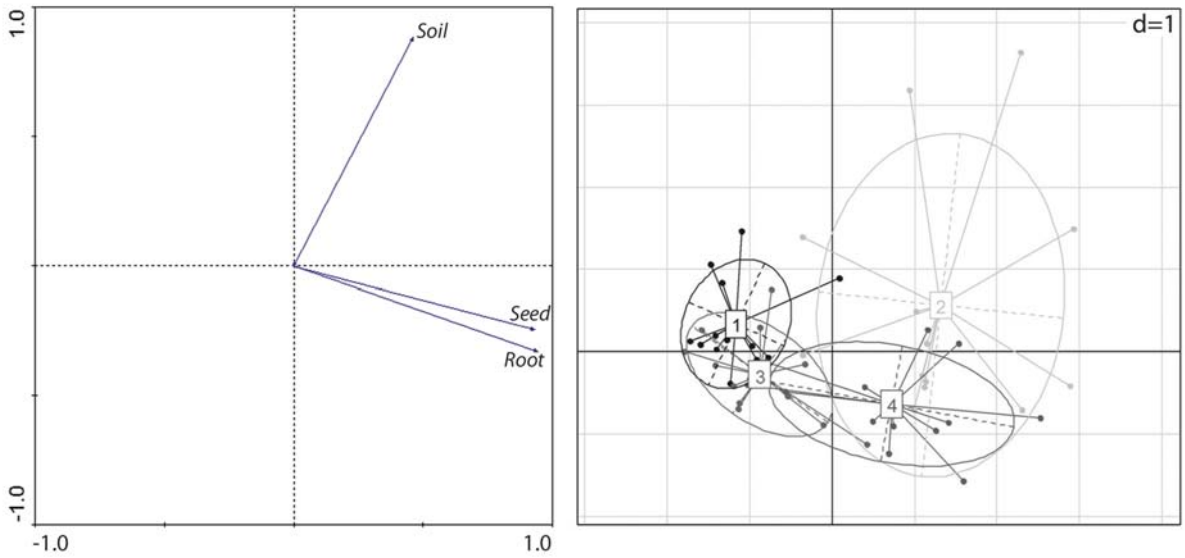

Figure 1. Principal component analysis (PCA1) of infections by *F. oxysporum* (1), *F. solani* (2), *Macrophomina phaseolina* (3) and *Rhizoctonia solani* (4) in bean root and seed, and field soil according to pathogen.

and soil samples collected from the three study regions (Abhar, Kheirabad, Khodabandeh). According to the second PCA (PCA2), the infection of root, seed and soil significantly differed among the three main bean growing Zanjan's regions studied (Monte Carlo permutation test; $P = 0.079$; Fig. 2). The horizontal axis (plant infection indicator) of PCA2 distinguished Abhar and Khodabandeh regions from Kheirabad, indicating more frequent isolations of the pathogens from root and seed samples collected from Abhar and Khodabandeh when compared to Kheirabad region. In addition, the highest and lowest plant infections were detected for Khodabandeh and Kheirabad regions, respectively. Based on the vertical axis (soil infestation indicator), Abhar region was distinguished from Khodabandeh and Kheirabad regions. This indicated denser soil populations of the root rot pathogens in the soil of Abhar's bean fields compared to Khodabandeh and Kheirabad. Among the regions studied, the densest and sparest populations of the pathogens in the soils were sampled from bean fields located in Abhar and Kheirabad, respectively.

Association of infection with agro-ecosystem

According to the third PCA, the five principal components accounted for 75.1% of total data variance (Table 2). The first PC of PCA3 determined the associations of bean class and herbicide application with frequencies of *F. oxysporum* isolated from root and *F. solani*, *M. phaseolina* and *R. solani* isolated from seed samples. PC2 indicated linkages of bean class, P-fertilizer application and soil EC to frequencies of *F. oxysporum* and *F. solani* isolated from bean seed, and soil populations of *F. oxysporum* and *R. solani*. Based on loading values obtained for PC3, planting depth corresponded with isolation frequencies of *F. solani*, *M. phaseolina* and *R. solani* from bean root and soil population of *R. solani*. PC4 described relationships between planting depth, soil pH, urea application, frequency of *F. oxysporum* isolated from seed, and soil populations of *F. oxysporum* and *M. phaseolina*. Herbicide application, planting date and previous crop were associated with soil populations of *F. solani* and *R. solani* according to PC5 loadings.

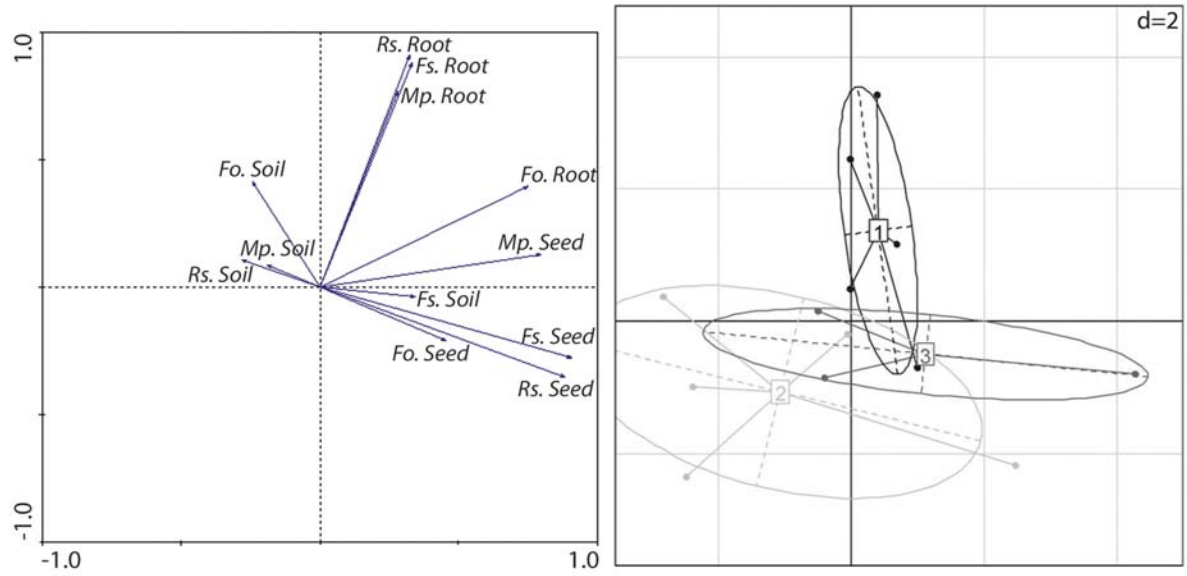

Figure 2. Principal component analysis (PCA2) of infections by *F. oxysporum* (Fo), *F. solani* (Fs), *Macrophomina phaseolina* (Mp) and *Rhizoctonia solani* (Rs) in bean root and seed, and field soil collected from Abhar (1), Kheirabad (2) and Khodabande (3) according to region.

Table 2. Principal component analysis of infections by *F. oxysporum* (Fo), *F. solani* (Fs), *Macrophomina phaseolina* (Mp) and *Rhizoctonia solani* (Rs) in bean root and seed, and field soil in association with agronomic and soil properties.

Variables	Principal components				
	1	2	3	4	5
Bean market class	**0.28**[a]	**-0.28**	-0.23	-0.11	0.13
Crop grown before bean	0.08	0.05	-0.20	0.03	**0.30**
Date of planting	-0.07	-0.12	0.04	0.00	**-0.58**
Depth of planting	-0.17	-0.00	**0.27**	**-0.39**	0.00
EC of field soil	-0.04	**-0.36**	0.04	0.20	-0.20
Herbicide application	**0.31**	0.11	-0.19	0.05	**-0.28**
P-fertilizer application	0.13	**-0.39**	-0.15	-0.15	0.05
PH of field soil	-0.09	0.07	0.10	**0.34**	0.14
Sand content	0.14	-0.13	0.18	0.24	-0.21
Urea application	-0.07	-0.24	0.23	**0.29**	-0.12
Fo in root	**0.34**	0.01	0.24	0.23	0.14
Fo in seed	0.19	**0.26**	-0.04	**-0.27**	-0.05
Fo in field soil	0.17	**-0.26**	-0.19	**-0.31**	-0.10
Fs in root	0.21	-0.23	**0.38**	-0.05	0.02
Fs in seed	**0.35**	**0.27**	-0.03	0.21	0.02
Fs in field soil	0.20	-0.21	-0.15	-0.07	**-0.29**
Mp in root	0.14	-0.05	**0.42**	-0.07	0.17
Mp in seed	**0.39**	0.17	0.09	-0.14	-0.04
Mp in field soil	-0.02	-0.19	-0.20	**0.41**	0.12
Rs in root	0.23	-0.19	**0.35**	-0.12	0.16
Rs in seed	**0.34**	0.21	-0.08	0.18	-0.13
Rs in field soil	0.08	**-0.30**	**-0.26**	0.01	**0.39**
Eigenvalues	4.4	4.0	3.5	2.6	2.1
Percentage variation	19.8%	18.2%	15.8%	11.8%	9.5%

[a] Loading values were regarded significant (bold), if > 0.25.

Discussion

Comprehensive understanding of parameters that influence the patterns of pathogens spread within cropping systems provide the essential epidemiological information to develop more efficient disease management strategies and subsequently improve productivity. In field surveys like that performed by Sahile *et al.* (2008) in northern Ethiopia, influences of crop management practices on faba bean chocolate spot (*Botrytis fabae*) and soil population fluxes were determined. Bruton and Reuveni (1985) and Höper *et al.* (1995) looked at soil populations of *M. phaseolina* and *F. oxysporum,* respectively. Mengistu *et al.* (2009) also examined the combined effects of tillage, cover crop and herbicide use on root, stem and soil infestations by *M. phaseolina* at plot scale. However, no previous study has compared the relative infectivity of different bean root rot pathogens to plant and soil. In fact, all the previous studies looked at individual root rot pathogen, and thus, the joint interactions of four major root rot

pathogens with the bean cropping system are little understood.

Comparative studies such as the present macro-scale research improve our understanding of pathogens prevalence in the crop and soil depending on pathogen and soil. Although Naseri and Mousavi (2015) reported interrelationships among root rot pathogens infecting root, seed and soil, the effects of pathogen and region on such associations remained little understood. The findings of the present study evidenced that specifications regarding pathogen prevalence for each inoculum source and cultivation area are required to be considered in organizing the integrated management of bean root rots as a complex disease. Such information may lead us to draw up specific root-rot-control programs corresponding to the pathogen and region, e.g. with avoiding unnecessary fungicide applications.

Although all four major bean-root-rot pathogens were isolated from root, seed and soil samples collected from the 13 experimental sites studied, their isolation frequencies from the root, seed and soil differed by pathogen based on our PCA results. The first principal component explained 58% of variance in the data on pathogens prevalence in bean roots and seeds, and field soils. The PC1 as plant infection factor also recognized root and seeds infections as more important descriptors of the bean-root-rot pathosystem compared to soil population. A large number of previous studies focused on evaluating patterns of *F. solani, F. oxysporum, M. phaseolina* and *R. solani* populations in the soil. For instance, Lodha *et al.* (1990) and Amir and Alabouvette (1993) looked at soil populations of *M. phaseolina* and *F. oxysporum*, respectively. According to the present findings, the assessment of root and seed infected by the pathogens under agro-ecological conditions should provide more accurate representations of these rhizospheric events for yield-estimate, disease-control, and resistance-screening purposes. Thus, estimating root and seed infections according to bean root rot intensity not only simplifies pathologists' tasks to quantify seed-borne inocula, but also improves our epidemiological insight for management purposes. Although the first part of this statement was achieved by Naseri and Mousavi (2015), the second aim was approached with the help of the present findings.

The similar PC1 loadings for the frequencies of pathogens isolated from roots and seeds evidenced a close importance of these two plant parts in being infected by the four pathogens. One may conclude that the level of bean root rots caused by *F. solani*, *F. oxysporum*, *M. phaseolina* and *R. solani* corresponds strongly to their seed infections. Provided that future small-scale experimentation confirms our observation, the infection of seeds could be estimated by detecting the frequency of pathogens isolated from bean roots at pod maturity. Considering the close linkage of bean root rot epidemics to root and seed infections (Naseri and Mousavi, 2015), it is possible to estimate infections of the root and seed according to an easier field-assessment of the disease. For instance, fungicidal treatment of seeds produced under severe bean-root-rot epidemics should be performed for a safe cultivation or consumption in sustainable agriculture.

Furthermore, PC2 recognized the soil population as the third important criterion of bean-root-rot pathogens prevalent in bean crops. The remainder of our multivariate analysis demonstrated significant differences in infections of root, seed and soil among the four major root rot pathogens. The plant infection axis evidenced greater colonization of bean roots and seeds by *F. solani* and *R. solani* compared to *M. phaseolina* and *F. oxysporum*. Under agronomic and environmental conditions encountered in this research, *F. solani* was identified as the most pathogenic causal agent of bean root and seed rots followed by *R. solani*, *M. phaseolina* and *F. oxysporum*. Considering the significant effect of Fusarium root rot caused by *F. solani* on bean production (Naseri and Mousavi, 2015), the priority of developing an integrated root-rot-control program should be set on those strategies that

restrict *F. solani* colonization in beans. Furthermore, the soil infestation axis indicated denser soil populations of *F. solani* and *F. oxysporum* than *R. solani* and *M. phaseolina* in the bean-root-rot pathosystems studied. This time, the priority of soil management methods should be given to lowering the two Fusarium species prevalence in field soil. To the best of our knowledge, this is the first record of a joint comparison of *F. solani*, *F. oxysporum*, *M. phaseolina* and *R. solani* infecting root, seed and soil at a large scale of bean crops. This study added new information on comparisons of root and seed infections, and soil populations according to the pathogen and region factors supplementing existing knowledge reported by Naseri and Mousavi (2015).

Isolations of *F. solani*, *R. solani*, *M. phaseolina* and *F. oxysporum* from root, seed and soil samples were recorded for the three regions, Abhar, Kheirabad and Khodabandeh studied here. It was of interest to detect more prevalent infection court in each main bean growing area for setting research and disease-control priorities in accordance with regional infection levels. According to our PCA analysis, the intensity of plant and soil infestations varied depending on the region. The plant infection axis evidenced more frequent isolations of the root rot pathogens from roots and seeds collected from Abhar and Khodabandeh than from Kheirabad. The soil infestation axis indicated denser soil populations of the root rot pathogens in the soil of Abhar compared to Khodabandeh and Kheirabad. Thus, root-rot-control and soil-management strategies should be organized to limit the prevalence of *F. solani* and *F. oxysporum* more effectively than *R. solani* and *M. phaseolina* in bean plants of Khodabandeh and field soils of Abhar. Among the regions studied, the lowest plant infections and sparest soil populations of the pathogens were detected in Kheirabad.

The identification of agronomic and soil characteristics causing such regional differences in the prevalence of pathogens within the plant and soil could provide valuable information for developing disease control programs. The present results linked the pathogen populations in the soil to bean market class (Pinto, Red and White), herbicide and urea applications, P-fertilizer, planting date and depth, previous crop, soil EC and pH. Seed infections were associated with bean class, herbicide and urea applications, P-fertilizer, planting depth, soil EC and pH. Root infections also corresponded to bean class, herbicide application, and planting depth. Therefore, soil, seed and root infections by root rot pathogens were related to nine, seven and three agro-ecological variables tested in this study, respectively. To the best of our knowledge, this is the first report of comparing infections of root, seed and soil by bean root rot pathogens in association with a number of agronomic and soil variables under commercial field conditions across different geographical areas of Iran.

In conclusion, the agro-ecosystem was less influential on pathogens infecting the root when compared with pathogens occurring in the soil and the bean seed. Moreover, the significant contributions of bean class and planting depth into root, seed and soil infestations indicated the promising potential of these two environmental-friendly agronomic practices to control bean root rots. Lowering pathogens populations appears as important as root rot control in bean crops. For instance, the biocontrol of bean root rot pathogens in field soil may reduce crop damage as effective as disease control in terms of genotypic resistance or fungicide application. The linkages of soil suppressiveness to herbicides for *F. solani,* soil EC for *F. oxysporum* f. sp. *lactucae*, texture for *R. solani*, pH for *F. oxysporum* f. sp. *lini*, and rotation for *F. solani* f. sp. *phaseoli* and *R. solani* have been reported previously (Burke and Kraft, 1974; Höper *et al.,* 1995; Toubia-Rahme *et al*., 1995; Otten and Gilligan, 1998; Chitarra *et al*., 2013). However, this study advanced the current knowledge via evidencing the joint associations of agro-ecological characteristics with root rot pathogens infecting bean root and seed, and field soil. Further research could include more agro-

ecological descriptors and their mechanisms to be examined in the bean-root-rot pathosystem.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Σήψεις ριζών σε καλλιέργεια φασολιού σε σχέση με την περιοχή, το είδος του παθογόνου και το αγρο-οικοσύστημα

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Η κατανόηση της επίδρασης του αγροοικοσυστήματος σε παθογόνα των φυτών είναι ιδιαίτερα σημαντική όταν εφαρμόζονται μέθοδοι καταπολέμησης με στόχο την παρεμπόδιση της επικράτησης των παθογόνων πριν αυτά να μολύνουν τα φυτά. Για το λόγο αυτό μελετήθηκαν η συχνότητα εμφάνισης σοβαρών παθογόνων που απομονώθηκαν από ρίζες και σπόρους φασολιού και προκαλούν σήψεις ριζών, καθώς και οι πληθυσμοί αυτών στο έδαφος καλλιεργούμενων χωραφιών με φασόλια. Πολυμεταβλητή στατιστική ανάλυση έδειξε μεγαλύτερη συχνότητα απομονώσεων των παθογόνων μυκήτων *Fusarium solani* και *Rhizoctonia solani* από τις ρίζες και τους σπόρους σε σύγκριση με τους μύκητες *Macrophomina phaseolina* και *Fusarium oxysporum.* Δύο είδη του γένους *Fusarium* είχαν υψηλότερους πληθυσμούς στο έδαφος σε σύγκριση με αυτούς των μυκήτων *R. solani* και *M. phaseolina*. Τα παθογόνα απομονώθηκαν σε μεγαλύτερη συχνότητα από ρίζες και σπόρους που συλλέχθηκαν από τις περιοχές Abhar και Khodabandeh, Ιράν, σε σύγκριση με την περιοχή Kheirabad. Οι αγρονομικές και εδαφικές μεταβλητές συσχετίζονταν λιγότερο με τη μόλυνση των ρίζων σε σύγκριση με τη μόλυνση του εδάφους και των σπόρων. Η εμπορική ποιότητα των φασολιών, η εφαρμογή ζιζανιοκτόνων και το βάθος φύτευσης συσχετίζονταν με τις προσβολές στις ρίζες, τους σπόρους και το έδαφος. Οι παράμετροι αυτές αποτελούν αξιόπιστη βάση για την επιλογή της κατάλληλης στρατηγικής αντιμετώπισης αυτών των παθογόνων σε μια περιοχή, στο πλαίσιο της βιώσιμης γεωργίας.

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Impact of *Bacillus subtilis* strains on survival and reproduction of grapevine phylloxera

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Summary Many strains of Plant Growth Promoting Rhizobacteria (PGPR) have the ability to induce the systemic resistance in several pathosystems. This study investigates the protective effect of four strains belonging to *Bacillus subtilis* (Bs168, Bs2500, Bs2504 and Bs2508) on grapevine roots of the sensitive local grapevine cultivar "Helwani" against phylloxera. Fresh roots were immersed in bacterial suspensions 0, 3, 5 and 15 hours before infesting with phylloxera eggs. Results of biometric measurements showed significant differences in the life cycle of phylloxera between treated and untreated roots. *Bacillus* strains negatively affected phylloxera development and reproduction. The efficiency of treatment increased when root immersion time in the bacterial suspension increased. On the other hand, there were significant differences between strains in terms of their protective effect against phylloxera, but Bs2508 strain was the most effective especially when the roots were immersed for 15 hours. This study supports previous studies in order to employing PGPR strains as biocontrol agents against pests.

Additional keywords: Biocontrol, grapevine, PGPR, Phylloxera, resistance, rhizobacteria

Introduction

Grapevine phylloxera, *Daktulosphaira vitifoliae* (Hemiptera: Phylloxeridae), is a small aphid-like insect that lives and feeds exclusively on the roots of grapevines and occasionally in distinctive galls on grapevine leaves. It causes direct damage to grapevine by forming damaging root galls (Granett *et al.*, 2001). Since, there is not an effective control method for grape phylloxera, it is considered as one of the most important grapevine pests in the world. Several methods to control phylloxera were suggested such as insecticides, irradiation and hot water dips. Those could be economically very useful in quarantine treatments against phylloxera (Granett *et al.*, 2001; Makee *et al.*, 2010). The only way to manage an infestation in the long term is to replant the vineyard using vines grafted to an American phylloxera-resistant rootstock. Makee *et al.*, 2003 reported that there was a great variation between tested local varieties concerning phylloxera life cycle. However, for yet unknown reasons, some rootstocks lost their resistance to phylloxera after many years of use in several parts of the world (Granett *et al.*, 1983). For example, rootstock B41 has remained resistant in France while it is not resistant in Californian vineyards; therefore, farmers have to replant their vineyards with the appropriate resistant rootstocks (Song and Granett, 1990; De Benedictis and Granett, 1993).

Little information on biological control of grape phylloxera is available. Some plant growth promoting rhizobacterial (PGPR) are able to stimulate inducible defense mechanisms that render the host plant less susceptible to a subsequent pathogen attack (Van Wees *et al.*, 2008; De Vleesschauwer and Höfte, 2009; Mandal and Ray, 2011; Weller *et al.*, 2012). This phenomenon called induced systemic resistance (ISR) (Pieterse *et al.*, 2002; Kloepper *et al.*, 2004). Was demonstrated in many plant species against a broad spectrum of fungal, bacterial and viral diseases and against insects and nematodes (Van Loon *et al.*, 1998; Ramamoorthy *et al.*, 2001; Durrant and Dong, 2004; Verhagen *et al.*, 2004; Valenzuela-Soto *et al.*, 2010;

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Walters *et al.*, 2013). ISR can be the basis of integrated management strategies to plant pests (Ramamoorthy *et al.*, 2001; Zehnder *et al.*, 2001; Saravanakumar *et al.*, 2007).

In this domain, a non-pathogenic *Pseudomonas putida* strain BTP1 was shown to enhance the level of resistance in several pathosystems (Ongena *et al.*, 1999; Ongena *et al.*, 2004; Adam *et al.*, 2008). Adam *et al.* (2012) illustrated the influence of *P. putida* BTP1 on life cycle of grapevine phylloxera on fresh roots of the local grape variety Helwani (*V. vinifera*), and *in vitro* on Ru140 and B41 rootstock roots. In other PGPR strains of *Bacillus subtilis* the capacity to enhance resistance in tomato and barley against fungal diseases has been demonstrated (Ongena *et al.*, 2007; Adam *et al.*, 2019). The main goal of this work was to study the protective effect of four strains of *B. subtilis* (Bs168, Bs2500, Bs2504 and Bs2508) on fresh roots of the local grape variety Helwani, and determine the influence of root immersion time in the bacterial suspension on survival percentage reproduction and development of grapevine phylloxera.

Materials and Methods

Insects

Grapevine phylloxera was originally collected from phylloxera-infested grapevine roots from fields in south Syria. The phylloxera colony was established on fresh and healthy pieces of roots (4-7 mm in diameter and 5-7 cm long) of the sensitive local grapevine cultivar Helwani (*Vitis vinifera*) following similar procedures to those mentioned by Makee *et al.* (2003).

Microbial strains

Four *B. subtilis* strains (Bs168, Bs2500, Bs2504 and Bs2508) were kindly provided by Prof. P. Tonart- University of Liege - Belgium. Bacterial strains were maintained in Petri plates containing 868 medium (10 g/l Glucose, 10 g/l Peptone, 10 g/l yeast extract and 15 g/l agar) (Jacques *et al.*, 1999). Plates were incubated at 30 ± 1°C for 24 h. Bacterial cells were collected and resuspended in 10 mM $MgSO_4$ to a final density of $2x10^8$ CFU/ml before use.

Treatment with bacterial strains

Fresh and healthy pieces of roots (4-7 mm in diameter and 10 cm long) were washed with tap water and soaked in bacterial suspensions ($2x10^8$ CFU/ml) for different time intervals: 0, 3, 5 and 15 h. For the control treatment, roots were soaked in distilled water for the same time intervals. The root pieces were left to air-dry. Each root piece was infested with 20 newly-laid phylloxera eggs (<24 h old), kept in a plastic box with tightly fitting lids and incubated at 25±1°C, 70±5% RH in darkness. The experiments were repeated twice; five roots were used in each treatment and each time interval.

A daily microscope inspection of all phylloxera stages on all roots was carried out. The number of eggs hatched, feeding nymphs and adults were detected to calculate the survival (% percentage of emerged mature females) in each treatment. Fecundity (total number of eggs) of phylloxera was evaluated for five randomly chosen individuals of root-feeding phylloxera females on each root. Thus, 25 females were examined in each treatment. The eggs laid by each female were counted until the female's death. Additionally, the oviposition period (the time from the first laid egg to the natural death of individual females) was recorded.

In vitro assay on grapevine plants with Bs2508 strain

Induced resistance assay was carried out on in vitro grapevine plants using the most effective strain (Bs2508) against phylloxera following similar procedures to those mentioned by Adam *et al.* (2013). Five plantlets (growing in tissue culture with two or three roots) were selected. One root of each plant was kept out of the medium within the tube while the other root remained in the medium. The one root was treated with 1 ml of bacterial suspension (10^8 CFU ml^{-1}) of Bs2508 strain or by distilled water for the control plantlets. Seven days later, the second

root was infested with sterile three-day-old eggs of phylloxera (20 eggs). The number of hatched eggs, nymphs and adults were recorded to determine the survival percentage (%) and development time (egg to egg). Five females were selected on each plant (25 females per treatment) to determine the mean oviposition period and fecundity of phylloxera.

Effect of Bs2508 strain on egg fertility of phylloxera

Two hundred phylloxera eggs were soaked in bacterial suspension of Bs2508 strain or in sterilized water for different immersion times (0, 3, 5, 15, 24, 48 and 72 h). The eggs were then placed on sterile filter paper in plates and incubated at 25±1°C, 70±5% RH in darkness until hatching.

Statistical analysis

All statistical analyses were performed using STATISTIC program version 6 (Statsoft, Inc. 2003) at 5% level (p= 0.05). Data were subjected to analysis of variance (ANOVA) for the determination of differences in means between treatments at each immersion time. Means were separated using Fisher's PLSD test.

Results

Effect of *B. subtilis* strains on survival and reproduction of phylloxera

There were significant differences in survival and reproduction of phylloxera on immersed roots between treatments in bacterial suspensions of Bs168, Bs2500, Bs2504 and 2508 strains compared to the control roots (Table 1). The immersion of grapevine roots in water for 3, 5 and 15 h led to a significant decrease in survival percentage (%) of mature females, which was more obvious at 15h (20%) compared to the control roots (0h). In bacterial suspensions of Bs2500 and Bs2508 strains, the survival percentage of phylloxera was significantly lower compared to control roots at different immersion time intervals ($F = 24.24$; df = 3, 16; $P < 0.0001$ and $F = 383.29$; df = 3, 16; $P < 0.0001$, respectively) (Table 1). Bs2508 was the most effective strain on the survival percentage especially at 15 hours. Bs168 and Bs2504 strains did not exhibit any effect on the survival percentage of phylloxera at all immersion time intervals.

The mean number mean of eggs differed significantly between treatments Bs168, Bs2500 Bs2504, Bs2508 strains and the control ($F = 466$; df = 3, 96; $P < 0.0001$) (Table 2). The fecundity of phylloxera decreased significantly with the increase of immersion time intervals in all treatments compared to the untreated roots (zero time) ($F = 400$; df = 3, 96; $P < 0.0001$) (Table 2). Bs2508 was the most effective strain on fecundity of phylloxera at 15 hours.

The oviposition period significantly decreased with the increase of immersion time intervals in bacterial suspension of Bs2500, Bs168 Bs2504 and Bs2508 strains compared to the control ($F = 168$; df = 3, 96; $P < 0.0001$) (Table 3). Bs2508 was the most effective strain on oviposition period of phylloxera as it declined the oviposition period about 24%

Table 1. Effect of the time of root immersion of grapevine cv. Helwani in bacterial suspension of *Bacillus subtilis* Bs168, Bs2500 Bs2504 and Bs2508 strains on survival percentage (%) of grape phylloxera *Daktulosphaira vitifoliae*.

Time (h)	Control	Bs168	Bs2500	Bs2504	Bs2508
0	87.5 ± 1.7 aA	86.4 ± 2.31 aA	72 ± 3.66 aB	87 ± 2.12 aA	87.1 ± 2.37 aA
3	78.2 ± 0.75 bA	74.46 ± 1 b A	48.2 ± 3.73 bB	75.8 ± 2.58 bA	36.8 ± 1.23 bC
5	76.6 ± 1.3 bA	73.2 ± 1.07 b A	50.6 ± 0.6 bcB	74 ± 2.47 bA	24.9 ± 1.32 cC
15	70 ± 0.91 cA	71.8 ± 1.07 b A	40.6 ± 1.5 cB	71.8 ± 1.39 bA	22.3 ± 0.78 cC

Percentages followed by the same capital letters (rows) and by the same small letters (columns) are not significantly different at $p < 0.05$ (Fisher's PLSD test).

compared to the control roots at 15 hours ($F = 232$; $df = 3, 96$; $p<0.0001$) (Table 3). Moreover, the oviposition period of phylloxera decreased significantly with the increase of immersion time interval in water.

Effect of *B. subtilis* Bs2508 strain on survival and reproduction of phylloxera on grapevine plantlets *in vitro*

The most efficient *B. subtilis* strain was selected for resistance inducing assays. In *in vitro* conditions, survival (%) ($F = 78.4$; $df = 1, 8$; $p <0.0001$), fecundity ($F = 123$; $df = 1, 48$; $p <0.0001$), oviposition period ($F = 303.9$; $df = 1, 48$; $p <0.0001$) and development period ($F = 355.6$; $df = 1, 48$; $p <0.0001$) of phylloxera significantly decreased in Bs2508-treated plantlets compared to those of the control (Table 4).

The fertility of phylloxera eggs were negatively affected when immersed in *B. subtilis* Bs2508 strain suspension. The percentage of egg fertility significantly decreased with the increasing of the immersion time interval in both Bs2508-treated and untreated eggs. A significantly lower percentage (39%) *of* hatched eggs was recorded at 15h immersing time interval, compared to the control (Figure 1).

Table 2. Effect of the time of root immersion of grapevine cv. Helwani in bacterial suspension of *Bacillus subtilis* Bs168, Bs2500 Bs2504 and Bs2508 strains on fecundity (total number of eggs) of grapevine phylloxera *Daktulosphaira vitifoliae*.

Time (h)	Control	Bs168	Bs2500	Bs2504	Bs2508
0	64.4 ± 0.74 aA	63.5 ± 1.09 aA	61.6 ± 1.26 aA	62.52 ± 1.14 aA	63.7 ± 1.43 aA
3	42.6 ± 0.61 bA	30.2 ± 0.81 bC	31.7 ± 1.16 bC	39.48 ± 1.11 bB	38 ± 0.46 bB
5	42.1 ± 0.66 bA	29.4 ± 0.75 bB	26.7 ± 0.67 cC	32 ± 0.39 cB	25 ± 0.7 cC
15	33 ± 0.65 cA	29 ± 0.68 bA	27 ± 0.66 cB	32.6 ± 0.57 cA	19.8 ± 0.26 dC

Percentages followed by the same capital letters (rows) and by same the small letters (columns) are not significantly different at $p< 0.05$ (Fisher's PLSD test).

Table 3. Effect of the time of root immersion of grapevive, cv. Helwani in bacterial suspension of *Bacillus subtilis* Bs168, Bs2500 Bs2504 and Bs2508 strains on the oviposition period (days) of grapevine phylloxera *Daktulosphaira vitifoliae*.

Time (h)	Control	Bs168	Bs2500	Bs2504	Bs2508
0	16.7 ± 0.2 aB	15.8 ± 0.73 aB	19.3 ± 0.55 aA	16.1 ± 0.42 aB	15.4 ± 0.41 aB
3	14.3 ± 0.16 bB	11.8 ± 0.18 bC	15.1 ± 0.18 bA	10.0 ± 0.21 bE	10.6 ± 0.16 bD
5	12.3 ± 0.12 cA	10.4 ± 0..22 cB	10.4 ± 0.27 cB	9.8 ± 0.38 bC	9.4 ± 0.16 cD
15	11.0 ±0.16 dA	9.5 ± 0.22 cB	9.0 ± 0.2 dC	8.5 ± 0.25 cD	7.6 ± 0.19 dE

Means followed by the same capital letter (rows) and by the same small letter (columns) are not significantly different at $p< 0.05$ (Fisher's PLSD test).

Table 4. Effect of *B. subtilis* Bs2508 strain on survival (%), fecundity, oviposition period and development period of grapevine phylloxera *Daktulosphaira vitifoliae, in vitro* in treated grapevine plants cv. Helwani.

Treatment	Fecundity (eggs)	Oviposition (days)	Survival (%)	Development period (day)
Bs2508	19.8 ± 0.6B	7.7 ± 0.2B	47.9 ± 3.5B	30.9 B
Control	36.1 ± 0.8A	11 ± 0.1A	76.4 ± 4.1A	35 A

Percentages followed by different letters (columns) are significantly different at $p < 0.05$ (analysis of proportions).

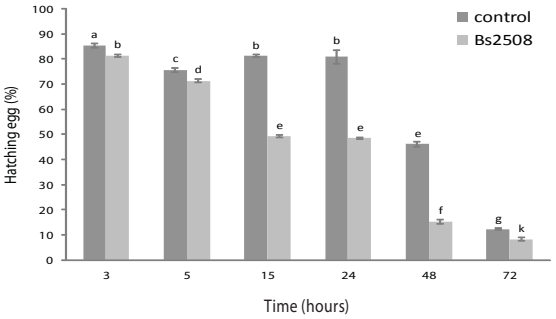

Figure 1. Effect of *B. subtilis* Bs2508 strain on the percentage of egg hatching of grapevine phylloxera *Daktulosphaira vitifoliae*. Each column represents data of five samples, each of 100 eggs. Data were subjected to ANOVA analysis and the differences between means were tested for significance using Tukey HSD test (values with different letters are significantly different at $P<0.001$).

Discussion

Gapevine phylloxera is considered as the most important pest in grapevine fields. To our knowledge, there is no effective method to control phylloxera (Makee *et al.*, 2010). However, the biological control by non-pathogenic rhizobacteria could be an alternative and supplemental control method (Ramamoorthy *et al.*, 2001). The current study aimed to evaluate the effect of four bacterial strains *B. subtilis* (Bs168, Bs2500 Bs2504 and Bs2508) against phylloxera by using the immersion method of grape roots in bacterial suspension. Our results illustrated that survival and reproduction parameters of phylloxera were decreased significantly in bacteria-treated roots, in particular by *B. subtilis* Bs2508 strain at 15 h. The reproduction and development of phylloxera decreased with the increase of immersion time interval suggesting that the control practice of immersing of grapevine roots in water could be enhanced by PGPR strains.

Our findings are in agreement with precedent studies using *P. putida* BTP1 which impacted negatively the ability of phylloxera to develop and increased grapevine resistance and tolerance toward this pest in bacteria-treated plants (Adam *et al.*, 2012; Adam *et al.*, 2013). Other studies indicated a positive effect of the dip technique in hot water to control phylloxera, black foot *Cylindrocarpon* sp. disease and the nematode *Radopholus similis* in dormant nursery grapevines and palm plants, respectively (Granett *et al.*, 2001; Tsang *et al.*, 2003; Arcinas *et al.*, 2005; Bleach *et al.*, 2013; Gramaje *et al.*, 2014).

The resistance of grapevine roots to phylloxera may be due to poor nutrition and a decline in the productivity of the insect (Granett *et al.*, 1983) indicating that in the present study phylloxera could not survive and feed on the treated roots by *B. subtilis* strains. Georgiev *et al.* (2014) showed that the temporary immersion in water could stimulate the production of secondary metabolites as the secoiridoid glycosides accumulation which are known to exhibit a wide array of biological and pharmacological properties. These secondary metabolites may play a major role in the reinforcement of the cell wall and serve as defense compounds against pathogens (Underwood, 2012; Michael Wink, 2015).

Future studies would be necessary to investigate the induced defense mechanism against control phylloxera by a PGPR, i.e. whether it is antibiosis due the interaction between the two organisms, antixenosis due the decrease of plant attractiveness to phylloxera (Granett *et al.*, 2001) or the produce of toxic compounds in plant tissues against the pest (Qingwen *et al.*, 1998; Zehnder *et al.*, 2001; Vijayasamundeeswari *et al.*, 2009). Moreover, further research is needed to determine the efficacy of the immersion of grapevine roots in bacterial suspensions of *B. subtilis* before using under field conditions.

In conclusion, this study showed a good efficiency of *B. subtilis* strains, in terms of lower survival, reproduction and development, to control phylloxera on fresh roots and grapevine plants *in vitro* especially by *B. subtilis* Bs2508 *strain* at 15 h of immersing time interval. Thus, it might contribute together with previous studies using PGPR strains to develop a biocontrol method against grape phylloxera in quarantine centers and nurseries.

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Επίδραση στελεχών του βακτηρίου *Bacillus subtilis* στην επιβίωση και την αναπαραγωγή της φυλλοξήρας της αμπέλου

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Περίληψη Πολλά στελέχη ριζοβακτηρίων που ενισχύουν την ανάπτυξη των φυτών (PGPR) έχουν την ικανότητα να επάγουν διασυστηματική ανθεκτικότητα σε αρκετά παθοσυστήματα. Η παρούσα εργασία διερευνά την προστατευτική δράση τεσσάρων στελεχών του βακτηρίου *Bacillus subtilis* (Bs168, Bs2500, Bs2504 και Bs2508) στις ρίζες αμπέλου της ευαίσθητης τοπικής ποικιλίας Helwani έναντι της φυλλοξήρας της αμπέλου. Παραγματοποιήθηκε εμβάπτιση ριζών σε βακτηριακά εναιωρήματα 0, 3, 5 και 15 ώρες πριν την έκθεση σε ωά της φυλλοξήρας. Τα αποτελέσματα των μετρήσεων βιομετρικών χαρακτήρων έδειξαν σημαντικές διαφορές στον κύκλο ζωής της φυλλοξήρας μεταξύ των ριζών που είχαν δεχτεί την επέμβαση και αυτών που δεν είχε γίνει επέμβαση. Τα βακτηριακά στελέχη επηρέασαν αρνητικά την ανάπτυξη και την αναπαραγωγή της φυλλοξήρας. Η αποτελεσματικότητα των επεμβάσεων αυξάνονταν με τον χρόνο εμβάπτισης στο βακτηριακό εναιώρημα ενώ παρατηρήθηκαν σημαντικές διαφορές στη δράση μεταξύ των στελεχών. Το στέλεχος Bs2508 ήταν το πιο αποτελεσματικό ειδικά όταν ο χρόνος εμβάπτισης των ριζών ήταν 15 ώρες. Τα ευρήματα υποστηρίζουν προηγούμενες μελέτες για τη χρήση στελεχών PGPR ως παραγόντων βιολογικής αντιμετώπισης εχθρών των καλλιεργειών.

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Toxicity of gaseous ozone to the different life stages of cowpea beetle, *Callosobruchus maculatus* (Coleoptera: Bruchidae), under laboratory conditions

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Summary The cowpea beetle, *Callosobruchus maculatus* (Fabricius), is recognized as a common insect pest of cowpea worldwide. The present study aimed to evaluate the insecticidal activity of ozone gas against all life stages (egg, larva, pupa and adult) of *C. maculatus* at a concentration of 600 ppm (1.2 g/m^3) after 6 exposure times of 0.5, 1, 2, 3, 4 and 5 h under laboratory conditions. The mortality percentages of all life stages were significantly increased with increasing exposure time. Similarly, longer exposure time to ozone caused higher reduction of adult emergence from eggs, larvae and pupae. Treatment with ozone for 5 h caused egg mortality of 72.3%. Total mortality of adults was observed 5 days after treatment with ozone for 0.5 h. Moreover, treatment with ozone for 5 h resulted in 75, 100 and 94.1% reductions in adult emergence from eggs, larvae and pupae, respectively. The results also indicated that the larvae and adults were more susceptible to ozone gas than the eggs and pupae. Based on the findings, ozone showed promising insecticidal activity against all stages of *C. maculatus* and could be used as potential fumigant for management of this insect.

Additional keywords: Cowpea beetle, fumigation, insecticidal activity, stored product insect

Introduction

Cowpea (*Vigna unguiculata* Linnaeus) is a multifunctional legume crop that plays an important role in both human food and animal feed. The high nutritional value of cowpea is due to the high content of proteins in dry seeds (Hall, 2004). *Callosobruchus maculatus* (Fabricius) is the most important and common pest of stored cowpea seeds in different regions of the world as well as in Egypt. This insect attacks stored cowpea and other legumes, contaminates infested seeds with its faeces and causes physical damage through its post-harvest feeding and reproductive activities (Ali *et al*., 2005; Musa and Adeboye, 2017). Generally, management of *C. maculatus* in stored products is mainly based on the use of chemical control means, such as fumigants and synthetic insecticides (Southgate, 1978; Akinkurolere *et al.,* 2006). However, the use of these chemicals nowadays is under argument because of environmental and safety concerns.

Recently, researchers have focused on screening and developing less hazardous and less expensive alternatives for controlling stored product insects. Among alternatives, ozone is currently attracting attention because of the inherent advantages of this gas (Mendez *et al*., 2003; Maier *et al.,* 2006). Ozone (O_3) is an allotrope of oxygen and is formed by subjecting air to an electrical discharge which makes ozone production a method of high sustainability. Ozone has a half-life of 20-50 min, rapidly decomposing to diatomic oxygen which is a natural component of the atmosphere (Kells *et al.,* 2001). Because ozone can be easily generated at the treatment site using only electricity and air, it offers several safety advantages over conventional post-harvest insecticides and fumigants. First, there are no stores of toxic chemicals or chemical mixing hazards, and there is no need to transport fumigant feedstock to a site or to consider its post-fumiga-

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tion disposal or collection. Second, because of its short half-life, ozone reverts back to naturally occurring oxygen, leaving no residue on the stored products (Law and Kiss, 1991). The US Food and Drug Administration (FDA) classified ozone for treating bottled water as ''generally recognized as safe'' (GRAS) (FDA, 1982) and has also approved its use as a direct additive for food treatment, storage and processing (FDA, 2001). However, discrepancies regarding the efficacy of ozone on stored products require further research for safe conclusions on its wider use, including the cost of its application, its ability to penetrate stored product grains and its use as an alternative to traditional fumigants (Jian *et al,*. 2013; Isikber and Athanassiou, 2015).

The effect of ozone gas on stored-product insects, such as *Sitophilus oryzae* (Linnaeus), *Ephestia kuehniella* Zeller and *Rhyzopertha dominica* (Fabricius), has been evaluated in several laboratory and field studies (Isikber *et al.,* 2007; Subramanyam *et al.,* 2014). In those studies, ozone showed toxicity and good potential for the control of stored-product insects, mostly focused on stored grain insects. However, there is limited information on the insecticidal activity of ozone against stored-legume insects, such as *C. maculatus*. The overall objective of the present study was to determine the effectiveness of ozone in the suppression of the cowpea beetles and its potential as an alternative to chemical insecticides. The specific objectives of the work were to determine: 1) the required ozone exposure time to achieve satisfactory mortality of the eggs, immature stages within cowpea, and adults of *C. maculatus*, 2) the relationship between mortality and time after ozone treatment, and 3) the effect of ozone treatment on adult emergence from immature stages (eggs, larvae and pupae).

Materials and Methods

Test insect

The cowpea beetle, *Callosobruchus maculatus* (F.), colony was originally obtained from the Plant Protection Research Institute, Dokki, Giza, Egypt. The beetles were reared on sterilized cowpea grains (*Vigna unguiculata* var. Cream 7) in 1-L wide-mouth glass jars in an incubator at 25 ± 2°C, with 65±5% relative humidity and a 12-h: 12-h light-dark photoperiod as described by Shu *et al.* (1996). The insect was maintained for several years without exposure to insecticides at the Department of Plant Protection, Faculty of Agriculture, Al-Azhar University, Cairo, Egypt.

Ozone generation technique

A laboratory corona discharge ozone generator (model OZO-6VTT) was provided by Ozomax Inc., Canada (http://www.ozomax.com). Ozone gas was generated from purified extra-dry oxygen feed gas. The generator was designed to produce up to 30 g of ozone per hour. A 3-liter test chamber was made of glass jar and equipped with two inlet and outlet valves and a pressure gauge to give pressure indication. The chamber was connected to the generator and ozone analyzer to measure the ozone concentration output from ozone generator (Ozone Analyzer BMT 964, BMT Messtechnik, Berlin, Germany).

Fumigation chamber

The fumigation chamber consisted of 3-L glass jar capped with a metal stopper equipped with tubes to allow the ozone gas to enter from the ozone generator and another to allow the air to exit the chamber. Two pieces of rubber tubing [5 cm long and 6.2 mm internal diameter (ID)] were attached to the other tubes and sealed with pinch clamps. The jars were sealed with silicone vacuum grease.

Exposure of different life stages of *C. maculatus* to ozone gas

The susceptibility of four stages (eggs, 4th instar larvae, pupae, adults) of *C. maculatus* exposed to 600 ppm (1.2 g/m^3) of ozone after six exposure time intervals was investigated at 25 ± 2°C. Stages of *C. maculatus* were obtained from stock cultures as fol-

lows: Five pairs were let to oviposit onto cowpea grains; grains bearing two to four eggs each, were placed in wire-mesh cages (3 cm ID × 5 cm length) to obtain a total of 50 eggs per cage. As larvae of *C. maculatus* feed and develop internally, radiography was used to follow larval and pupal development at 25±2°C and 65±5% r.h. (Mbata and Reichmuth 1996, Mbata *et al.,* 2000). Eggs hatched into first instars after 2 days. After hatching, the colour of the egg changed from white to cream white because of frass deposition in the eggshell. The fourth larval instar was attained between 14 and 16 days after egg laying, whereas the pupal stage was attained between 19 and 21 days. Thus, in these experiments, 14-day-old developing individuals were assumed to be fourth instar larvae. Moreover, grains infested with pupae could be identified as the pupae could be seen through an opaque pre-emergence "window" in the cotyledons of the grains. Ten g of cowpeas bearing a certain life stage of *C. maculatus* (fifty 24h-old eggs, 14-day-old larvae, 48h-old pupae, twenty 24 h-old adults) were placed in each wire-mesh cage and exposed to ozone concentration of 600 ppm (1.2 g/m^3) for 0.5, 1, 2, 3, 4 and 5 h in a fumigation chamber. Each treatment was replicated four times. The cages were kept in a climatic chamber at 25 ± 2°C with 65±5% r.h. and a 12-h: 12-h light-dark photoperiod.

After treatment, the eggs, neonates and adults were examined daily to determine the mortality percentages over 7 days. The mortality percentages were corrected with Abbott's formula (Abbott, 1925). The treated cowpeas infested with larvae or pupae were transferred to glass jars (50 ml), incubated under the same conditions and the number of emerged adults was recorded daily until no more adults emerged. The reduction percentages of adults emerged from eggs, larvae and pupae were calculated according to the formula of Henderson and Tilton (1955).

Data analysis

The mortality percentages of egg, neonate and adult and reduction from immature stages were subjected to Arcsin transformation prior to analysis. Analysis of variance (ANOVA) was performed and the means were compared with the Duncan's New Multiple Range Test (DMRT) with a significance level <0.05. Data analysis was conducted using SPSS 21.0 (SPSS, Chicago, IL, USA). The mortality percentages of eggs, neonates and adults after different exposure times were subjected to probit analysis (Finney, 1971) to calculate LT_{50} values and the respective 95% confidence intervals using the same SPSS software program.

Results

Effect of ozone gas on mortality of eggs and neonates

The mortality rates of eggs and neonates exposed to ozone concentration of 600 ppm (1.2 g/m^3) for different exposure times (0.5, 1, 2, 3, 4 and 5 h) are shown in Table 1. All exposure times caused significant mortality of eggs compared to the control treatment. Also, the egg mortality percentages gradually increased with increasing time of expo-

Table 1. Mortality percentages of eggs and neonates of *Callosobruchus maculatus* seven days after treatment with 600 ppm (1.2 g/m^3) of ozone for different exposure times[a].

Exposure time (h)	Egg mortality (% ±SE)	Neonate mortality (% ±SE)
0	0.0 ± 0.0 e[b]	0.0 ± 0.0 d
0.5	39.9 ± 0.9 d	30.7 ± 1.3 c
1	53.2 ± 0.9 c	31.4 ± 1.3 c
2	54.8 ± 1.1 c	32.2 ± 2.7 c
3	64.3 ± 2.1 b	37.5 ± 2.0 b
4	67.3 ± 0.6 b	39.8 ± 2.1 b
5	72.3 ± 1.3 a	57.1 ± 0.9 a
F	223.2	33.9
P	<0.01	<0.01
LT_{50} (h) (Confidence limits)	1.0 (0.6-1.4)	7.6 (4.2-48.5)

[a] Data are expressed as mean values ± SE from experiments with four replicates of 50 eggs each.
[b] Mean values within a column sharing the same letter are not significantly different (P < 0.05).

sure to ozone gas; egg mortality percentage was 39.9 % at exposure time of 0.5 h and reached 72.3 % at exposure time of 5 h (F = 223.2; df = 6.0; P <0.01). Moreover, exposure to ozone also resulted in significant mortality among the newly hatched larvae (neonates). The highest mortality of neonates (57.1%) was observed after 5 h of ozone exposure (F = 33.9; df = 6.0; P <0.01). The LT_{50} values were 1.0 and 7.6 h for eggs and neonates, respectively.

Effect of ozone gas on adult emergence

Table 2 and Figure 1 show the reduction percentages of adult emergence from *C. maculatus* eggs, larvae and pupae after treated with 600 ppm (1.2 g/m^3) of ozone at different exposure times (0.5, 1, 2, 3, 4 and 5 h). All the exposure times induced significant reduction in adult emergence of *C. maculatus* from eggs, larvae and pupae compared to the untreated control. Furthermore, the adult emergence declined drastically with the increase of the exposure time. The larvae were more susceptible to treatment with ozone than pupae and eggs, particularly at the longer exposure times of 2, 3, 4 and 5 h (eggs: F = 65.9 ; P <0.01; df = 6.0; larvae: F = 668.3 ; P <0.01; df = 6.0; pupae: F = 123.4 ; P <0.01; df = 6.0). The treatment of larvae, pupae and eggs with ozone induced 100, 94.04 and 75.0%, reduction in adult emergence, respectively, after 5 h exposure time.

Table 2. Reduction percentages of adult emergence of *Callosobruchus maculatus* from different life stages treated with 600 ppm (1.2 g/m^3) of ozone for different exposure times.[a]

Exposure time (h)	Reduction (% ± SE)		
	Egg	Larva	Pupa
0	0.0 ± 0.0 g[b]	0.0 ± 0.0 g	0.0 ± 0.0 g
0.5	13.6 ± 2.3 f	16.8 ± 2.1 f	16.7 ± 4.2 f
1	38.6 ± 1.8 e	33.0 ± 0.9 e	45.2 ± 3.4 e
2	43.2 ± 2.7 d	77.1 ± 1.6 d	65.5 ± 0.6 d
3	54.5 ± 0.9 c	82.6 ± 0.6 c	75.0 ± 0.8 c
4	56.8 ± 4.2 b	95.4 ± 0.7 b	82.1 ± 2.1 b
5	75.0 ± 1.9 a	100.0 ± 0.0 a	94.1 ± 0.8 a
F	61.9	668.3	123.4
P	<0.01	<0.01	<0.01

[a] Data are expressed as mean values ± SE from experiments with four replicates.
[b] Mean values within a column sharing the same letter are not significantly different (P <0.05).

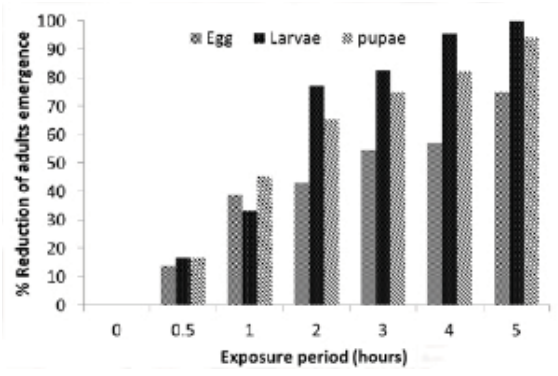

Figure 1. Reduction percentages of adult emergence of *Callosobruchus maculatus* from different life stages treated with 600 ppm (1.2 g/m^3) of ozone for different exposure times.

Effect of ozone gas on adult mortality

The effects of ozone treatment on the adult mortality of *C. maculatus* exposed to 600 ppm (1.2 g/m^3) for different exposure times are summarized in Table 3. Complete mortality (100%) of adults was observed 1 day after treatment with ozone for exposure times of 4 and 5 h (F = 100.9 df = 6.0; P <0.01). Moreover, exposure of adults to ozone for 2 and 3 h caused complete mortality (100%) 3 days after treatment (F = 246.9; df = 6.0; P <0.01), while exposure for 0.5 and 1 h resulted in 100% mortality after 5 days. Values of LT_{50} of adults treated with 600 ppm (1.2 g/

Table 3. Mortality percentages of *Callosobruchus maculatus* adults exposed to 600 ppm (1.2 g/m^3) ozone for different exposure times[a].

Exposure time (h)	Mortality (% ± SE)				
	DAA1	DAA2	DAA3	DAA4	DAA5
0	0.0 ± 0.0 f[b]	0.0 ± 0. 0 d	0.0 ± 0.0 d	0.0 ± 0.0 b	0.0 ± 0.0
0.5	25.9 ± 2.6 e	44.4 ± 3.8 c	81.5 ± 2.6 c	96.0 ± 2.5 a	100 ± 0.0
1	38.4 ± 2.2 d	61.6 ± 2.3 b	88.4 ± 1.1 b	96.3 ± 1.3 a	100 ± 0.0
2	57.9 ± 1.6 c	88.4 ± 0.3 a	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0
3	73.3 ± 2.4 b	93.3 ± 2.3 a	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0
4	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0
5	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0
F	100.9	132.1	246.9	511.6	
P	<0.01	<0.01	<0.01	<0.01	
LT50 (h) (Confidence limits)	1.4 (1.1-1.7)	0.6 (0.5-0.7)	-	-	-

[a] Data are expressed as mean values ± SE from experiments with four replicates of 20 adults each.
[b] Mean values within a column sharing the same letter are not significantly different (P < 0.05).
DAA: days after treatment.

m^3) ozone were 1.4 and 0.6 h, 1 and 2 days after treatment.

Discussion

The present study showed that ozone is effective against the different life stages of *C. maculatus*. Treatment with ozone at 600 ppm (1.2 g/m^3) for 5 h caused 72.3% mortality in eggs and 57.1% mortality in neonates. Also, the egg and neonate mortality percentages gradually increased as exposure to ozone gas increased. Similar results were obtained by other researchers addressing the effects of ozone in terms of egg mortality of other stored product insect species. For example, Isikber *et al*. (2007) found that treatment of *E. kuehniella* eggs with 300 ppm ozone gas for 2 h exposure caused 85.1% mortality. Similarly, Isikber and Oztekin (2009) investigated the susceptibility of *Tribolium confusum* Jacquelin du Val life stages to treatment with ozone at 13.9 mg/l[1] (6,482 ppm) and found that the egg mortality reached 62.5% at 2-h exposure time. Keivanloo *et al.* (2014) stated that treatment with ozone resulted in 56.6% egg mortality of *Plodia interpunctella* (Hübner) at 5 ppm. In contrary with our results, Bonjour *et al.* (2011) reported that treatment with ozone at a concentration of 70 ppm for 4 days did not cause significant egg mortality of *P. interpunctella*.

The results of the present study indicated different susceptibility of the biological stages: *C. maculatus* eggs were less susceptible to the ozone treatment than larvae and adults. In agreement with these findings, Wood (2008) found that the eggs of *Galleria mellonella* (Linnaeus) were more tolerant to ozone gas than larvae. This may be due to the barriers preventing ozone from reaching the target sites inside the egg. Insect eggs have low respiration and metabolic rates, and ozone needs to cross through the egg's outer shell to come into contact with the insect embryo (McDonough *et al*., 2011).

Larvae of *C. maculatus* were more susceptible to ozone than pupae and this could be attributed to the outer layer of pupae that provides some additional barriers to ozone and may cause a more rapid decomposition (McDonough *et al*., 2011). These results are in agreement with Osman (2009) who investigated the efficacy of ozone against *E. kuehniella* larvae at 1 g/m^3 concentration for exposure times of 0.5, 1, 2, 3, 4 and 5 h and reported that larvae were more susceptible than pupae to treatment with ozone. Larvae of *G. mellonella* were also more suscep-

tible than pupae to ozone treatment (James, 2011). Jemni *et al.* (2015) studied the effect of ozone on *Ectomyelois ceratoniae* (Zeller) larvae on intentionally infested dates. They found that the mortality of the larvae depended on the ozone concentration and the exposure period. Treatment of larvae with 170.7 ppm ozone for 80 min caused ten-fold higher mortality (82.0%) than that in control samples (8.0%). Moreover, Gad (2017) stated that the exposure of *Tribolium castaneum* (Herbst) larvae and pupae to high concentrations of ozone (1500 ppm) for 5 h resulted in complete mortality in both stages, and low ozone concentrations also caused a significant reduction in adult emergence.

The reduction in adult emergence of *C. maculatus* from larvae and pupae exposed to ozone gas significantly increased with the increase of exposure time and reached 100 and 94.1% after 5 h of exposure, respectively. Similar findings have been described by Kells *et al.* (2001) and Leesch (2002) on the effect of ozone treatment with 50 and 300 ppm on *P. interpunctella* larvae and pupae. Additionally, Isikber and Oztekin (2009) showed that *T. confusum* larvae were susceptible to ozone exposure, and mortality reached 86.3% at 13.9 mg/l[1] (6,482 ppm) at 4 h exposure time.

The adult mortality rate of *C. maculatus* increased with increasing ozone exposure times, reaching total mortality 5 days after exposure to ozone for 0.5 h. These findings are in agreement with the study of Osman (2009) on the efficacy of ozone at 1 g/m^3 concentration for different exposure times of 0.5, 1, 2, 3, 4 and 5 h against the adult stages of *T. castaneum*, *S. oryzae*, *Sitophilus granaries* (Linnaeus), *R. dominica* and *Oryzaephilus surinamensis* (Linnaeus). The highest mortality rate was recorded for *O. surinamensis* adults 2 days after exposure to ozone, followed by adults of *S. oryzae* 3 days after exposure. McDonough *et al.* (2011) noticed that exposure of *P. interpunctella* adults to 500 ppm ozone caused 100% mortality within 60 min. Silva *et al.* (2016) evaluated the toxicity of ozone (750.8 ppm) against *R. dominica* adults on wheat grains and found that the periods of ozone exposure required to cause mortalities of 50 and 95% ranged from 8.7 to 13.1 h and from 11.3 to 18.1 h, respectively.

Mortality from ozone gas has been attributed to the fact that ozone is a powerful oxidizing agent that can react directly with proteins, DNA and double bonds of polyunsaturated fatty acids (PUFA) and that during degradation of ozone to dioxygen, free radicals (reactive oxygen species; ROS) may be formed which can also cause peroxidation of PUFAs and destroy vital molecules such as DNA and proteins (Hermes-Lima, 2004; Korsloot *et al*., 2004). Thus, it is likely that such effects, in isolation or together, result in cellular injuries and eventually death of the insect exposed to ozone gas (Holmstrup *et al*., 2011).

In conclusion, treatment with ozone at 600 ppm (1.2 g/m^3) caused high mortality rates in eggs, neonates and adults of *C. maculatus* and effectively reduced adult emergence from eggs, larvae and pupae. The adults and larvae were more susceptible to ozone gas than eggs and pupae. Therefore, our findings indicated that ozone gas has a potential to be used as fumigant for the management of *C. maculatus* on cowpea grains.

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Εντομοκτόνος δράση του αερίου όζοντος στα διάφορα βιολογικά στάδια του *Callosobruchus maculatus* (Coleoptera: Bruchidae), υπό εργαστηριακές συνθήκες

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Περίληψη Ο βρούχος *Callosobruchus maculatus* (Fabricius) αναγνωρίζεται ως ένας κοινός εντομολογικός εχθρός του φασολιού παγκοσμίως. Η παρούσα μελέτη εξετάζει την εντομοκτόνο δράση του αερίου όζοντος έναντι όλων των βιολογικών σταδίων (ωό, προνύμφη, πλαγγόνα, ενήλικο) του *C. maculatus* σε συγκέντρωση 600 ppm (1,2 g/m^3) μετά από έξι χρονικά διαστήματα έκθεσης, 0,5, 1, 2, 3, 4 και 5 ώρες υπό εργαστηριακές συνθήκες. Τα ποσοστά θνησιμότητας όλων των βιολογικών σταδίων αυξήθηκαν σημαντικά με την αύξηση του χρόνου έκθεσης. Ομοίως, ο μεγαλύτερος χρόνος έκθεσης στο όζον προκάλεσε μεγαλύτερη μείωση της εμφάνισης ενηλίκων από ωά, προνύμφες και πλαγγόνες. Η έκθεση στο όζον για πέντε ώρες προκάλεσε θνησιμότητα σε ποσοστό 72,3% στα ωά. Ολική θνησιμότητα στα ενήλικα παρατηρήθηκε πέντε ημέρες μετά την έκθεση στο όζον για μισή ώρα. Επιπλέον, η έκθεση στο όζον για πέντε ώρες είχε ως αποτέλεσμα τη μείωση κατά 75, 100 και 94,1% στην εμφάνιση ενηλίκων από ωά, προνύμφες και πλαγγόνες, αντίστοιχα. Οι προνύμφες και τα ενήλικα ήταν πιο ευαίσθητα στο όζον από τα ωά και τις πλαγγόνες. Με βάση τα αποτελέσματα της μελέτης, το αέριο όζον έχει καλή εντομοκτόνο δράση σε όλα τα βιολογικά στάδια του *C. maculatus* και θα μπορούσε να χρησιμοποιηθεί ως υποκαπνιστικό για την αντιμετώπισή του.

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SHORT COMMUNICATION

Insecticidal effect of deltamethrin, dinotefuran and spiromesifen against the sugarcane whitefly *Neomaskellia andropogonis* on CP69-1062 sugarcane cultivar

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Summary The sugarcane whitefly, *Neomaskellia andropogonis* Corbett (Homoptera: Aleyrodidae), is one of the important pests of sugarcane in Iran. The use of selective chemical insecticides helps to suppress whitefly infestations. Although several insecticides from various groups have been registered to control whiteflies, this is the first study to test the efficacy of deltamethrin, dinotefuran and spiromesifen insecticides against eggs, second instar nymphs and pupae of the sugarcane whitefly on CP69-1062 sugarcane cultivar. Five concentrations of the tested insecticides were applied in a leaf dipping bioassay under laboratory conditions. Probit analysis indicated that deltamethrin and dinotefuran with LC_{50} values of 50.1 and 49.5 ppm were the most toxic insecticides against eggs of *N. andropogonis*. Deltamethrin controlled nymphal and pupal stages more effective than the other two tested insecticides and the LC_{50} values were 49.7 and 5.44 ppm on nymphs and pupae, respectively. The LC_{50} values of dinotefuran on second instar nymphs and pupae were 564.7 and 78.7 ppm and the values were 270.9 and 18.3 ppm for spiromesifen, respectively. The results support the use of the insecticides in rotation according to their different mode of action in integrated pest management programs of the sugarcane whitefly *N. andropogonis*.

Additional keywords: Chemical control, insecticides, life stages, sugarcane whitefly

Introduction

Sugarcane (interspecific hybrids of *Saccharum officinarum* L.) is a tropical perennial grass cultivated in many countries for production of sugar and biofuel as energy cane (James, 2014). It is an economic important component of industrial crops in Iran, where it is cultivated in the south west part, in Khuzestan province, on more than 120,000 hectares (Sadeghzadeh-Hemayati *et al*., 2011). Its mono-culture system of planting may multiply the risk of damage caused by several arthropod herbivores and impose economic loss in field and factory conditions (Nikpay and Goebel, 2016).

The sugarcane whitefly, *Neomaskellia andropogonis* Corbett (Homoptera: Aleyrodidae), was first identified in Iran in 2006 (Askarianzadeh and Manzari, 2006) and now it is classified as emerging pest since its damage is increasing continuously in the recent years. *Neomaskellia andropogonis* lays its eggs under the leaves of sugarcane, the nymphs suck phloem sap and cause reduction of photosynthesis rate. In heavy infestation the sugar purity and brix are greatly decreased. The excreted honeydew serves as a medium for sooty mold (Nikpay and Goebel, 2016) (Fig. 1).

Possible control strategies of the whitefly include chemical, biological and cultural control (Pandya, 2005; Koohzad-Mohammadi *et al*., 2017; Nikpay, 2017). Chemical control of the sugarcane whitefly includes application of thiamethoxam (Actara 25WG)

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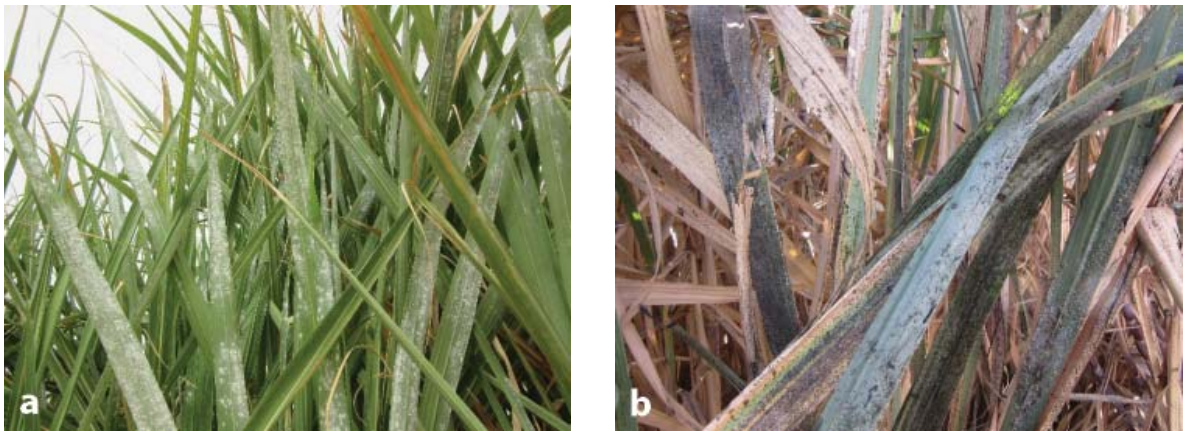

Figure 1. Population of whitefly on sugarcane CP69-1062 variety (a), and sooty mold grows on plants with heavy whitefly damage (b).

(Vijayaraghavan and Regupathy, 2006), imidacloprid (Bhavani and Rao, 2013), deltamethrin, dinotefuran and spiromesifen insecticides (Koohzad-Mohammadi *et al*., 2017) under field conditions and Sivanto®, Oberon Speed® (Saeedi and Ziaee, 2018) and thiacloprid + deltamethrin, pyriproxyfen, spirotetramat insecticides (Behnam-Oskuyee *et al*., 2020) under laboratory conditions. In Iran, the main control strategies are based on resistance cultivars, cultural practices such as removing highly infested leaves, nutrition management via use of rationale fertilizers and appropriate irrigation intervals and conservation of biological control parasitic wasps and native predators such as spiders and lacewings (Koohzad-Mohammadi *et al*., 2019). To date, no chemical control is applied for this pest in sugarcane fields in Iran. However, damages on sugarcane by *N. andporogonis* have been increasing recently due to outbreaks of the pest (Askarianzadeh and Minaeimoghadam, 2018; Nikpay, 2017), making research on effective chemical insecticides highly important for the management of this pest in near future.

According to Koohzad-Mohammadi *et al.* (2017), the most effective control was succeeded by dinotefuran (more than 65% control in all biological stages) and deltamethrin (75% egg control, 48% nymph, 70% pupae and 87% adult control) after 30 days from the foliar application on the cultivar IRC99-02 (Koohzad-Mohammadi *et al*., 2017). The percentage mortality of different biological stages of sugarcane whitefly in cultivar CP69-1062 was significantly lower than that of IRC99-02 (Koohzad-Mohammadi *et al*., 2017). Flupyradifurone (Sivanto® 200 SL) against the insect nicotinic acetylcholine receptor and spiromesifen + abamectine (Oberon Speed® 24 SC) on different biological stages of *N. andropogonis* in two cultivars, IRC99-02 and CP69-1062, at concentrations of 20, 60, 100, 200 and 400 ppm, showed that the concentration of 400 ppm of both insecticides controlled well adult, nymph and pupae of the whitefly (Saeedi and Ziaee, 2018). Oberon Speed® 24 SC had less effect on the egg stage (Saeedi and Ziaee, 2018).

Nowadays, selection of suitable pesticides which are compatible with biological control is essential for the success of integrated pest management. Deltamethrin is a non-systemic insecticide from the group of pyrethroids (Rehman *et al*., 2014). Dinotefuran is a systemic insecticide from the group of neonicotinoids with an oral-contact effect (Sreenivas *et al.,* 2015). Spiromesifen is a non-systemic insecticide from a new group of tetronic acids (Mann *et al.,* 2012). The aim of this study was to assess the lethal concentration (LC_{50}) of deltamethrin, dinotefuran and spiromesifen insecticides on the biological stages of egg, nymph and pupa of *N. andropogonis* on the commercial CP69-1062 sugarcane cultivar.

Materials and Methods

Sugarcane plants

Sugarcane plants for the experiments were cultivated in buckets in the greenhouse. Buckets (8-liter) were filled in a 1: 1: 1 ratio of sand, soil and animal manure and placed in the greenhouse of Shahid Chamran University of Ahvaz in mid-June 2017. Two to three sugarcane cuttings of the cultivar CP69-1062 (Koohzad-Mohammadi *et al*., 2017), each with a healthy bud, were planted into the bucket. The sugarcane cuttings were irrigated once every three days and after they reached the five-leaf stage, urea fertilizer was applied to the soil.

Insects

All biological stages of the sugarcane whitefly, *N. andropogonis*, were obtained from sugarcane infested fields during the growing season from early September to early November and then the whitefly adults were released on the sugarcane plants cultivated in the buckets in the greenhouse.

Laboratory bioassays

The efficacy of three insecticides, deltamethrin (Decis® 2.5%EC, Belgian Agrifar Company), dinotefuran (Starkle® 20%WDG, Mitsui Chemicals), and spiromesifen (Oberon® SC240, Bayer, Germany), were tested against eggs, second instar nymphs and pupae of the whitefly. Different sets of bioassays were performed in the laboratory for each life stage, using the leaf dipping method. According to the results of previous study (Koohzad-Mohammadi, 2018), five concentrations of each compound were used: 10, 30, 50, 100 and 200 ppm for deltamethrin; 100, 200, 300, 500 and 750 ppm for dinotefuran; 100, 200, 400, 500 and 800 ppm spiromesifen. Tween 20 (1%) was added to the insecticides solution. The leaves were dipped in the insecticide or control solutions for 10 sec and then were individually placed in transparent plastic bottles (5 cm in diameter and 20 cm in height). The bottles were covered with muslin cloth for ventilation and kept in incubators set at 30 ± 1°C, 60 ± 5% relative humidity (r.h.) and a photoperiod of 8:16 h (L:D). Distilled water (2 ml) was poured daily on the bottom of the plastic bottles to keep the leaves moisten. All experiments were performed with 6 replicates and distilled water and 1% Tween 20 were used as control.

For egg testing, infested leaves (15 cm in length) from the cultivation buckets were cut and transferred to the laboratory. Then, 30 adults of *N. andropogonis* (1-d old) were introduced on the leaves. After 24 h, adults were removed and the number of eggs laid on each leaf was counted by a Heerbrugg Switzerland Wild M3C stereomicroscope. Egg hatch was determined 8 days after treatment based on the duration of the 6-8 day duration of *N. andropogonis* egg stage by Minaei-Moghadam *et al.* (2009). The whitefly eggs were considered dead in the event of loss of swelling and drying of the cuticle structure as described by Qian *et al.* (2012).

For second instar nymphs, infested leaves (15 cm in length) were cut and transferred to the laboratory. Twenty-five first instar nymphs were kept on each leaf using a stereomicroscope. Given that the duration of the first instar is 2 days (Minaei-Moghadam *et al*., 2009), the leaves were treated after 2 days. The leaves were checked daily up to five days after treatment using stereomicroscope and the mortality was reported. Based on Sohrabi *et al.* (2011) the nymphs that were dry and detached from the leaf when probed were considered dead.

To assess the insecticidal efficacy on sugarcane whitefly pupal stage, sugarcane leaves (15 cm in length) were cut and checked under a stereomicroscope to keep only third instar nymphs removing all other stage using a brush. After 24 h, 25 pupae were kept per leaf and considered as 1-d old pupae. The leaves were observed daily and overall pupal mortality was determined according to Sohrabi *et al.* (2011).

The LC_{50} value, 95% confidence interval and relative toxicity were calculated by probit analysis (Finney, 1971). The LC_{50} ratio of lethal concentrations was used to determine the relative toxicity of two pesticides or to investigate the relative sensitivity of

different biological stages to one or more insecticides. The relative toxicity and their confidence limits were calculated by using statistical software SPSS 16 (SPSS, 2007).

Results and Discussion

The LC_{50} values of deltamethrin, dinotefuran, and spiromesifen against eggs, second instar nymphs and pupae of *N. andropogonis* are presented in Table 1. Overall, deltamethrin had the highest insecticidal efficacy on eggs, second instar nymphs, and pupae on the sugarcane cultivar CP69-1062. Abou-Yousef *et al.* (2010) found that the pyrethroids deltamethrin and alpha-cypermethrin were effective against a resistant lab-strain of the whitefly *Bemisia tabaci* (Genn.) to neonicotinoids (dinotefuran, acetamiprid, imidacloprid). Evaluation of a 'thiacloprid+deltamethrin' mixture (Proteus®) in comparison with spirotetramat and pyriproxifen against different life stages of *N. andropogonis* under laboratory conditions on the sugarcane cultivars CP69-1062 and IRC99-02 clearly showed that the thiacloprid+deltamethrin mixture was the most toxic against all life stages and reduced efficiently percentage fertility of *N. andropogonis* on both sugarcane treated cultivars (Behnam-Oskuyee *et al.*, 2020).

Relative toxicity of the tested insecticides on the whitefly life stages showed that the toxicity of deltamethrin was 9.2 and 9.13 times higher on the pupal than on the egg and nymphal stage, respectively, while it did not differ between eggs and second instar nymphs, indicating higher biological action on the pupal stage. The high toxicity rate of deltamethrin on sugarcane whitefly eggs might be due to the effect of pyrethroid insecticides on embryonic development and inhibition of larval hatch as indicated by Buczek *et al.* (2019) for deltamethrin and alpha-cypermethrin on *Ixodes ricinus* (L.) (Acari: Ixodidae) at sublethal concentrations. Both substances disturbed the embryonic development in *I. ricinus* occurring in hindrance of the earliest stage of embryogenesis, as well as impediment hatch of larvae.

The insecticidal activity of spiromesifen was 16.2 and 14.8 times higher on pupae than on eggs and second instar nymphs, respectively, while on second instar nymphs was significantly higher than that on eggs (Table 2). Thus, according to our findings spiromesifen had a little effect on eggs and

Table 1. LC_{50} values of deltamethrin, dinotefuran and spiromesifen insecticides against eggs, second instar nymphs and pupae of the sugarcane whitefly *Neomaskellia andropogonis* on the sugarcane cultivar CP69-1062.

Insecticide	LC_{50} (ppm)	95% Confidence limits (ppm)		Slope ± SE	χ^2
		Lower	Upper		
Eggs					
Deltamethrin	50.1	42.5	58.0	1.95 ± 0.09	0.45
Dinotefuran	49.5	37.9	60.4	1.95 ± 0.16	0.65
Spiromesifen	296.0	270.4	323.2	1.34 ± 0.08	1.87
2nd instar nymphs					
Deltamethrin	49.7	23.8	79.4	0.65 ± 0.04	11.4
Dinotefuran	564.7	514.7	564.2	0.87 ± 0.09	0.39
Spiromesifen	270.9	253.2	288.2	1.34 ± 0.06	0.908
Pupae					
Deltamethrin	5.44	0.06	16.41	0.28 ± 0.04	8.46
Dinotefuran	78.7	70.7	86.4	1.64 ± 0.06	6.99
Spiromesifen	18.3	2.71	42.8	0.25 ± 0.05	2.26

Five concentrations for each insecticide; χ^2: Chi-square test for linearity of concentration–mortality response.

Table 2. Relative toxicity of LC_{50} values of deltamethrin, dinotefuran and spiromesifen insecticides against different life stages of sugarcane whitefly *Neomaskellia andropogonis* on the sugarcane cultivar CP69-1062.

Insecticide	Relative toxicity		
	Nymphs to eggs	Eggs to pupae	Nymphs to pupae
Deltamethrin	1.00 (0.73 - 1.78)	9.20 (3.53 - 708.3)	9.13 (4.83 -396.66)
Dinotefuran	11.41 (9.34 - 13.58)	0.63 (0.54 - 0.70)	7.17 (6.53 - 7.28)
Spiromesifen	0.91 (0.89 - 0.94)	16.17 (7.55 - 99.78)	14.80 (6.73 - 93.43)

the highest insecticidal activity on the pupal stage. Our results on the eggs are in compliance with findings on other whitefly species. Liu (2004) found that spiromesifen was more effective than thiamethoxam (neonicotinoid) and buprofezin (inhibitor of chitin synthesis) against different biological stages of the cotton whitefly *B. tabaci* on cabbage and melon but eggs were the least susceptible stage to spiromesifen. In studies by Mann *et al.* (2012) on the toxicity of spiromesifen on susceptible population of *B. tabaci* biotype B in benthos plants in the USA, spiromesifen had a little effect on the whitefly's eggs, but it controlled more than 70% of the nymph population and 40% of adults 48 h after treatment. In field trials conducted in Arizona, USA, spiromesifen and buprofezin insecticides significantly controlled the population of nymphs and adults of *B. tabaci* on melon and prevented the decline of melon fruit quality (Palumbo, 2009).

In the case of dinotefuran, the toxicity on eggs and pupae was 11.41 and 7.17 fold higher than that on nymphs, respectively, indicating high insecticidal activity on *N. andropogonis* eggs, which is very important as high mortality in the egg stage can reduce the population at the beginning of whitefly generation. Ovicidal effect of other neonicotinoid active substances has been demonstrated on other pests. Moscardini *et al.* (2013) showed that imidacloprid in a mixture with spirotetramat had some ovicidal activity (based on egg hatching) on the predator *Orius insidiosus* (Say) (Hemiptera: Anthocoridae), given that spirotetramat acts on juvenile stages of sucking insect pests (inhibition of lipid biosynthesis) (Prabhaker *et al*., 2014; Salazar-López *et al.*, 2016). Sreenivas *et al.* (2015) investigating the management of cotton sucking pests using third-generation neonicotinoid molecules, found that dinotefuran at 30 g/ha significantly reduced the population of *B. tabaci* cotton whitefly and had a significant effect on the control of the pest population. Qu *et al.* (2017) reported that dinotefuran had the highest insecticidal activity among six tested compounds against *B. tabaci* [Middle East-Asia Minor1 (MEAM1 biotype B) and Mediterranean (MED or biotypeQ)]. Furthermore, our findings that dinotefuran was more toxic to pupae than nymphs are in accordance with those by Qu *et al.* (2017). According to their results, no significant difference was recorded in the survival rate of 3rd instar nymphs between the control and the dinotefuran treatment at sublethal concentration (LC_{25}: 170 mg/L), while the survival rates of pupae and adults of *B. tabaci* MEAM1 after treating with dinotefuran significantly decreased by 9.81 and 11.07, respectively, in comparison with the control. On the other hand, the low susceptibility of *N. andropogonis* nymphs to dinotefuran may be attributed to its influence on their feeding behavior as Miao *et al.* (2014) reported at low concentrations (LC_{10} and LC_{50}) of dinotefuran on the wheat aphid *Sitobion avenae* (Fabricius) (Hemiptera: Aphididae) where the percentage of non-probing interval time increased and the phloem sap ingestion decreased.

The results of this study showed that all three insecticides with different mode of action are effective for the control of sugarcane whitefly under laboratory conditions, supporting a selection based on the target growth stage and an alternate use to minimize the risk for resistance development,

which is crucial for this pest as well as a pivotal concept of successful integrated pest management program.

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ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Δράση εντομοκτόνων με δραστικές ουσίες deltamethrin, dinotefuran και spiromesifen κατά του αλευρώδη *Neomaskellia andropogonis* στην ποικιλία CP69-1062 του ζαχαροκάλαμου

P. Koohzad-Mohammadi, M. Ziaee και A. Nikpay

Περίληψη Ο αλευρώδης του ζαχαροκάλαμου, *Neomaskellia andropogonis* Corbett (Homoptera: Aleyrodidae), είναι ένας από τους σημαντικούς εντομολογικούς εχθρούς του ζαχαροκάλαμου στο Ιράν. Η χρήση εκλεκτικών εντομοκτόνων συμβάλλει στην αντιμετώπιση προσβολών από αλευρώδεις. Αν και υπάρχουν αρκετά εγκεκριμένα εντομοκτόνα με δραστικές ουσίες διαφορετικών χημικών ομάδων για αλευρώδεις, αυτή είναι η πρώτη μελέτη εκτίμησης της δράσης εντομοκτόνων με τις δραστικές ουσίες deltamethrin, dinotefuran και spiromesifen εναντίον ωών, νυμφών δεύτερης ηλικίας και πλαγγόνων του αλευρώδη του ζαχαροκάλαμου στην καλλιεργούμενη ποικιλία CP69-1062. Δοκιμάστηκαν πέντε συγκεντρώσεις των εντομοκτόνων με βιοδοκιμές εμβάπτισης φύλλων υπό εργαστηριακές συνθήκες. Η ανάλυση probit έδειξε ότι τα εντομοκτόνα με δραστικές ουσίες deltamethrin και dinotefuran, με τιμές LC50 50,1 και 49,5 ppm, αντίστοιχα, ήταν πιο δραστικά εναντίον των ωών του *N. andropogonis*. Το

εντομοκτόνο με δραστική deltamethrin είχε πιο αποτελεσματική δράση εναντίον των νυμφών και των πλαγγόνων (με τιμές LC50 49,7 και 5,44 ppm, αντίστοιχα) συγκριτικά με τα άλλα δύο εντομοκτόνα. Οι τιμές LC50 του dinotefuran στις δεύτερης ηλικίας νύμφες και τις πλαγγόνες ήταν 564,7 και 78,7 ppm και αυτές του spiromesifen ήταν 270,9 και 18,3 ppm, αντίστοιχα. Τα αποτελέσματα υποστηρίζουν τη χρήση των δοκιμασθέντων εντομοκτόνων, με εναλλαγή σύμφωνα με τον τρόπο δράσης τους, σε ολοκληρωμένα προγράμματα αντιμετώπισης του αλευρώδη *N. andropogonis*.

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