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Aspect of the degradation and adsorption kinetics of atrazine and metolachlor in andisol soil

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Summary The degradation kinetics and sorption characteristics of atrazine and metolachlor in Japanese andisol soil were evaluated using laboratory incubation of soil samples. The water content of the soil was set to field capacity while three different temperatures (5, 25 and 35°C) were considered for the experiment. First order model fitted the degradation kinetics of both herbicides under the investigated temperature range with half-lives ranging from 19.2 to 46.9 days for atrazine and from 23.4 to 66.9 days for metolachlor, respectively. The activation energies (*Ea*) of atrazine and metolachlor calculated using Arhenius equation were 21.47 and 23.91 kJ mol^{-1}, respectively. The soil sorption study was conducted using the batch equilibrium process. The adsorption behaviors of atrazine and metolachlor were investigated using linear, Freundlich and Langmuir isotherms although the linear and Freundlich isotherms gave relatively high correlation coefficient (R^2) and very low standard error of estimate (*SEE*). The free energy ($\Delta G°$) values were in the range -30.6 to -32.0 kJ/mol, and -32.1 to -41.5 kJ/mol for atrazine and metolachlor, respectively. Thermodynamic parameters indicated that the adsorption is spontaneous, endothermic accompanied by increase in entropy. The understanding of atrazine and metolachlor sorption processes is essential to determine the pesticide fate and availability in soil for pest control, biodegradation, runoff and leaching.

Additional keywords: adsorption isotherm, atrazine, degradation kinetics, metolachlor, temperature

Introduction

Pesticides mainly enter the environment through agronomic applications. Their interactions with soil depend on their physicochemical properties and on the nature and composition of the soil (Rodriguez-Liebana *et al.,* 2011). Atrazine [2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine] and metolachlor [2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1- methylethyl)- acetamide] (Figure 1a and b) are two commonly used herbicides for controlling the pre- and post-emergence of annual grasses and broad-leaved weeds in many crops, including maize, sorghum, and turf grasses (Tomlin, 2006). Atrazine was reported as commonly contaminating surface water and groundwater in the United States because of its relatively high water solubility and its widespread use (U.S. Geological Survey 1999). Similarly, metolachlor and its metabolites have been detected in streams, rivers, ponds, and wells (Rebich *et al.,* 2004; Kalkhoff *et al*., 1998).

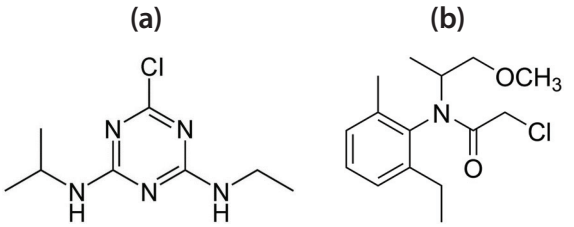

Figure 1. Chemical structures of (a) atrazine and (b) metolachlor.

Once pesticides are applied to agricultural land as they were designed, they adsorb to solids (plants and soil particles) in a dynamic process (ElShafei *et al*., 2009). The sorption processes play an important role in the fate and movement of agricultural pesticides. Hall *et al*. (2015) reported that, the most commonly used parameters to evalu-

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ate pesticide leaching to underground water are the chemical half-life (*DT50* day) and the sorption distribution coefficient (K_d, L kg^{-1}). Since the range of the sorption distribution coefficient for a given pesticide depends on the organic carbon of the soil, the sorption parameters reported in the literature are generally normalized in respect to the soil organic carbon content (K_{oc}, L kg^{-1}) (Oliveira *et al.,* 2013). Because pesticide fate and transport is largely controlled by the sorption behavior of the chemical, accurate site-specific K_{oc} values are essential for evaluating the potential leaching risk caused by pesticides in soil (Chirukuri and Atmakuru, 2015). Sorption is a dynamic process in which molecules are continually transferred between the bulk liquid and solid surface and influenced by the physicochemical properties of the pesticide itself, as well as the properties of the soil. The adsorption–desorption process of pesticide in soil affects not only the movement, volatilization, and degradation behaviors of pesticides, but also their bioavailability, transformation by biotic agents, and the possibility of contamination of underground water or surface water (Patakioutas and Albanis, 2002). In addition, Scribner *et al*. (1992) reported that, the observed differences in adsorption between the organic compounds in the same soil are because of difference in the physical and chemical characteristics of the compounds. Local climatic conditions can strongly influence the aforementioned soil properties and in turn, the sorption of applied herbicides (Langenbach *et al.,* 2001). Therefore, investigation of herbicide behaviors in agricultural soils under different temperatures is required to improve the risk assessment of aquatic ecosystem.

To evaluate the risk caused by atrazine and metolachlor in regards to water contamination, this study focuses on evaluating the dissipation behavior and sorption characteristics of atrazine and metolachlor in andisol soil. The aim was to identify the factors influencing the degradation, and sorption of atrazine and metolachlor. The effect of temperature on the degradation and adsorption of atrazine and metolachlor in andisol soil was also considered.

Materials and methods

Materials

All organic solvents and atrazine and metolachlor reference standards (purity 99.5%) were of analytical grade and purchased from Wako Pure Chemical Industries (Osaka, Japan). Water was produced with a Milli-Q Water Purification System (Millipore, Billerica, MA, USA). Glass filters and syringe filters were from Whatman (Maidstone, UK). Macroporous Diatomaceous earth (MDE) column (Chem Elut, 20 mL) was purchased from Varian (USA), Supelclean ENVI-18 and Graphitized Carbon Black (GCB) SPE cartridge (1000 mg, 6 mL) was purchased from Agilent (USA).

Experimental procedures

Soil characterization. Soil samples were collected from the experimental farm of Tokyo University of Agricultural and Technology, located on Fuchu, Tokyo, Japan. Soil was gathered from the surface up to 10 cm deep and was not previously treated with atrazine or metolachlor. The soil taxonomic order is andisol. In general, andisol soils are rich in organic matter (OM), with high specific surface area, and contain short-range ordered minerals. Physicochemical characteristic of the soil used in this study was described in details by Jaikaew *et al*. (2015). The soil had the following physicochemical properties: pH 5.8, organic carbon content 6.95%, and cation exchange capacity 34.1 (Table 1).

Dissipation in soil. After collecting the soil from the experimental farm, it was air-dried and sieved (2 mm diameter). Then, the soil was transferred into vials which contained each 10 g of soil. The soil samples (vials) were wetted and kept at 84% of the water holding capacity. This level of water content was the average condition observed during a field experiment (Jaikaew *et al*., 2015). Since water was re-introduced in the samples, mi-

Table 1. Physicochemical properties of surface soil (0-5 cm) (Jaikaew *et al.,* 2015).

pH (H_2O)	5.8
Organic carbon content (%)	6.95
Cation exchange capacity (cmol/kg)	34.1
Specific gravity (mg/m^3)	2.50
Core Sand, 2.0-0.2mm (%)	13.7
Fine Sand, 0.2-0.02mm (%)	29.5
Silt, 0.02-0.002mm (%)	33.4
Clay, ≤ 0.002mm (%)	23.4
Soil texture (ISSS)	Clay Loam (CL)
Average porosity (%)	0.79

crobial activity was reestablished. The samples were pre-incubated in dark incubators at 5, 20 and 35°C for 14 days before fortification with 3.0 mg kg^{-1} of atrazine and metolachlor. They were then incubated at 5, 20 and 35°C in the dark again during the experiment. The moisture content of the samples was adjusted every 2 days at 35°C and every 2 weeks at 20 and 5°C. The amount of water to replenish was determined by comparing the weights of the pre-incubated and incubated bottles. At predetermined intervals (0, 7, 14, 30, 60 and 120 days after treatment) triplicate soil samples were removed to determine the residues of atrazine and metolachlor.

Jaikaew *et al.* (2015) reported that the half-life values for atrazine and metolachlor significantly reduced during winter time having an average temperature of 5°C. The soil microbial activities were assumed to be insignificant as soil temperature approached to near zero °C. The effect of reduced microbial activity on dissipation of pesticide in soil was examined at temperature 5°C by conducting experiment on sterile and non-sterile soils. The sterilization was carried out by three consecutive autoclaving (TOMY 970 mm (SX-500)) for 20 min at 120°C with an interval of 3 h. The soil samples underwent the same procedure as that described above. Freshly bidistilled water was used for maintaining humidity of the sterile soil samples.

Adsorption in soil. The batch equilibrium technique recommended by OECD (2000) was used to determine the soil adsorption constants of atrazine and metolachlor in the andisol soil type at 5, 20 and 35°C. Before initiation of the experiment, the soil samples were sterilized as previously described. Sterilization was performed to restrain (prevent the biodegradation of atrazine and metolachlor) microbial degradation, and 50 mL polypropylene centrifuge tubes were filled with 25 g of sterile soil. An aliquot of 50 mL of 0.01 M $CaCl_2$ solution was added to each vial to produce soil solution ratio of 1:2 and equilibrated for 4 h at 5, 20 and 35°C. To the test vessels, 0.1, 0.5, 1.0, 3.0 and 5.0 µg g^{-1} of atrazine and metolachlor were added and they were placed in a horizontal shaker at 50–60 rpm for 24 h. The vials were removed from the rotator and centrifuged for 5 min at approximately 3000 RPM in a cooling centrifuge at 5–10°C. After centrifugation, the supernatant was transferred for determination of equilibrium concentration (q_e) of herbicide as discussed later. The adsorption experiments were triplicated. One blank (without herbicides) and one control (without soil) were included in each sample batche to assure the quality control of the experiments.

Herbicides extractions. Water sample: water samples were extracted using solid phase extraction technique. Briefly, water samples were filtered through a Glass Fiber filter and then adjusted to pH = 2.5 by phosphoric acid before extraction. Water samples were applied to preconditioned Supelclean ENVI-18 solid phase extraction cartridges under vacuum at a flow rate of 4 mL min^{-1}. After the whole sample had passed through, the cartridge was dried under vacuum for 5 min and herbicides were eluted with 6 mL of acetonitrile at a rate of 1 mL min^{-1}. The eluate was evaporated to dryness under a gentle stream of nitrogen (40–45°C) and the dry residue was re-suspended into 1 mL of acetonitrile, filtered through 0.22-µm PTFE filter which then was analyzed in an HPLC-Diode Array Detection (DAD) system as is described below.

Soil sample: Atrazine and metolachlor were

extracted from soil samples using the organic solvent acetone and then underwent solid phase extraction as described by Jaikaew *et al*. (2015). The water and soil extracts were re-suspended with 2 mL of acetonitrile filtered through 0.22-mm PTFE filter (Millipore, Billerica, MA) and transferred to a glass vial for final determination.

HPLC determination. HPLC analyses were conducted using a SHIMADZU VP series liquid chromatography, equipped photodiode array detector (DAD). A VP-ODS analytical column (150 mm × 4.6 mm id, 4.6 µm particle size) was used. The mobile phase was acetonitrile: water, 35: 65 (v/v) for atrazine and acetonitrile: water (0.1% acetic acid) 20:80 (v/v) for metolachlor, with a flow rate of 1 mL min^{-1} and the oven temperature during the analysis was 40°C. The injection volume was 20 µL. Detection wavelengths for atrazine and metolachlor were set at 220 and 204 nm, respectively.

Data analysis

Method validation. The method was validated by assessing linearity, recovery, precision, and specificity of peak areas. Samples of untreated water and soil were spiked with atrazine and metolachlor standard solutions at two fortification levels (LOQ and 10 x LOQ). Quantification was accomplished by using a calibration curve prepared by serial dilutions (concentration 0.05-5.0 µg ml^{-1}) of the stock solution prepared in mobile phase. Based on a signal to noise ratio of 3:1, the limit of detection of the instrument was established.

Dissipation kinetics and thermodynamic analysis. The dissipation processes of atrazine and metolachlor in soil were assumed to follow the first-order kinetic. The degradation rate constant and half-life were calculated using first-order rate equation:

$$C_t = C_0 e^{-kt} \quad (1)$$

where C_t represents the concentration of the herbicide residues at the time of *t*, C_0 represents the initial concentration after application, and *k* is the degradation rate constant in day^{-1}. The half-life (DT_{50}), defined as the time required for the herbicide residue level to fall to half of the initial residue level after application, was calculated from the degradation rate constant for each experiment using eqn. 2.

$$DT_{50} = \frac{\ln(2)}{k} \quad (2)$$

The degradation rate constant and the temperature influences on the kinetic analysis of degradation of atrazine and metolachlor in soil under the effects of temperatures were expressed by Arrhenius relation as follows:

$$k = Ae^{\frac{-Ea}{RT}} \quad (3)$$

where k is the first-order degradation rate constant, *A* is the pre-exponential factor (day^{-1}), *Ea* is the herbicide degradation activation energy expressed in kJ mol^{-1}, *R* is the universal gas constant equal to 8.31 x 10^{-3} (kJ mol^{-1}), and *T* is the absolute temperature expressed in Kelvin. Using the propriety of natural logarithm in regard to linearization, eqn. 3 becomes:

$$\ln(k) = \ln(A) - \frac{Ea}{RT} \quad (4)$$

The advantage of eqn. 4 is that plotting -ln(*k*) against the reciprocal of absolute temperature (1/*T*) results in straight lines. Therefore, the activation energy of atrazine and metolachlor can be extracted from the slope of the linear plot created for atrazine and metolachlor, respectively.

In this study, the temperature influence on the rate of degradation was quantified using the parameter Q_{10} which can be extracted from the following equation:

$$k_T = k_{Tref} \cdot Q_{10} \frac{(T - T_{ref})}{10} \quad (5)$$

where k_T (day^{-1}) is the degradation rate at temperature T (°C), k_{Tref} (day^{-1}) is the degradation rate at a reference temperature *Tref* (°C). The Q_{10} value is usually obtained from laboratory incubation studies under controlled temperature and soil moisture regimes.

Adsorption analysis. The amount of atrazine and metolachlor adsorbed after equilibrium was calculated according to the difference between the initial and the final equilibrium solution concentrations by Eqn. (6) as follows:

$$q_e = \frac{(C_0 - C_e) \cdot V}{m} \quad (6)$$

where q_e (mg kg^{-1}) is the amount of atrazine and metolachlor adsorbed by the soil, and C_0 and C_e (mg L^{-1}) are the initial and equilibrium aqueous concentrations, respectively. V (L) is the volume of the solution, and m (kg) is the mass of the soil.

The sorption behaviors of atrazine and metolachlor were further analyzed using linear, Freundlich and Langmuir equations. The linear model relates the sorbed-phase concentration to the aqueous concentration as:

$$q_e = K_d \cdot C_e \quad (7)$$

where K_d, is the soil sorption coefficient (L kg^{-1}). It incorporates both adsorption at the mineral surface and partitioning into any natural organic matter (ElShafei *et al*., 2009). Organic matter (OM) greatly affects the adsorption process of the pesticides in the soil, mainly because the particles of organic matter or clay provide the soil with an increased number of adsorptive sites onto which pesticides molecules can bind (Rani and Sud Sant, 2014). Therefore, K_d is usually normalized with respect to the soil organic matter content (Eqn. 8):

$$K_{oc} = \frac{K_d}{f_{oc}} \quad (8)$$

where K_{oc} is the soil organic carbon sorption coefficient (kg L^{-1}) and f_{oc} is the amount of organic matter in the soil (g g^{-1}). Next, the experimental data were tested using the Freundlich equation which is related to non-ideal, reversible, and multilayer adsorption with non-uniform distribution of adsorption heat and affinities over the heterogeneous surface (Bajeer *et al*., 2012). The equation was used in its log-transformed form (Eqn. 9):

$$\log(q_e) = \log(K_f) + \frac{1}{n}\log(C_e) \quad (9)$$

where K_f (L kg^{-1}) is the adsorption coefficient characterizing the adsorption capacity and n is the Freundlich equation exponent related to the adsorption intensity, which is used as an indicator of the adsorption isotherm nonlinearity. The Langmuir equation is valid when the adsorption involves the attachment of only one layer of molecule to the surface and the surface has a specific number of sites where the solute molecules can be attached (Giles *et al.,* 1960). The Langmuir equation is (Eqn. 10):

$$\frac{1}{q_e} = \frac{1}{Q_0} + \frac{1}{bQ_0C_e} \quad (10)$$

where Q_0 and b are Langmuir constants related to maximum monolayer adsorption capacity and energy of adsorption, respectively. The coefficient b reflects the equilibrium constant for the adsorption process and is an indication of the affinity of the adsorbent for pesticides (Gupta *et al.,* 2006).

The fitting of the isotherm models was checked by the coefficient of determination (R^2) and the standard error of estimate (*SEE*). The *SEE* value was computed as:

$$SEE = \sqrt{\frac{\sum (q_m - q_e)^2}{n-2}} \quad (11)$$

where q_m and q_e are the measured and calculated adsorbed amount of pesticide in soil, respectively and n is the number of measurement. Using the Langmuir constant, the enthalpy of adsorption ($\Delta H°$), the entropy of adsorption ($\Delta S°$), and the free energy of ad-

sorption ($\Delta G°$) can be calculated using the following equations:

$$\Delta G° = -RT \ln b \quad (12)$$

$$\ln \frac{b_2}{b_1} = -\frac{\Delta H°}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right) \quad (13)$$

$$\Delta G° = \Delta H° - T \cdot \Delta S° \quad (14)$$

Where all parameters were previously defined.

Results and discussion

Method performance

The mean recoveries in water samples for atrazine and metolachlor ranged from 97.02% to 102.2% and 95.15% to 99.33%, while in soil samples ranged from 96.08% to 98.33% for atrazine and from 98.75 to 99.78 for metolachlor (Table 2). The RSD ranged from 0.75 to 3.34% for atrazine and from 1.75 to 4.12% for metolachlor, respectively. Recovery rates and their RSD were acceptable. The LODs (limits of detection) and LOQs (limit of quantification) were found to be 0.01 mg kg^{-1} and 0.05 mg kg^{-1} respectively, for both herbicides in both matrices. These results demonstrate the good performance of the method. The matrix effect of this method was investigated by comparing standards in solvent with matrix-matched standards for five replicates at 1 mg kg^{-1}. Good linearity was obtained over the concentration range (0.05-5 µg mL^{-1}) with $R^2 > 0.999$ for atrazine and metolachlor under these conditions.

Dissipation of atrazine and metolachlor

The herbicides that are used for weed control and more generally to protect plants usually come into contact with soil, where their fate and transport processes are affected by a variety of processes (de Wilde *et al.*, 2008). Temperature is a very important factor governing the rate of degradation in soil.

In the present study, the monitored temperatures were 4.8 ± 0.3, 20.3 ± 0.3, and 35 ± 0.1°C, which are close to the targeted temperatures of 5, 20, and 35°C. In the following discussion these targeted temperatures are therefore used. The concentrations of atrazine 1 hour after the application of herbicides were 2.78 ± 0.04, 2.74 ± 0.06, 2.79 ± 0.02 mg kg^{-1} for the samples kept at 5, 20, and 35°C, respectively. In the samples where soil was sterilized and kept at 5°C, average atrazine concentration was equal to 2.84 ± 0.08 mg kg^{-1}. The concentrations of metolachlor were in the same ranges: 2.89 ± 0.03, 2.81 ± 0.01, 2.85 ± 0.06 mg kg^{-1} for the samples kept at 5, 20, and 35°C, respectively. The average concentration of metolachlor in the samples that were sterilized and kept at 5°C was 2.86 ± 0.07 mg kg^{-1}. At the end of the experiment, 120 days after the application of herbicides, the concentrations of atrazine were as low as 0.49 ± 0.06, 0.09 ± 0.02 and 0.04 ± 0.01 mg kg^{-1} (17.5%, 3.4% and 1.6% of initial concentration, respectively), while the concentrations of metolachlor were 0.74 ± 0.01, 0.10 ± 0.01 and 0.08 ± 0.01 mg kg^{-1} (26%, 3.5% and 2.9% of initial concentration, respectively), for temperatures of 5, 20, and 35°C, respectively. The final concentration of herbicides in the samples that were sterilized and stored at 5°C were 0.54 ± 0.01 and 0.81 ± 0.02 mg kg^{-1} for atrazine and metolachlor, respectively. These concentrations were similar to that of the samples kept at 5°C and not sterilized (Table 3).

The dissipation of atrazine and metolachlor in soil kept at 5, 20, and 35°C is displayed in Figure 2. In general, the dissipations of both atrazine and metolachlor increased with increasing temperature (Figure 2a and b). The dissipation trends of the sterilized and unsterilized samples kept at 5°C were similar (for both atrazine and metolachlor). The degradation rate constants ranged from 0.014 to 0.036 day^{-1} for atrazine and from 0.010 to 0.028 day^{-1}, for metolachlor, respectively.

The corresponding half-lives ranged from 19.2 to 46.9 days for atrazine and from 23.4 to 66.9 days for metolachlor, respectively (Table 3). The significance of difference in

Table 2. Recovery percentage of atrazine and metolachlor.

Matrix name	Fortification levels (mg kg^{-1}) (n* = 5)	Atrazine		Metolachlor	
		Recovery (%)	RSD**(%)	Recovery (%)	RSD**(%)
Water	0.05	97.02	3.34	95.15	4.12
	0.5	102.2	0.75	99.33	1.98
Soil	0.05	96.08	1.63	98.75	2.74
	0.5	98.33	1.52	99.78	1.75

* number of replicates
** Relative Standard Deviation

Table 3. Half-lives and rate equation of atrazine and metolachlor in soil.

Temperatures	Atrazine			Metolachlor		
	Rate equation	DT50 (days)	CI* (days)	Rate equation	DT50 (days)	CI* (days)
5°C	$C_t = 2.538e^{-0.014t}$	46.9	43.2-49.6	$C_t = 2.568e^{-0.011t}$	62.0	61.4-63.0
5°C**	$C_t = 2.6421e^{-0.014t}$	49.7	48.3-51.0	$C_t = 2.603e^{-0.010t}$	66.9	65.7-68.0
20°C	$C_t = 2.388e^{-0.029t}$	23.5	22.4-24.5	$C_t = 2.749e^{-0.028t}$	24.7	24.3-25.0
35°C	$C_t = 2.487e^{-0.036t}$	19.2	18.1-20.2	$C_t = 2.404e^{-0.025t}$	23.4	23.1-24.0

* Lower and upper 95% confidence intervals of the half-life
** Sterilized samples

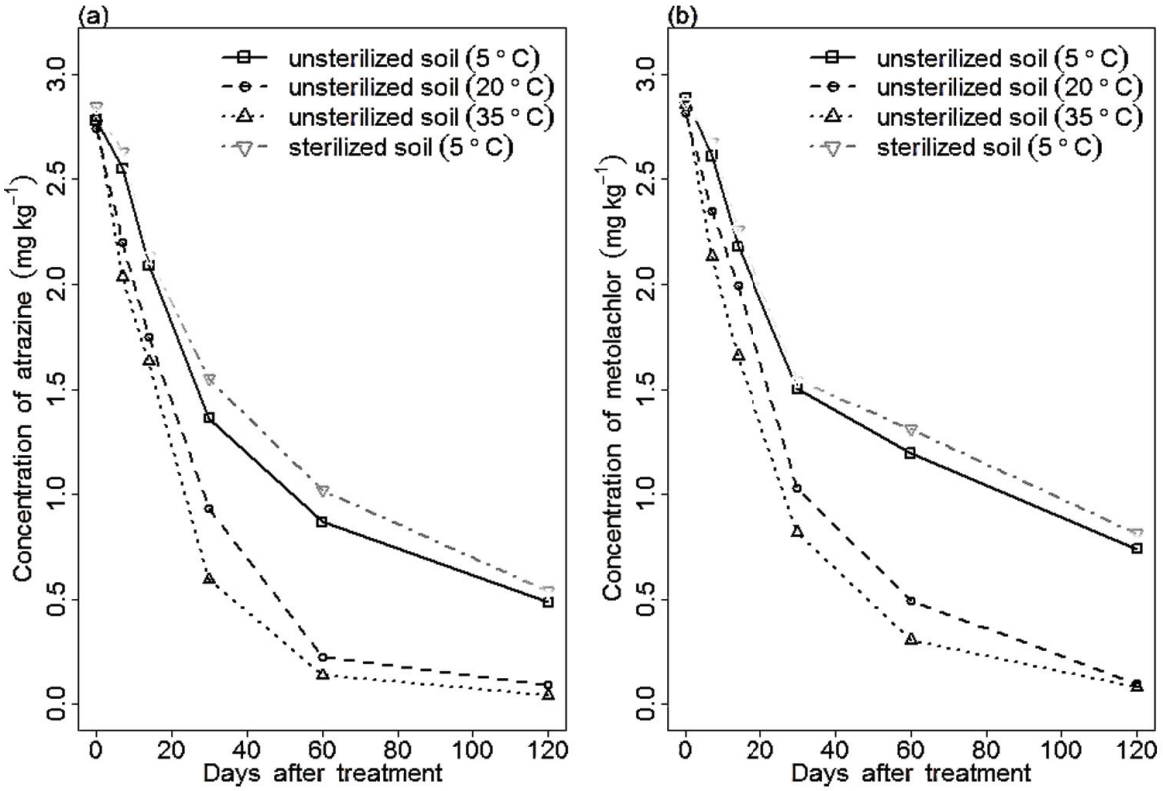

Figure 2. Dissipation of the herbicides (a) atrazine and (b) metolachlor at different soil temperatures.

half-life values at different temperatures was investigated for all combinations of temperatures for atrazine and metolachlor (Student's t test, α=0.05). The results were identical for both herbicides; there were no significant differences between the half-lives computed at 35 and 20°C. In addition, sterilized and unsterilized samples at 5°C also yield statistically identical half-lives. Pesticide degradation was reported to be optimal at mesophilic temperature range (Topp *et al.,* 1997). The half-life values of atrazine and metolachlor confirm this statement as there were significant differences between the half-lives computed at 5°C (sub-optimal conditions) and 20 or 35°C (optimal temperature conditions). The results regarding half-life calculated for the samples kept at 5°C supported that, in those samples, biodegradation was limited while the major dissipation pathway was assumed to be identified as being hydrolysis. For all the other combinations of temperatures, the computed half-life of atrazine and metolachlor were significantly different. Consequently, the dissipation rates of both herbicides were significantly affected by temperature ranging from 20 to 5°C. Vryzas *et al*. (2012) reported that the DT50 ranged from 5 to 18 days for atrazine and 56 to 72 days for metolachlor, respectively. Another study by Gaynor *et al.* (1998) stated that the DT50s of atrazine and metolachlor were similar and ranged from 31 to 66 days. In general, atrazine and metolachlor are considered to be persistent in soils and their half-life ranged from 15 to more than 60 days depending on the soil physicochemical properties (Byer *et al.,* 2011). The low half-life values reported in the literature (Barriuso and Houot, 1996; Vanderheyden *et al.,* 1997; Singh *et al*., 2003; Yassir *et al*., 1999) were related to (1) high soil pH which support higher bacterial biomass, (2) soil exposed to repeated applications of herbicides, and (3) specific management practices . The half-life of atrazine and metolachlor computed in this study are in agreement with the literature (Gaynor *et al.,* 1998) and a previous field monitoring study (Jaikaew *et al.,* 2015). The long half-lives of atrazine and metolachlor calculated for 5°C were similar to those observed in the field during the winter season while the shorter half-lives at 20 and 35°C were similar to those observed in the field during the summer season (Jaikaew *et al.,* 2015).

The activation energies of atrazine and metolachlor calculated using Arhenius equation (Eqn. 4) were 21.47 and 23.91 kJ mol^{-1}, respectively. The *Ea* of atrazine and metolachlor in this study is smaller than that reported in EFSA (2005). The Q_{10} approach gives a quantitative measure of the dissipation response to temperature. The average Q_{10}s computed for atrazine and metolachlor were 1.35 ± 0.22 and 1.42 ± 0.41, respectively. It can be interpreted as an increase of 10°C in temperature would increase atrazine degradation by 1.35 fold while the degradation of metolachlor would increase by 1.42 fold. The Q_{10}s of atrazine and metolachlor were both larger than that reported for the fungicide azoxystrobin (Purnama *et al*., 2014) but lower than the default value of 2.58 proposed by the FOCUS Work Group (FOCUS, 2011). In this study, the soil moisture content of the soil was kept at about 84% of the field capacity which probably explain the differences in half-life. Topp and Smith (1998) reported that the Q_{10} of atrazine and metolachlor was typically greater is soils with higher soil moisture content, while the average Q_{10} for three soil types were 2.52 and 1.77 for atrazine and metolachlor, respectively.

Adsorption equilibrium of atrazine and metolachlor

The present investigation primarily focuses on the effect of temperature with respect to adsorption. The adsorption isotherms obtained for atrazine and metolachlor were characterized by Giles *et al*. (1960) as C-shaped (Figure 3). This type of isotherm is characterized by the constant partition of solute between solution and substrate, until the maximum possible adsorption, where an abrupt change to a horizontal plateau occurs. In this study, the plateau is not visible, therefore the isotherms can be classified as C-1 following the classification established by Giles *et al.* (1960).

This indicates that within the concentration range used for atrazine and metolachlor, the complete saturation of the soil surface has not been reached. The linearity of the adsorption process indicate that the number of sites for adsorption remains constant: as more solute is adsorbed, more sites must be created (Giles *et al.,* 1960). The sorption of atrazine and metolachlor in this andisol soil was moderate, as indicated by the low K_f and K_d values presented in Table 4. The sorption of both herbicides was well fitted by the linear, Freundlich and Langmuir isotherm (R^2 > 0.98). However, the *SEE* were the highest using the Langmuir equation (Table 4). Therefore, the adsorption data can be explained better using the linear and Freundlich isotherm at all experimental temperatures. The sorption isotherms of atrazine were close to being linear since *n* was equal to unity for the experiments kept at 5 and 35°C while it was equal to 0.99 for the experiment kept at 20°C (Table 4). C-type (n=1) isotherms have been also described for the sorption of atra-

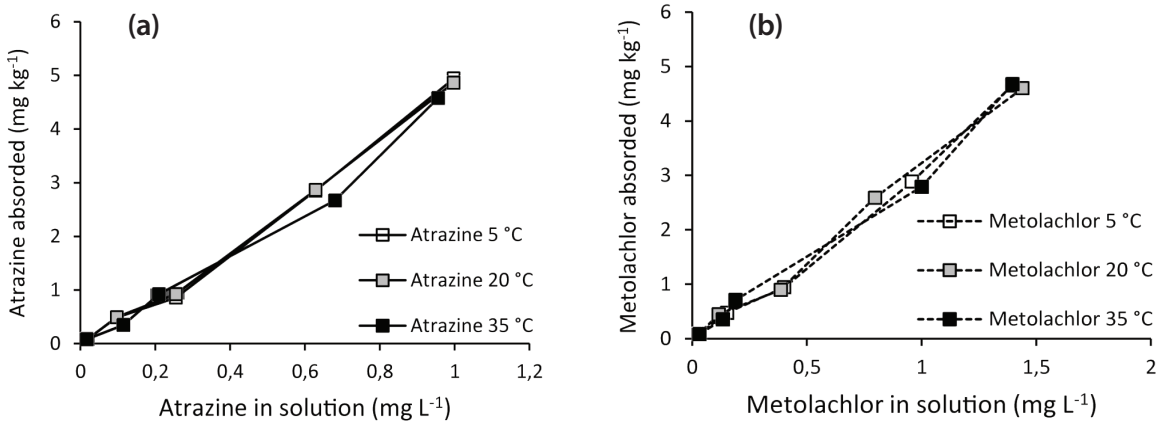

Figure 3. Adsorption isotherm of (a) atrazine and (b) metolachlor at different temperatures (error bars shows standard deviation).

Table 4. Linear, Freundlich and Langmuir isotherms and adsorption coefficients of atrazine and metolachlor.

Models	Parameters	Atrazine			Metolachlor		
		5°C	20°C	35°C	5°C	20°C	35°C
Linear	K_d (*CI*) (L kg^{-1})	4.52 (3.47-5.06)	4.51 (3.70-5.07)	4.18 (3.11-4.79)	3.12 (2.39-3.80)	2.94 (2.40-3.31)	3.05 (2.65-3.72)
	R^2	0.99	0.99	0.98	0.98	0.99	0.98
	SEE	0.60	0.25	0.36	0.25	0.36	0.29
Freundlish	K_f (*CI*) (L kg^{-1})	4.41 (4.38-4.45)^a	4.52 (4.47-4.55)^b	4.11 (3.91-4.34)^a	2.98 (2.96-2.99)^a	3.04 (3.00-2.99)^b	3.15 (3.05-3.25)^{a,b}
	n	1.00	0.99	1.00	0.98	1.00	0.97
	R^2	0.99	0.99	0.98	0.99	0.98	0.99
	SEE	0.35	0.24	0.39	0.30	0.28	0.26
Langmuir	*b* (L mol^{-1})	0.95	3.86	6.42	1.6E^{-2}	1.86	6.5E^{-1}
	Q_0 (mol g^{-1})	4.88E^{-3}	1.13E^{-3}	7.51E^{-4}	1.78E^{-1}	1.57E^{-3}	7.51E^{-3}
	R^2	0.99	0.99	0.99	0.99	0.99	0.99
	SEE	0.65	1.55	1.60	0.66	1.07	0.43

CI: Upper and lower 95% confidence interval
a, b: Assess the significance of the K_f, K_d and computed at different temperatures (two tailed t-test, α=0.05)

zine on soils (Celis *et al.,* 1997). The exponent 1/*n* of metolachlor was less than 1 for temperature of 5 and 35°C which indicating that metolachlor adsorbed to the andisol soil decreased slightly as the initial concentration increased (Flores *et al.,* 2009; Seybold and Mersie, 1996). Adsorption of atrazine and metolachlor can be considered to be linear over the concentration range evaluated. The significance in difference of K_f and K_d at different temperatures was investigated using two tails *t*-test, assuming equal variance in the data for all combination of temperatures (Table 4). The results were contrasted, and no temperature effect was detected with the adsorption of atrazine and metolachlor. In general, decreasing herbicide adsorption at higher temperatures has been observed and correlated to the increase of the solubility of herbicide (Kovaios *et al.,* 2006). Atrazine solubility in water was reported to be 33 mg L^{-1} at 20°C while the solubility of metolachlor in water was 530 mg L^{-1} at 20°C (Nemeth-Konda *et al*., 2002). In addition, at higher temperatures, the bond between component atoms and soil surface might be weaker so that herbicides can easily move from soil to water solute, resulting in a decrease of sorption with increasing temperatures. These trends were however not observed in the reported experiments.

The sorption results of atrazine and metolachlor were similar to those reported in the literature. The K_f values reported for atrazine include 0.2-4.2 L kg^{-1} (Brouwer *et al.,* 1990), 3.8-6.5 L kg^{-1} (Sharon and Koskinen, 1990), 0.4-3.1 L kg^{-1} (Moreau and Mouvet, 1997), and 1.5-2.0 L kg^{-1} (Seybold and Mersie, 1996). For metolachlor, K_f ranging from 3.72 to 6.61 and *n* ranging from 0.97 to 1.17 were reported by Krutz *et al.* (2004). The K_d values determined for metolachlor in this paper are also within the range of the K_d reported in the literature (Krutz *et al*., 2004).

To compare our result with other studies, the adsorption coefficients were normalized with respect to the organic carbon content of the soil used in this experiment (Eqn. 8); for this andisol soil, the K_{oc} varied between 59.7 and 64.0 L kg^{-1} for atrazine and between 43.1 and 45.7 L kg^{-1} for metolachlor, respectively. K_{oc} was reported to be negatively correlated with the aqueous solubility of chemicals (Seybold and Mersie, 1996). This trend was confirmed by this experiment as the solubility of atrazine is lower than that of metolachlor and the average K_{oc} of atrazine was higher than that of metolachlor.

Adsorption thermodynamic of atrazine and metolachlor

The effect of temperature and moisture on the mass transfer of solutes is particularly complex, where solubility of compounds in water, transport to the binding sites via diffusion and chemical sorption reactions are enhanced at higher temperatures (Tripathi *et al.,* 2015; Chirukuri and Atmakuru, 2015). The enthalpy and entropy values can give some indication of the type of mechanism involved in the sorption process. For the enthalpy ($\Delta H°$), Van der Waals interactions prevail at low energy level while H bonds are the main interactions in the range of 8-40 kJ mol^{-1} (DiVincenzo and Sparks, 1997). Chemical sorption was reported to be associated with enthalpy higher than 40 kJ mol^{-1} (Flores *et al.,* 2009; Rani and Sud Sant, 2014). The enthalpy of adsorption of atrazine and metolachlor calculated for this study were 45.5 and 82.9 kJ mol^{-1}, respectively (Table 5). The value for atrazine indicated H bond interactions between atrazine and soil functional groups. The sorption of s-triazines on

Table 5. Thermodynamic parameters for adsorption of atrazine and metolachlor in andisol soil.

	$\Delta H°$ (kJ/mol)	$\Delta S°$ (J/mol K)	$-\Delta G°$ (kJ/mol)		
			278 K	293 K	308 K
Atrazine	45.5	77.2	32.0	31.8	30.6
Metolachlor	82.9	409.1	41.5	32.1	36.5

organic matter was indeed governed by H bonds and proton transfer between s-triazines and acidic groups of humic substances (Barriuso *et al.,* 1997). For metolachlor, our findings suggest that sorption is mainly driven by chemical interactions. In addition, the adsorption of atrazine and metolachlor on andisol soil was confirmed as being endothermic by the positive Δ*H*° (Table 5). The entropy (Δ*S*°), of atrazine and metolachlor is positive, resulting in an increase in the disorder which have been interpreted by ElShafei *et al.* (2009) and Gurses *et al.* (2004) as the increasing degree of freedom of the water molecules as the molecules of herbicides decrease. The positive value of entropy resulted in negative values for Δ*G*° which indicates spontaneous adsorption processes and that adsorption occurs through a bonding mechanism (ElShafei *et al.,* 2009; Gupta *et al.,* 2006; Shariff, 2011). In general, the value of Δ*G*° for adsorption decreased with increasing temperature indicating that the interaction of pesticide was spontaneous with high preference of the soil surface.

Conclusion

In conclusion the half-lives of atrazine were 46.9, 23.5, and 19.2 days at 5, 20, and 35°C, respectively. The half-lives of metolachlor were 62.0, 24.7, and 23.4 days for 5, 20, and 35°C, respectively. The dissipation rate of atrazine and metolachlor were significantly affected by temperature in the 5-20°C range. Investigation of the adsorption behaviors of atrazine and metolachlor using linear, Freundlich and Langmuir isotherms, showed that the linear and Freundlich isotherms well fitted the experimental data. The range of the parameters were similar to those reported in the literature; K_{oc} of atrazine ranged from 59.7 and 64.0 L kg^{-1} while that of metolachlor ranged from 43.1 and 45.7 L kg^{-1}. Temperature did not affect the adsorption of atrazine and metolachlor. The sorption of both herbicides was highlighted as being endothermic by calculating the enthalpy of adsorption which was positive. In addition, the range of the parameter suggested that atrazine and metolachlor adsorbed to soil majorly due to H bond interactions and chemical interactions, respectively, between herbicides and soil functional groups.

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Κινητική αποδόμησης και ρόφησης των ζιζανιοκτόνων ατραζίνη και metolachlor σε έδαφος ηφαιστειακής τέφρας

P. Jaikaew, F. Malhat, J. Boulange και H. Watanabe

Περίληψη Στην παρούσα εργασία αξιολογήθηκε η κινητική αποδόμησης και τα χαρακτηριστικά ρόφησης των ζιζανιοκτόνων ατραζίνης και metolachlor σε ιαπωνικό έδαφος ηφαιστειακής τέφρας (andisol). Η κινητική της αποδόμησης διερευνήθηκε με επώαση των δειγμάτων εδάφους στο εργαστήριο. Η περιεκτικότητα σε νερό ορίσθηκε στην υδατοϊκανότητα του εδάφους, ενώ για το πείραμα λήφθηκαν υπόψη τρεις διαφορετικές θερμοκρασίες (5, 25 και 35°C). Η κινητική αποδόμησης και των δύο ζιζανιοκτόνων αποδόθηκε χρησιμοποιώντας κινητική πρώτης τάξης στο συγκεκριμένο εύρος θερμοκρασίας, με χρόνους ημιζωής που κυμαίνονταν από 19.2 έως 46.9 ημέρες για την ατραζίνη και από 23.4 σε 66.9 ημέρες για το metolachlor, αντίστοιχα. Οι ενέργειες ενεργοποίησης (*Ea*) της ατραζίνης και του metolachlor υπολογίστηκαν χρησιμοποιώντας την εξίσωση Arhenius και ήταν 21.47 και 23.91 kJ mol^{-1}, αντίστοιχα. Η μελέτη ρόφησης στο έδαφος πραγματοποιήθηκε χρησιμοποιώντας τη μέθοδο batch equilibrium. Η συμπεριφορά προσρόφησης της ατραζίνης και του metolachlor διερευνήθηκαν χρησι-

μοποιώντας τις ισόθερμες Γραμμική, Freundlich και Langmuir. Από αυτές, οι ισόθερμες Γραμμική και Freundlich έδωσαν σχετικά υψηλό συντελεστή συσχέτισης (R^2) και πολύ χαμηλό τυπικό σφάλμα εκτίμησης (*SEE*). Η ελεύθερη ενέργεια ($\Delta G°$) κυμάνθηκε για την ατραζίνη από -30.6 έως -32.0 kJ mol^{-1}, και για το metolachlor από -32.1 έως -41.5 kJ mol^{-1}. Η αξιολόγηση των θερμοδυναμικών παραμέτρων έδειξε ότι η προσρόφηση είναι αυθόρμητη, ενδόθερμη και συνοδεύεται από αύξηση της εντροπίας. Η κατανόηση των διαδικασιών ρόφησης της ατραζίνης και του metolachlor είναι απαραίτητη στο καθορισμό της τύχης και συμπεριφοράς των φυτοφαρμάκων και της διαθεσιμότητάς τους στο έδαφος, για τον έλεγχο των φυτοπαρασίτων, τη βιοαποδόμηση, την απορροή και την έκπλυση.

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Efficiency of sweet flag and curly parsley volatile oils compared with synthetic insecticides against *Ceroplastes rusci* on Ruellia plants

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Summary *Ruellia simplex* plant is grown for its aesthetic features including flowers, leaves and overall foliage appearance. The fig wax scale *Ceroplastes rusci* L. (Hemiptera: Coccidae) was detected for the first time in Egypt on *R. simplex*. Mineral oil, diazinon, thiamethoxam + chlorantraniliprole, and essential oils extracted from *Acorus calamus* and *Petroselinum crispum,* were compared for their ability to control the insect. Results indicated that reduction percentage increased gradually until day 7 after the treatment regarding adults, nymphs and their total. The maximum efficacy of the mineral oil, and thiamethoxam + chlorantraniliprole, was noticed 21 days after treatment, followed by *A. calamus* oil. Efficacy of *P. crispum* oil and diazinon reached more than 86% after 21 days and more than 90% 28 days after treatment. At 28 days, *A. calamus* oil reached its maximum efficacy. Plants treated with thiamethoxam + chlorantraniliprole were the tallest plants and possessed significantly higher number of branches and leaves, and leaf pigments followed by those treated with mineral oil or *A. calamus* oil. *A. calamus* oil and thiamethoxam + chlorantraniliprole were proved as promising compounds tested for the first time in controlling *C. rusci*.

Additional keywords: Acorus calamus, essential oil, fig wax scale insect, *Petroselinum crispum*, *Ruellia simplex*

Introduction

One of the newly introduced flowering ornamental plants into Egypt is *Ruellia simplex* C. Wright 'Katie', syn. *Ruellia brittoniana* Leonard 'Katie' known as Dwarf Mexican Petunia. It is a very popular landscape plant, effectively grown as an annual flowering plant as a pond marginal, in beds and borders, as free-blooming plant in large containers; it may be grown indoors as a houseplant (Gilman, 1999; Ezcurra and Daniel, 2007; Anonymous, 2015a).

The fig wax scale, *Ceroplastes rusci* L. (Hemiptera: Coccoidea: Coccidae), is a polyphagous pest that attacks a wide range of plants from at least 21 different families of fruits, ornamental plants and high economic value trees. It is well known as a serious pest of figs (*Ficus* spp.) and other commercial fruit crops in the Mediterranean region and in several agricultural regions of the world. It attacks almost all parts of the host plant and can cause death of the weakened and heavily infested leaves and shoots. Sooty moulds developing on the scale honeydew can reduce photosynthetic activity of leaves (Al-Momany and Al-Antary, 2008; Vu *et al*., 2006; Deng *et al*., 2015; Ismail *et al*., 2015). In addition to the direct plants physical damage, *C. rusci* is known to carry plant viruses (La Notte *et al*., 1997).

Chemical control is the main and effective tool used worldwide for controlling scale insects and mealybugs (Franco *et al.,* 2009). Mineral oils have been proved highly effective for controlling scale insects in a variety of fruits and orrnamental plants all over the world including Egypt (Ismail *et al*., 2015). Various organophosphorous compounds such as diazinon were also found to be effective against scale insects (Abd-

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Rabou *et al*., 2012). However, some of these compounds exhibited harmful effect on the natural enemies of the pests and other non-target organisms (Satar *et al*., 2013). Recently, a new insecticide was developed by mixing thiamethoxam and chlorantraniliprole. Thiamethoxam is a neonicotinoid insecticide found highly effective in controlling scale insects, mealybugs and other sucking pests (Abbas *et al*., 2014; Mohamed *et al*., 2015). Chlorantraniliprole is a relatively new anthranilic diamide systemic insecticide which has a potent insecticidal activity against a wide range of pests including Hemiptera (Sattelle *et al*., 2008).

Increasing emphasis is being placed on the potential usage of natural products such as essential oils as more environmentally friendly pesticides alternative to synthetic insecticides. Concerns about phytotoxicity of these oils and lack of confidence in their efficacy are the main reasons beyond the reluctance of many growers to incorporate such compounds in their pest management programs (Miller and Uetz, 1998). Essential oils from different plants have been known to show insecticidal, bactericidal, fungicidal and herbicidal properties (Bakkali *et al*., 2008). Of these compounds, volatile oil from the perennial plant *Acorus calamus* L. (Araceae), common name sweet flag, and *Petroselinum crispum* (Mill.) Fuss var. *crispum* (Apiaceae), common name curly parsley, are considered in the current research. The essential oil of sweet flag has been found by several authors such as Liu *et al.* (2013) to exhibit toxicity, chemosterilant, antifeedant and growth inhibitory effects against a variety of insect pests. Asarone as ether is the main component of the oil which is used for the control of pests and bacteria (Abdul-Hafeez and Egorov, 2012). Parsley essential oil is obtained from the leaves and seeds of curly parsley (Petropoulos *et al*., 2004). It was found by many authors such as Mulugeta *et al*. (2015) to have insecticidal, nematicidal, antimicrobial and antiradical activities.

Accordingly, the main objective of the current research was to compare the efficiency of natural volatile oils of both *A. calamus* and *P. crispum* with famous synthetic insecticides viz; mineral oil, thiamethoxam + chlorantraniliprole, diazinon, as foliar application for controlling the fig wax scale on *R. simplex* plants.

Material and Methods

Pot plants of *R. simplex* heavily infested with the fig wax scale were used. The plants were grown in 20-cm pots and kept under lathhouse conditions (70% shade) over the period of the experiment. The current study was conducted at the Experimental Farm of the Department of Ornamental Plants and Landscape Gardening, Assiut University, Egypt during 2014 and 2015.

Tested compounds

The insecticides tested were 20% thiamethoxam + 20% chlorantraniliprole (Voliam flexi 40% WG obtained from Syngenta Crop Protection, Switzerland); diazinon (Diazin 60% EC obtained from Medmac for Manufacturing Agricultural Chemicals and Veterinary Products Company Ltd. Jordan) and KZ mineral oil 95% EC (Kafr El-Zayat Co., Egypt). The essential oils of *A. calamus* and *P. crispum* were extracted by the hydro-distillation method using a Clevenger-type Apparatus (Clevenger, 1928) at the Laboratory of Medicinal Plants, Faculty of Agriculture, Assiut University. Roots of *A. calamus* were collected from Kazan state (Tatarstan Republic, Russia). The vegetative parts were collected from parsley plants grown at the Floriculture Experimental Farm, Assiut University. Samples were shredded into small pieces and distilled. The essential oil was collected in dark glass vessels and kept at -40°C until it was used.

Bioassays

Both essential oils were tested at the rate of 2ml/l water and emulsified with 1ml of Tween-80. The rates of the commercial formulations were 0.4 g thiamethoxam + chlorantraniliprole/l water, 5ml diazinone/l water and 15 ml mineral oil/l water. Also a

non-treated control was used. Each treatment comprised of 3 plants and was replicated 3 times. The insecticides and essential oils were sprayed using a hand laboratory sprayer equipped with a flat-fan nozzle. Data regarding the number of live nymphs and adults of *C. rusci* were recorded before application and after 1, 3, 7, 15, 21, 28 and 35 days. Population reduction percentage was calculated according to Henderson and Tilton's formula (1955). Upon completion of the experiment, vegetative growth characteristics were recorded i.e. plant height, branch number/plant and leaf number/plant. In addition, leaf content of chlorophyll a, b and carotenoids was determined as mg/g fresh weight according to the acetone incubation method described by Krishnan *et al.* (1996) and Dere *et al*. (1998).

Statistical analysis

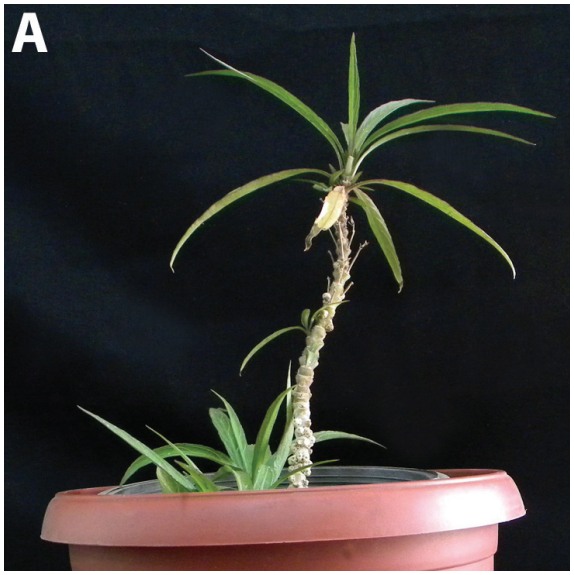
Data were statistically analyzed using two-way split-plot ANOVA (Snedecor and Cochran, 1989) where time of observations was considered as sub-plot and tested compounds as main-plot. Means were separated by least significant difference (L.S.D.) at 5% level of significance according to Steel and Torrie (1982). Statistical analysis was performed using Statistix 8.1 program.

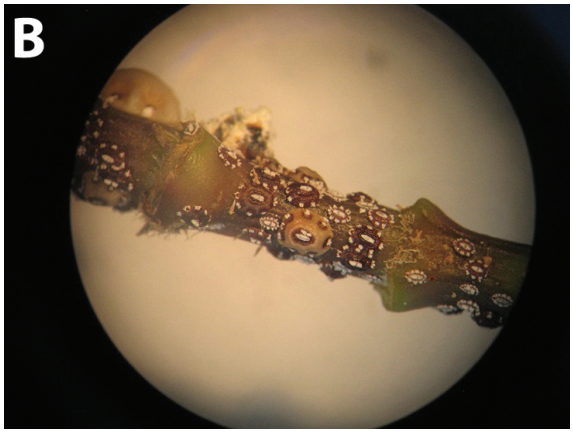
Results

Reduction % of *C. rusci*

Ceroplastes rusci was detected and recorded on *R. simplex* plants grown at the Floriculture Experimental Farm, Faculty of Agriculture, Assiut University, Egypt. To our knowledge, this is the first report of *C. rusci* on *R. simplex* plants in Egypt. Figure 1 illustrates an infested plant and different insect stages on stems and leaves.

Results revealed that one day after treatment, all tested compounds induced reductions of nymph population of *C. rusci* (2.08 -6.09 %), which did not significantly differ among them (Table 1). Among all tested compounds, only *A. calamus* oil showed mortality against the adult stage, which





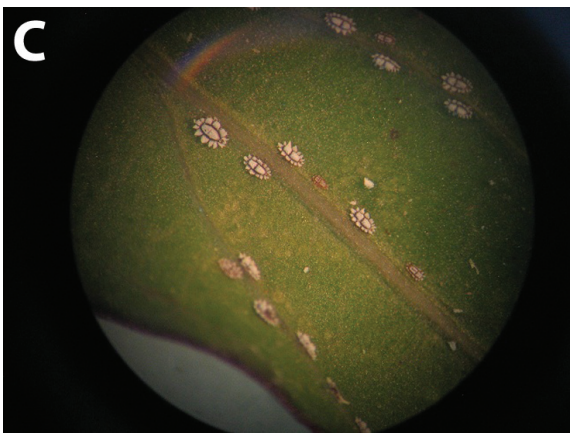


Figure 1. Non-treated *Ruellia simplex* plants infested with nymphs and adults of *Ceroplastes rusci*. A: infested whole plant, B: infested stem with all insect stages (eggs, nymphs and adults), C: infested leaf. Images B and C are under binocular stereoscope (magnification 10X).

Table 1. Effect of synthetic insecticides and volatile oils on the reduction percentage of nymph, adult and total populations of *Ceroplastes rusci* on *Ruellia simplex* plants.

Days after treatment	Compounds	Percentage of Reduction (%)		
		Nymphs	Adults	Total
1	Thiamethoxam + chlorantraniliprole	4.71	0.00	2.36
	Diazinon	2.08	0.00	1.04
	Mineral Oil	5.57	0.00	2.79
	Petroselinum crispum Oil	4.38	0.00	2.19
	Acorus calamus Oil	6.09	0.39	3.24
3	Thiamethoxam + chlorantraniliprole	30.92	34.66	32.79
	Diazinon	13.85	20.19	17.02
	Mineral Oil	33.37	33.54	33.46
	Petroselinum crispum Oil	23.05	13.37	18.21
	Acorus calamus Oil	34.99	45.58	40.29
7	Thiamethoxam + chlorantraniliprole	66.22	60.75	63.48
	Diazinon	40.62	48.38	44.50
	Mineral Oil	76.13	76.14	76.13
	Petroselinum crispum Oil	48.92	33.95	41.43
	Acorus calamus Oil	73.81	75.28	74.55
15	Thiamethoxam + chlorantraniliprole	90.88	88.33	89.61
	Diazinon	67.37	76.30	71.84
	Mineral Oil	91.92	93.99	92.95
	Petroselinum crispum Oil	69.25	57.48	63.37
	Acorus calamus Oil	93.00	91.22	92.11
21	Thiamethoxam + chlorantraniliprole	98.02	99.34	98.68
	Diazinon	87.56	92.18	89.87
	Mineral Oil	98.59	99.64	99.11
	Petroselinum crispum Oil	89.68	86.63	88.16
	Acorus calamus Oil	96.15	97.45	96.80
28	Thiamethoxam + chlorantraniliprole	97.82	96.22	97.02
	Diazinon	93.79	92.87	93.33
	Mineral Oil	93.69	95.73	94.71
	Petroselinum crispum Oil	95.32	90.54	92.93
	Acorus calamus Oil	98.02	99.35	98.68
35	Thiamethoxam + chlorantraniliprole	90.66	92.01	91.34
	Diazinon	89.31	84.75	87.03
	Mineral Oil	85.71	83.83	84.77
	Petroselinum crispum Oil	89.00	86.98	87.99
	Acorus calamus Oil	94.57	95.76	95.17
LSD 0.05	Time	2.38	5.40	2.87
	Compounds	1.93	2.76	2.03
	Time × Compounds	5.10	7.29	5.38

gave 0.39 % reduction. These results indicate that nymphs of *C. rusci* were more susceptible to the all tested compounds than the adult stage one day after treatment.

Three days after treatment, *A. calamus* provided the highest reduction percentage against nymphs and adults, followed by the mineral oil and thiamethoxam + chlo-

rantraniliprole (Table 1, Figure 2). Diazinon was found to be the least toxic compound, caused the least reduction against nymphs and adults (Table 1).

Seven days after treatment, a slight change in the efficacy order of the tested compounds was noticed; the mineral oil was the most toxic compound on nymphs and adults followed by *A. calamus* oil, and then thiamethoxam + chlorantraniliprole. Both diazinon and *P. crispum* oil were found comparatively the least effective compounds (Table 1).

Fifteen days after treatment, the evaluated compounds revealed the same efficacy order as at three days after treatment for adults and in total (nymphs and adults). For nymphs, the *A. calamus* oil showed the highest reduction percentage, followed by the mineral oil and thiamethoxam + chlorantraniliprole with non significant differences among the three compounds. However, they significantly differed from diazinon and the *P. crispum* oil, which recorded the least reduction percentages (Table 1).

Twenty one days after the treatment, the mineral oil, thiamethoxam + chlorantraniliprole and *A. calamus* oil exhibited the greatest potent against nymphs, adults and in total, where they gave 96.8-99.11 % reduction with non-significant differences between them. Diazinon and *P. crispum* oil had the least effect (Table 1).

Twenty eight days after treatment, *A. calamus* oil and thiamethoxam + chlorantraniliprole recorded the highest reduction on nymphs and adults (over 96%). The *P. crispum* oil, the mineral oil and diazinon showed also high reduction percentages (over 90%) of nymphs, adults and in total.

Thirty five days after treatment, *A. calamus* oil and thiamethoxam + chlorantraniliprole induced the highest reduction in the nymph, adult and total populations, with non-significant differences between them. Non-significant differences were noticed between both *P. crispum* oil and diazinon reduction percentages of adult, nymph and total populations. The mineral oil exhibited the least efficacy on all insect stages.

In general, adult, nymph and total reduction percentage of *C. rusci* increased gradually until day 21 after the treatment. After 7 days, *A. calamus* oil, thiamethoxam + chlorantraniliprole and mineral oil exhibited the best results while *P. crispum* oil and diazinon

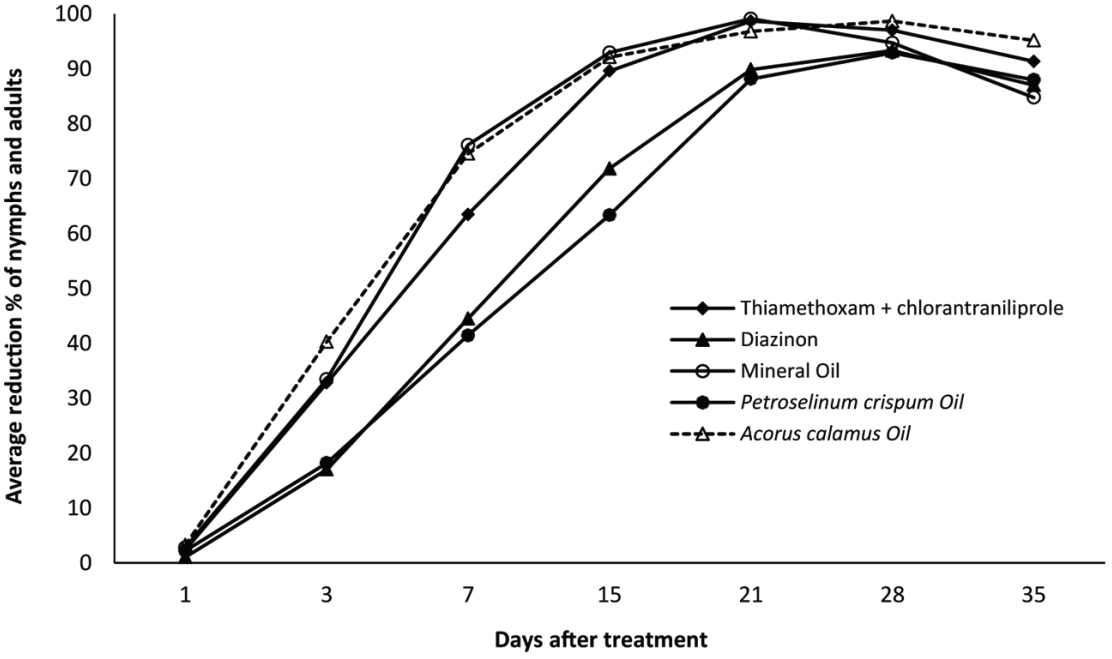


Figure 2. Effect of synthetic insecticides and volatile oils on total reduction percentage of nymphs and adults of *Ceroplastes rusci* on *Ruellia simplex* plants.

showed the lowest ones. The highest reduction percentage was recorded with the same three compounds between day 15 and 28, reaching more than 90% in all cases except the reduction percentage in adult and total with thiamethoxam + chlorantraniliprole after 15 days. The maximum efficacy of mineral oil, and thiamethoxam + chlorantraniliprole, was noticed after 21 days (more than 98%), followed by *A. calamus* oil (more than 96%) with no significant differences. Efficacy of both *P. crispum* oil and diazinon increased gradually and reached more than 86% after 21 days and more than 90%, 28 days after the treatment. At 28 days, *A. calamus* reached its maximum efficacy (more than 98%). The effect of all tested compounds started to decrease gradually after 28 days.

Plant growth

Data presented in Table 2 show some vegetative characteristics of Ruellia plants infested with *C. rusci* after the treatment with pesticides or volatile oils. Plants treated with thiamethoxam + chlorantraniliprole were the tallest ones and possessed the highest number of branches and leaves followed by those treated with the mineral oil or *A. calamus* oil. Moreover, the plants treated with *A. calamus* oil exhibited slight leaf yellowing suggesting an evidence of slight phytotoxicity, which completely recovered about two weeks after the treatment.

Leaf content of chlorophyll a, b and carotenoids further support the superiority of thiamethoxam + chlorantraniliprole and *A. calamus* oil in alleviating the negative impact of the insect on growth quality of Ruellia plant. Chlorophyll a reached the highest values in the plants treated with thiamethoxam + chlorantraniliprole, *A. calamus* oil and mineral oil, respectively. While chlorophyll b had the same trend, carotenoids' content was found significantly higher in *A. calamus* oil, mineral oil and *P. crispum* oil (Table 2).

Discussion

Ruellia plant is grown for its aesthetic features including flowers, leaves and overall foliage appearance, which was found to be highly affected by the infestation with *C. rusci*. Different compounds were tested for their ability to control insect and alleviate its negative influence on plant growth and appearance.

Mineral oil, the only recommended pesticide for *C. rusci* in Egypt, exhibited high efficacy in controlling *C. rusci* nymphs and adults; the highest efficacy was recorded 21 days after treatment which decreased after 28 days of the treatment. Inhibition of insect

Table 2. Final quality parameters and leaf content of chlorophyll a, b and carotenoids of *Ruellia simplex* plants after the treatment with synthetic insecticides and volatile oils.

Treatments	Measurements					
	Plant height cm	Branch number/ plant	Leaf number/ plant	Chl. "a" mg/g FW	Chl. "b" mg/g FW	Carotenoids mg/g FW
Control (untreated)	15.00 c	3.67 b	33.33 d	0.222 d	0.072 d	0.154 c
Thiamethoxam + chlorantraniliprole	16.90 a	5.57 a	55.23 a	0.427 a	0.127 ab	0.173 bc
Diazinon	16.73 a	3.77 b	38.16 c	0.285 c	0.072 d	0.157 c
Mineral Oil	15.83 b	5.77 a	52.53 a	0.397 a	0.117 bc	0.214 a
Petroselinum crispum Oil	15.93 b	3.50 b	45.17 b	0.327 b	0.102 c	0.192 ab
Acorus calamus Oil	16.50 ab	5.67 a	48.43 b	0.422 a	0.138 a	0.223 a
LSD 0.05	0.79	0.99	3.90	0.038	0.017	0.033

* Means in the same column followed by the same letter are not significantly different at $p=0.05$, LSD test.

growth was strongly related with significant improvement in plant growth characteristics including plant height, number of branches and leaves, and leaf content of chlorophylls a and b. In agreement with our results, several previous studies revealed that mineral oils application gave satisfactory results in controlling scale insects such as *C. rusci* in figs (Ismail *et al*., 2015), soft wax scale *Ceroplastes destructor* Newstead on citrus (Blank *et al*., 1997), the guava soft scale *Pulvinaria psidii* Mask on guava (Aly *et al*., 1984), the mango soft scale *Klifia acuminata* Signoret and *P. psidii* on mango (Nada *et al*., 1990), and the nigra scale *Parasaissetia nigra* Nietner and the cottony camellia scale *Pulvinaria floccifera* Westwood on guava and mango (Abd-Rabou *et al*., 2012). It was also proved effective against scale insects in Egypt such as olive scale (*Parlatoria oleae* Colvee) (Ibrahim, 1990). The mode of action of mineral oils is usually assumed to be suffocation (Davidson *et al*. 1991). In some cases, oils also may act as poisons through their interacting with the fatty acids of the insect and their interfering with normal metabolism (Helmy *et al*., 1992; Cranshaw and Baxendale, 2011). Nevertheless, other compounds showed promising results which significantly surpassed those of the mineral oil.

The volatile oil of *A. calamus* exhibited significantly high efficacy against nymphs and adults of *C. rusci*, exceeding 90% inhibition 21 days after treatment (more than 96%) and reaching its maximum efficacy (more than 98%) after 28 days; the efficacy declined after 35 days, yet it was still superior to other tested compounds. The decrease in insect populations led to a noticeable recovery in overall plant appearance as could be inferred from the data of plant vegetative characteristics. *A. calamus* oil has been shown by several previous studies to exhibit insecticidal activity caused by its active component (asarone) against several insect species of economic importance (Hossain *et al*., 2008; Liu *et al*., 2013). It induced antifeedant and growth inhibitory effects in *Peridroma saucia* (Koul and Isman, 1990), *Spodoptera litura* Fab. (Thanapandian *et al*., 2011) and *Tribolium castaneum* (Chandel *et al*., 2001). Moreover, Saxena *et al*. (1977) found that asarone possessed insect chemosterilant properties. The active component of *A. calamus* (α- and β-asarone) inhibited both spermatogenesis and oogenesis causing sterility in a variety of insect species such as the melan fly *Bactrocera cucurbitae* (Nair *et al*., 2001) and the kelp fly *Coelopa frigida* (Ramos-Ocampo and Hsia, 1986).

Our results provided an evidence that thiamethoxam and chlorantraniliprole is a promising agent which exhibited excellent control of *C. rusci* insect on *R. simplex* plants with no evidence of phytotoxicity. Treated plants were characterized by the best growth characteristics including plant height, number of branches and leaves, and leaf content of chlorophylls a and b. This mixture is a new insecticide, containing active substances which belong to neonicotinoids and anthranilic diamide with different mode of actions (Saglam *et al*., 2013). Our findings are in accordance with those obtained by several previous studies such as Awamleh *et al*. (2009). Ahmed *et al*. (2005) and Taha *et al*. (2012) also demonstrated the efficacy of thiamethoxam on the green date palm pit scale (*Asterolicanium phoenicis* Rao) on date palm. Chlorantraniliprole is very effective against several Lepidopteran, Coleopteran and Hemipteran pests that attacked fruit, vegetable and ornamental plants (Sattelle *et al*., 2008). In addition, chlorantraniliprole may have some ovicidal effect against fig wax scale. The efficacy of chlorantraniliprole on egg hatching has been found in other pests such as the diamondback moth, *Plutella xylostella* L. (Han *et al*., 2012), and *Tribolium confusum* Jacquelin du Val (Saglam *et al*., 2013). Chlorantraniliprole has a novel mode of action that interrupts the normal muscle contraction causing impaired regulation, paralysis and ultimately death of sensitive insect species or causing feeding cessation, lethargy, and muscle paralysis, finally insect death (Lahm *et al*., 2005; Cordova *et al*., 2006).

In comparison with the other tested compounds, both *P. crispum* oil and diazinon exhibited the least reduction percentages of all insect stages. They had no signif-

icant effect on the insect until 15 days after the treatment then the effect increased at 21 days and reached the maximum at 28 days (more than 90%). The insecticidal action of *P. crispum* essential oil might be attributed to the toxicological properties of the chemical components of the oil that can have various effects on the respiratory and gastroenterology systems of the insects (Mahmoodi *et al*., 2014). Gruszecki (2009) reported that the main components of parsley leaf essential oil are α-pinene (25.5%), p-cymenene (17.7%), β-myrcene (16.9%), β-phellandrene (15.2%) and β-pinene (9.6%). Its efficacy has been reported against several insects such as adults of *Trialeurodes vaporariorum* (Westwood) (Mahmoodi *et al*., 2014).

Effectiveness of different organophosphate insecticides, including diazinon, has been evaluated against scale insects in several previous studies. Diazinon inhibits the cholinesterase in the target pests and shows satisfactory effect against scale insects in fruit, ornamental and cut flower crops (Kwaiz, 1999).

In conclusion, *A. calamus* oil and thiamethoxam + chlorantraniliprole oil were proved as promising compounds tested for the first time in controlling *C. rusci* on *R. simplex*, compared to the recommended compound of mineral oil.

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Αποτελεσματικότητα αιθέριων ελαίων των φυτών *Acorus calamus* και *Petroselinum crispum,* σε σύγκριση με συνθετικά εντομοκτόνα, κατά του κηροπλάστη, *Ceroplastes rusci*, σε φυτά *Ruellia simplex*

I.A. Mohamed, G.S. Mohamed, E.Y. Abdul-Hafeez και O.H.M. Ibrahim

Περίληψη Το φυτό *Ruellia simplex* καλλιεργείται για την καλλωπιστική αξία των ανθέων, των φύλλων και γενικότερα της εμφάνισης της κόμης του. Ο κηροπλάστης, *Ceroplastes rusci* L., (Hemiptera: Coccidae) καταγράφηκε για πρώτη φορά στην Αίγυπτο πάνω σε φυτά *R. simplex*. Η βιολογική δράση σκευασμάτων με δραστικές ουσίες παραφινικά λάδια, diazinon και thiamethoxam + chlorantraniliprole, και αιθέριων ελαίων από τα φυτά *Acorus calamus* (κν. άκορος κάλαμος) και *Petroselinum crispum* (κν. μαϊντανός) δοκιμάστηκε κατά του κηροπλάστη. Τα ποσοστό μείωσης του πληθυσμού σε ενήλικα και τις νύμφες του εντόμου αυξάνονταν σταδιακά έως την έβδομη ημέρα μετά την εφαρμογή. Η μέγιστη αποτελεσματικότητα των σκευασμάτων με παραφινικό λάδι, και thiamethoxam + chlorantraniliprole παρατηρήθηκε 21 ημέρες μετά την εφαρμογή, ακολουθούμενη από αυτή του αιθέριου ελαίου *A. calamus*. Η αποτελεσματικότητα του αιθέριου λαδιού του μαϊντανού και του diazinon έφτασε σε ποσοστό 86%, 21 ημέρες μετά την εφαρμογή, και ξεπέρασε το 90%, 28 ημέρες μετά την εφαρμογή. Στις 28 ημέρες, το λάδι του κάλαμου έδειξε τη μεγαλύτερη αποτελεσματικότητα. Τα φυτά που δέχτηκαν την επέμβαση του σκευάσματος με thiamethoxam + chlorantraniliprole ήταν τα υψηλότερα και έφεραν μεγαλύτερο αριθμό κλάδων και φύλλων, και χρωστικών στα φύλλα, ακολουθούμενα από αυτά στα οποία έγινε εφαρμογή με παραφινικό λάδι ή λάδι του *A. calamus*. Το αιθέριο έλαιο του κάλαμου και το σκεύασμα με thiamethoxam + chlorantraniliprole, που δοκιμάστηκαν πρώτη φορά κατά του κηροπλάστη, έδωσαν ενθαρρυντικά αποτελέσματα για τη βιολογική δράση τους κατά του εντόμου.

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Rhizobacteria cooperative effect against *Meloidogyne javanica* in rhizosphere of legume seedlings

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Summary Root-knot nematodes are major pests of legume fields in Iran. This research evaluated the effect of *Rhizobium leguminosarum* bv. *phaseoli* and *Pseudomonas fluorescens* CHA0 (stand alone and combination treatment) on galling and reproduction of root-knot nematode, *Meloidogyne javanica,* in legum (chickpea, bean, lentil, pea) seedling rhizosphere, and the growth properties of the host plants. The legumes seeds were sown in 1kg sterilized sandy loam soil. Inocula were 5 J_2/g of soil, in the case of the nematode, while considering the bacteria 1×10^7 cfu/kg soil. A treatment of nematicide (cadusafos) was performed, as a commonly used nematicide in Iran, at 2g/kg soil. Two months after inoculation, the following parameters were recorded: the number of knots, egg masses and reproduction factor of the nematode, bacterial nodules per root, and growth properties of seedlings in the treatments (control, nematode, nematode+nematicide, and nematode+rhizobacteria). The greatest bacterial effect on the control of the nematode was observed in the rhizosphere of the bean treatments. Inoculation with *Rhizobium* in the soil decreased the galling on the legumes' roots, and the combined inoculation with *Pseudomonas* and *Rhizobium* resulted in a higher decrease of the galling.

Additional keywords: antagonism, biocontrol, management, nodule, *Pseudomonas*, root-knot nematode

Introduction

Legumes are well recognized for their seeds, which are rich of proteins, minerals and vitamins (Bojnanska *et al*., 2012), and their significant role in maintaining productivity in agricultural systems (Dashadi *et al*., 2011). After cereals, leguminous crops are the most important crops in Iran (Alizadeh *et al*., 2013).

Root-knot nematodes (*Meloidogyne* spp.) are known as a major constraint to leguminous plants, causing yield losses in heavily infested fields (Onyeke and Akueshi, 2012). They are very important plant parasitic nematodes due to their worldwide distribution, wide host range, many species and interactions with root rot and wilt fungi and bacteria (Nicol, 2002; Perry and Moens, 2006). *Meloidogyne javanica* (Treub) Chitwood has a broad host range and great dispersal, making plant roots more vulnerable to root rot pathogens in Iran (Hosseini Nejad and Khan, 2001).

A number of biocontrol agents have demonstrated high potency in reducing damage by nematodes in different crops (Gupta *et al*., 2015). Natural enemies of nematodes are successful in reducing the activity of plant parasitic nematodes through parasitism, secretions (toxin, antibiotic and enzyme), competition, inducing systemic resistance and stimulation of host resistance (Tian *et al*., 2007). There are many examples of research in the field of biological control of plant parasitic nematodes, especially root-knot nematodes, due to the application of various microorganisms, including bacteria (Ashoub and Amara, 2010; Jang *et al*., 2016; Ntalli and Caboni, 2012; Saeedizadeh, 2016; Sokhandani *et al*., 2016).

In a rhizosphere, significant and intensive interactions take place between the plant, soil and microorganisms (Panizzon *et al*., 2016). Plant growth-promoting rhizobacteria (PGPR) are free-living bacteria that may impart beneficial effects on plants (Nihorimbere *et al*., 2011). PGPR enhance emergence, colonize roots and stimulate overall plant development. The presence of rhizobia in a

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rhizosphere may protect the host root from damage induced by pathogens (Lipiec and Glinski, 2011). Therefore, the handling of rhizosphere by inoculation of PGPR has shown considerable promise for biocontrol of plant pathogens (Ahemad and Kibret, 2014).

Rhizobacteria-nematodes interactions have been extensively considered to manage plant-parasitic nematodes (Shaikh *et al*., 2016; Siddiqui and Akhtar, 2009). Rhizobacteria genera such as *Pasteuria*, *Pseudomonas* and *Bacillus* have a respectable potential as biological control agents against plant parasitic nematodes (Beneduzi *et al.,* 2012). Most frequently studied antagonistic rhizobacteria to affect the nematodes are *Bacillus subtilis*, *Bacillus sphaericus* and *Pseudomonas fluorescens* (Ashoub and Amara, 2010; Muthulakshmi *et al*., 2010). On the other hand, infestation by nematodes is known to suppress bacterial nodulation and consequently decrease nitrogen fixation in leguminous plants (Siddiqui *et al*., 2013).

Rhizobium forms an endosymbiotic nitrogen-fixing association with roots of legumes and it can also act as a biocontrol agent on root-knot nematodes exhibiting the capacity to colonize plant roots and the nematode galls (Siddiqui and Akhtar, 2009). *P. fluorescens* CHA0 has been introduced as biocontrol agent of root-knot nematodes. The bacterium has been able to suppress the nematodes populations through the production of secondary metabolites (Siddiqui *et al*., 2006).

Taking into account the importance of root-knot nematodes and the need for biological control methods, the scope of the research was to evaluate the effect of *R. leguminosarum* bv. *phaseoli* and *P. fluorescens* CHA0 as rhizobacteria against *M. javanica* in legumes seedling rhizosphere, and growth properties of the plants.

Materials and Methods

Preparation of nematode inoculum

Infested roots with root-knot nematodes were sampled from a population maintained in the fields of tomato plants (cv. Walter) in the southern Tehran city, Iran. Extraction and preparation of *M. javanica* inoculum were applied according to the Hussey and Barker (1973) using the single egg mass method. According to the morphological and morphometrical characteristics of body and perineal pattern, the nematodes were initially identified (Aydinli and Mennan, 2016). The egg mass of the species was added to the rhizosphere of tomato seedling cv. Local; the nematode was multiplied and second stage juveniles (J_2) were finally obtained in a glasshouse (10 hours lighting and 25±2°C temperature). Infected tomato roots bearing large egg masses were incubated in water for three days at 28±2°C and hatched J_2 were collected and counted. The nematode inoculum level of the treatments was determined as 5 J_2/g of soil (Gharabadiyan *et al*., 2013).

Preparation of bacterial inoculum

P. fluorescens CHA0 and *R. leguminosarum* bv. *phaseoli* were obtained from the culture collection of the Department of Plant Pathology, Shahed University, Tehran, Iran.

P. fluorescens CHA0

The bacterium was prepared as a bacterial inoculum suspension according to Weller and Cook (1983). A full loop of 48-hour culture of the bacterium on King's medium B (King B) was transferred to a flask containing 100ml King B liquid medium and incubated for 48 hours on a shaker (120 RPM) at 27°C. Bacterial suspension was centrifuged for 10 min @ 6000g, and washed for 2-3 times with a natural salt solution (NaCl 0.14M) to remove residual nutrient medium. Bacterial cells were extracted by recentrifuging and suspending to a solution of 1×107cfu/ml, which was prepared using the standard curve spectrophotometrically in a carboxymethyl cellulose solution. To develop the optimal amount of inoculants, this solution was diluted by Tween80 (0.02%) (Weller and Cook, 1983).

R. leguminosarum bv. *phaseoli*

To activate the bacterium, 1ml of liquid

medium of YMA (Yeast Mannitol Agar) under sterile conditions was added to bacteria. For bacterial proliferation, one inoculation loop of the bacterium dissolved in 100ml of liquid YMA and incubated on an orbital shaker @ 200 RPM for 24 h. For concentration determination of the bacterial suspension, the Rhizobium was cultured in a liquid medium of YMA and incubated on an orbital shaker @ 200 RPM for 24 h at 25°C (Sadovinkova *et al*., 2003). The culture was centrifuged @ 1000g for 10 min and was resuspended with phosphate buffer. If the optical density (OD620) of this solution was 0.1, it means 108 cfu/ml. To develop the optimal amount of inoculants (107cfu/ml), this solution was diluted by phosphate buffer (Bai *et al*., 2003).

Plant material and inoculation

The seeds of four legumes, including chickpea (*Cicer arietinum* L., cv. Sefid), bean (*Phaseolus vulgaris* L., cv. Bahman), lentil (*Lens culinaris* Medikus, cv. Gachsaran), and pea (*Pisum sativum* L., cv. Shamshiri) were obtained from Department of Plant Protection, Shahed University, Tehran, Iran. They were surface-sterilized by 70% ethanol for 2 min and 1% sodium hypochlorite for 5 min, and rinsed 5 times with distilled water (Wang and Oyaizu, 2009). Four seeds were sown in 1kg steam-sterilized sandy loam soil in the greenhouse; and one week after germination thinning was done to save a single seedling per pot. The plants were inoculated at the trifoliate emergence stage. The seedlings were kept in a greenhouse (natural light and 25±2°C).

The nematode inoculum was set at 5 J_2/g soil, the bacteria at 1×10^7 cfu/kg soil, and the nematicide RUGBY® 10 G (Cadusafos), as a reference product, at 2g/kg soil (Safdar *et al*., 2012). The inocula were individually applied to the pots in a volume of 5ml suspension in a ring from around the plant root to a depth of 3cm. Control comprised of healthy untreated seedlings (free of the nematode and bacteria inocula). Each pot plant represented one replication.

Measurement of growth properties of the plants

Plants were uprooted 60 days after inoculation and the root systems were gently washed. The plants were cut with a knife above the base of the root emergence zone. Excess water was removed by blotting before weighing shoots and roots separately.

Evaluation of *M. javanica* activity in the plants

According to the proposed method of Hussey and Janssen (2002), activity of *M. javanica* was evaluated in the control and treated plants as the number of egg masses and knots per root, and final population and reproduction factor per pot. At first, the roots of the plants were washed with tap water and drained on blotting paper. To specify the number of egg masses, roots were divided into 3-4cm parts and egg masses were stained with Floxin solution B (0.15g/l of water), bleached with lactophenol and counted under a dissecting microscope (Hussey and Janssen, 2002). For determination of the nematode population in the pot soil, a 250g subsample of well mixed soil was processed according to Jenkins (1964), known as centrifuge or sugar flotation method. The number of nematodes in prepared suspension was used to calculate the population of nematodes per pot (1kg soil). To count the number of juveniles, eggs and females inside the root, 1g sub-sample of the root was macerated in a Waring blender and counts were done on the suspension thus obtained. The numbers of nematodes present in the root were calculated by multiplying the numbers of nematodes present in 1g of the root with the total weight of the root. The reproduction factor was calculated utilizing the equation RF = Pf/Pi, which is RF: reproduction factor, Pf: final population of the nematode, and Pi: primary population of the nematode (Osei *et al*., 2010).

Statistical analysis

This experiment was based on a Complete Randomized design with four legumes species and 12 treatments for the

management of *M. javanica*, including: control (with no the inoculum of the nematode and the bacteria), rhizobium (R), pseudomonas (P), nematode (N), rhizobium + pseudomonas (RP), rhizobium + nematode (RN), pseudomonas + nematode (PN), rhizobium + pseudomonas + nematode (RPN), rhizobium + nematode (a week later) (Rn), pseudomonas + nematode (a week later) (Pn), rhizobium + pseudomonas + nematode (a week later) (RPn), and nematode + nematicide (NN). The treatments were replicated four times. The data were subjected to one way analysis of variance (ANOVA). Mean treatments were compared using a Duncan multiple range test. All analyses were performed by using SAS software version 9.1.

Results

The experiment revealed significant differences between the various treatment and crop combinations (ANOVA, $p \leq 0.05$). The greatest bacterial effect on the control of the nematode was observed in bean treatments (Table 1). The plant roots in the *Pseudomonas* treatment produced less galls than those in the nematode treatment (Table 1) However, higher galling occurred on the roots treated with *Rhizobium*. Combined application of *Rhizobium* and *Pseudomonas* caused a lower reduction in galling and multiplication of the nematode than the nematicide (Table 1).

The level of the nematode activity (gall, egg mass and reproduction factor) was higher on the treatments of lentil plants followed by bean, pea and chickpea (Table 1). The lowest nematode activity was noted in the nematicide treatment (Table 1). Inoculation of *Pseudomonas* and *Rhizobium* reduced nematode activity, especially in the treatments Pn, Rn and RPn, where the inoculation with the nematode was conducted one week after the bacteria inoculation (Table 1). The treatments of *Pseudomonas* have been more effective than *Rhizobium* on reducing the nematode activity (Table 1).

The bacterial nodulation of *Rhizobium* on the legume roots infested by the nematode was lower than on those at the *Rhizobium* treatment (R) (Table 2). Also the infested roots by the nematode, in the treatments which received the co-inoculation of *Pseudomonas* and *Rhizobium,* produced fewer nodules than those in the stand alone treatment of *Rhizobium* (Table 2). The descending order of the number of bacterial nodules obtained was: chickpea>pea>bean>lentil (Table 2). The nematode was more effective in reducing bacterial nodulation by *Rhizobium* in comparison with *Pseudomonas*. The number of bacterial nodules obtained in the treatments was in descending order: R>RP>RPn>Rn>RPN>RN (Table 2).

Data analysis of plant growth properties indicated a difference between the fresh weight of root and shoot in all treatments, compared with the control (Figures 1 and 2); this difference was positive in all treatments, except the treatment with the nematode (N). The greatest increase (positive difference) of root and shoot fresh weight were obtained in the treatments with the bacteria (*Pseudomonas* and *Rhizobium*) inoculation, either with or without the nematode (*Meloidogyne*) inoculations (P, R, RP) (Figures 1, 2). The highest increase in fresh weight of root and shoot was observed in the treatments RP, R and P (descending order). The inoculation with the nematode resulted in the least weight of roots and shoots. The highest reduction in root and shoot weight was obtained in the nematode treatment of lentil and bean (Figures 1 and 2).

Discussion

Our results indicate that inoculation with *Rhizobium* and *Pseudomonas* in stand-alone application can reduce *Meloidogyne* activity in the rhizosphere of the legumes seedlings. *Rhizobium* decreases the nematode galling while the nematode reduces the bacterial nodules on the roots of the plants and consequently decreases nitrogen fixation in leguminous plants (Siddiqui *et al*., 2013). Other studies have also indicated an increased

Table 1. Activity of *Meloidogyne javanica* (galls, egg mass, reproduction factor) (mean ± StE) on root of legume seedlings (chickpea, bean, lentil, pea) after inoculation with *Rhizobium leguminosarum* bv. *phaseoli* and *Pseudomonas fluorescens* CHA0.

	Host	Treatment*							
		N	PN	Pn	RN	Rn	RPN	RPn	NN
Gall†	Bean	29.5±1.258 d•	24.5±0.957 gh	19.75±1.315 ijkl	27.5±1.19 def	22.5±0.866 hi	21.25±1.493 ij	15.75±0.854 nop	0.75±0.479 q
(No/root)	Chickpea	26.5±0.645 efg	18.25±1.109 klmn	15.5±0.645 op	20.75±1.25 ijk	17.5±0.645 lmno	17±0.816 mno	13.75±0.854 p	1.5±0.645 q
	Lentil	40.25±0.479 a	36.25±0.629 b	27.5±0.866 def	37.5±0.645 b	32.75±0.946 c	33.25±0.629 c	21.5±1.19 ij	1.5±0.645 q
	Pea	29±1.08 de	22.5±1.323 hi	19.25±1.315 jklm	26±0.408 fg	25.5±0.645 fg	20.5±0.645 ijk	16±0.707 nop	0.5±0.289 q
Egg mass	Bean	19.5±1.555 e	16±1.472 ghi	10.25±0.854 jk	18±0.707 efg	15.5±0.645 hi	15±0.408 i	7.75±0.479 lm	0.25±0.25 o
(No/root)	Chickpea	18.25±0.854 ef	8.5±0.645 klm	5±0.707 n	10.25±0.629 jk	7.75±0.946 lm	7±0.707 m	4.75±0.479 n	0.5±0.289 o
	Lentil	30.5±0.645 a	25.75±0.75 c	17.5±0.645 efgh	27.75±0.479 b	23±1.08 d	23.5±0.645 d	14.75±0.946 i	0.75±0.479 o
	Pea	19±1.08 ef	15.5±1.323 hi	9.75±0.629 jkl	17.75±0.854 efg	17.25±0.479 fgh	11.25±0.629 j	9±0.408 klm	0.25±0.25 o
RF	Bean	1.25±0.016 de	0.9125±0.042 gh	0.5975±0.015 lm	1.215±0.013 e	0.81±0.009 j	0.79±0.009 j	0.525±0.013 n	0.00375±0.004 p
	Chickpea	1.1575±0.039 f	0.5875±0.005 lm	0.22±0.011 o	0.5775±0.006 lmn	0.575±0.017 lmn	0.545±0.006 mn	0.2275±0.009 o	0.0075±0.005 p
	Lentil	1.9025±0.054 a	1.3475±0.023 c	0.9375±0.009 g	1.405±0.01 b	1.285±0.013 d	1.265±0.023 de	0.7225±0.049 k	0.00875±0.006 p
	Pea	1.2825±0.009 d	0.83±0.025 ij	0.6±0.011 lm	0.955±0.014 g	0.875±0.006 hi	0.6075±0.018 l	0.545±0.023 mn	0.0025±0.003 p

* Treatment abbreviations are as follows:
N (nematode), PN (pseudomonas + nematode), Pn (pseudomonas + nematode (a week later)), RN (rhizobium + nematode), Rn (rhizobium + nematode (a week later)), RPN (rhizobium + pseudomonas + nematode), RPn (rhizobium + pseudomonas + nematode (a week later)) and NN (nematode + nematicide.

† Activity of *Meloidogyne javanica* was evaluated as the number of galls (knots) and egg masses per root; and RF (reproduction factor) per pot.

• Values shown are the mean of four replications (n = 4) ± standard error. Means of each trait (Gall, Egg mass and RF) that do not share the same letter are significantly different ($P \leq 0.05$).

Table 2. Number of bacterial nodules of *Rhizobium leguminosarum* bv. *phaseoli* (Mean±StE) on root of legume seedlings.

Plant†	Treatment*					
	R	RN	Rn	RP	RPN	RPn
Bean	17±0.41 abc	9.25±0.63 k	13±0.91 fghi	14±0.41 defg	12.25±0.85 ghi	14.25±0.85 defg
Chickpea	19±0.91 a	11.75±0.85 hij	17.5±0.65 ab	17.5±0.87 ab	15.5±0.65 bcd	17±1.08 abc
Lentil	15±0.41 cdef	6.5±0.65 l	11±0.71 ijk	12.25±0.85 ghi	11.75±0.85 hij	12.25±0.85 ghi
Pea	17.5±0.65 ab	10±0.41 jk	12.75±1.11 ghi	15.5±0.65 bcde	13.75±0.63 efgh	16±0.41 bcd

* Treatment abbreviations are as follows:
R (rhizobium), RN (rhizobium + nematode), Rn (rhizobium + nematode (a week later)), RP (rhizobium + pseudomonas), RPN (rhizobium + pseudomonas + nematode) and RPn (rhizobium + pseudomonas + nematode (a week later)).

† Activity of *Rhizobium leguminosarum* bv. *phaseoli* was evaluated as the number of nodules per root.

• Values shown are the mean of four replications (n = 4) ± standard error. Means that do not share the same letter are significantly different ($p \leq 0.05$).

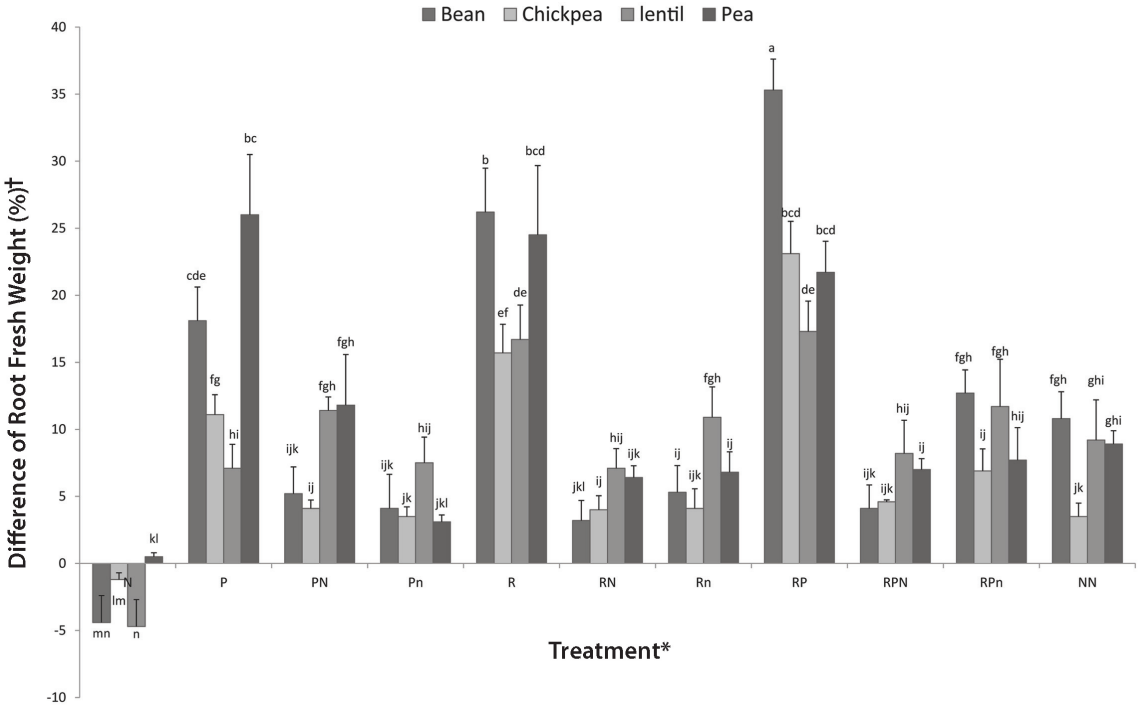

Figure 1. Effect of inoculation with *Rhizobium leguminosarum* bv. *phaseoli* and *Pseudomonas fluorescens* CHA0 on root fresh weight of legume seedlings infested with root-knot nematode *Meloidogyne javanica*, as compared with control♦

† Each value has been calculated using the equation: $\frac{\text{Treatment mean-control mean}}{\text{control mean}} \times 100$

* Completely randomized design with four replications. Treatment abbreviations are as follows:
N (nematode), P (pseudomonas), PN (pseudomonas + nematode), Pn (pseudomonas + nematode (a week later)), R (rhizobium), RN (rhizobium + nematode), Rn (rhizobium + nematode (a week later)), RP (rhizobium + pseudomonas), RPN (rhizobium + pseudomonas + nematode), RPn (rhizobium + pseudomonas + nematode (a week later)) and NN (nematode + nematicide).

♦ Control comprised of healthy untreated seedlings (free of the nematode and bacteria inocula).
Comparisons were carried out using Duncan test (α=0.05); means that do not share the same letter are significantly different ($p \leq 0.05$).

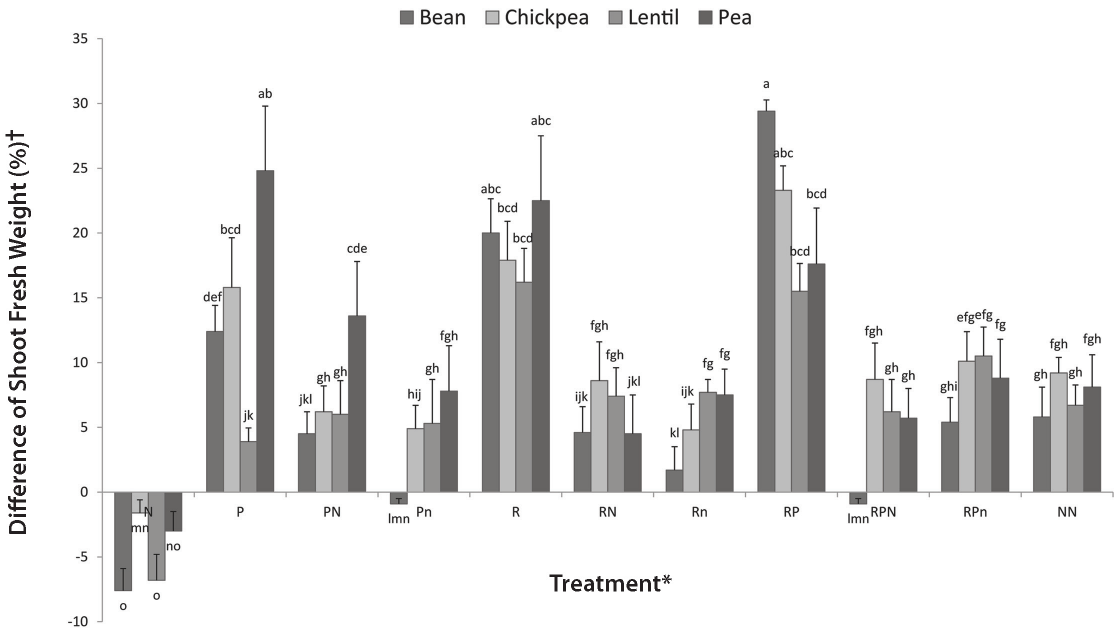

Figure 2. Effect of inoculation with *Rhizobium leguminosarum* bv. *phaseoli* and *Pseudomonas fluorescens* CHA0 on shoot fresh weight of legume seedlings infested with root-knot nematode *Meloidogyne javanica*, as compared with control♦

† Each value has been calculated using the equation: $\frac{\text{Treatment mean-control mean}}{\text{control mean}} \times 100$

* Completely randomized design with four replications. Treatment abbreviations are as follows: N (nematode), P (pseudomonas), PN (pseudomonas + nematode), Pn (pseudomonas + nematode (a week later)), R (rhizobium), RN (rhizobium + nematode), Rn (rhizobium + nematode (a week later)), RP (rhizobium + pseudomonas), RPN (rhizobium + pseudomonas + nematode), RPn (rhizobium + pseudomonas + nematode (a week later)) and NN (nematode + nematicide).

♦ Control comprised of healthy untreated seedlings (free of the nematode and bacteria inocula). Comparisons were carried out using Duncan test (α=0.05); means that do not share the same letter are significantly different ($p \leq 0.05$).

effect of nematodes on the bacterial nodulation on host roots (Ugwuoke and Eze, 2010). Rhizobia are reported to produce toxic metabolites inhibitory to many plant pathogens (Hmissi *et al*., 2011). However, in our case, it seems that competition for the host root tissue is the most important concern between the antagonizing organisms (*Rhizobium* and *Meloidogyne*).

Pseudomonas can inhibit galling and reproduction of the nematode. These findings coincide with other studies suggesting that *P. fluorescens* CHA0 can be considered as a common biological agent to root-knot nematodes (Saeedizadeh, 2016; Siddiqui *et al*., 2005).

The combined use of *Rhizobium* and *Pseudomonas* was more beneficial in increasing plant growth and reducing nematode multiplication, probably due to positive interaction. This positive interaction is evident by the increase in nodulation and the reduction in nematode multiplication. Some strains of *R. leguminosarum* and *P. flourescens* have been used in combination to control root-knot nematodes affecting legumes (Ashoub and Amara, 2010). In our trial, positive cooperative effect of *P. fluorescens* CHA0 and *R. leguminosarum* bv. *phaseoli* on the plants has been demonstrated by the suppression of activity of *M. javanica* as well as by the host growth enhancement of the legume seedlings. Bayat *et al.* (2014) showed that the root fresh weight of legumes increased significantly upon rhizobial inoculation. Similar results have been reported in *Vicia faba* (Dashadi *et al*., 2011). In faba bean, individual inoculation and co-in-

oculation of *Rhizobium* and *Azotobacter* increased most of growth indicators such as root dry weight. Co-inoculation of *Rhizobium* and *Azotobacter* could improve some of the faba bean growth properties under water stress conditions (Dashadi *et al*., 2011).

Several management strategies have been developed for *Meloidogyne* spp., nevertheless their usage in subsistence agriculture is restricted. Chemical compounds used to control nematodes are too expensive, and non-chemical practices, such as crop rotation, are limited because of the wide host range of the root-knot nematodes. Biological control agents, such as *Pseudomonas* and *Rhizobium*, can allow significant a reduction in the use of nematicides and chemical fertilizers through their numerous direct or indirect mechanisms of action.

In conclusion, according to our results, using a co-inoculum of *P. fluorescens* CHA0 and *R. leguminosarum* bv. *phaseoli* can be more effective against *M. javanica,* compared with stand-alone application of either bacterium, and will reduce the level of its pathogenic activity. Biological control of the nematode using the bacteria in a joint application cannot be as effective as the commercial synthetic pesticides in the greenhouse. However, the prolonged use of biocontrol agents will reduce the nematode population in long-term, minimizing the risk for development of nematicide resistance, adverse effects to the environment and humans, which are associated with the chemical control. Moreover, bacterial nodulation induced on the legumes root inoculated with *R. leguminosarum* can improve plant growth besides the contribution to biological control of the nematode on the legume roots.

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Συνδυαστική δράση ριζοβακτηρίων κατά του κομβονηματώδη *Meloidogyne javanica* στη ριζόσφαιρα ψυχανθών

F.-S. Tabatabaei και A. Saeedizadeh

Περίληψη Οι κομβονηματώδεις είναι σημαντικοί εχθροί στις καλλιέργειες ψυχανθών στο Ιράν. Η παρούσα εργασία αξιολόγησε την επίδραση των βακτηρίων *Rhizobium leguminosarum* bv. *phaseoli* και *Pseudomonas fluorescens* CHA0 (απλές και συνδυασμένες επεμβάσεις) στο σχηματισμό κόμβων και την αναπαραγωγή του κομβονηματώδη, *Meloidogyne javanica*, στη ριζόσφαιρα ψυχανθών (ρεβίθι, φασόλι, φακή, μπιζέλι), καθώς και στην ανάπτυξη των φυτών-ξενιστών. Οι σπόροι των ψυχανθών σπάρθηκαν σε 1kg αποστειρωμένου αμμοπηλώδους εδάφους. Έγινε εμβολιασμός με 5 προνύμφες δευτέρου σταδίου (J2) του νηματώδη/g εδάφους, και 1×10^7 CFU/kg εδάφους για τα βακτήρια. Πραγματοποιήθηκε μία εφαρμογή με νηματωδοκτόνο που περιέχει δ.ο. cadusafos, ως ένα συνήθως χρησιμοποιούμενο νηματωδοκτόνο στο Ιράν, σε 2g/kg εδάφους. Δύο μήνες μετά τον εμβολιασμό, καταγράφονταν οι ακόλουθες παράμετροι: ο αριθμός των κόμβων στις ρίζες και των ωόσακκων, ο ρυθμός αναπαραγωγής του νηματώδους, ο αριθμός φυματίων στη ρίζα, και διάφορες παράμετροι ανάπτυξης των φυτών στις επεμβάσεις (μάρτυρας, νηματώδης, νηματώδης + νηματωδοκτόνο, νηματώδης + ριζοβακτήριο). Η μεγαλύτερη βιολογική δράση των βακτηρίων κατά του νηματώδους παρατηρήθηκε στη ριζόσφαιρα των φυτών φασολιού. Ο εμβολιασμός με *Rhizobium* στο έδαφος μείωσε το σχηματισμό κόμβων στις ρίζες των ψυχανθών, και ο συνδυασμένος εμβολιασμός με *Pseudomonas* και *Rhizobium* οδήγησε σε μεγαλύτερη μείωση σχηματισμού κόμβων.

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Effect of several rhizobacteria strains on barley resistance against *Pyrenophora graminea* under field conditions

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Summary The effect of *Pseudomonas putida* BTP1, *Bacillus subtilis* Bs2500, Bs2504, and Bs2508 strains on the incidence (I) and severity (S) of barley leaf stripe disease caused by *Pyrenophora graminea* was evaluated under field conditions. Three barley cultivars varying in resistance level were used. The resistance achieved in our study was long-lasting. *P. putida* BTP1 and Bs2508 were in general the most effective strains in reducing significantly both I and S of barley leaf stripe disease vis-a-vis three cultivars in two growing seasons 2013/2014. The disease was reduced up to 66% in Arabi Abiad treated with *P. putida* BTP1. The susceptible landrace cultivar Arabi Abiad exhibited a significant induction of resistance by Bs2508 and BTP1. However, the resistant cultivar Banteng did not exhibit significant further increase in resistance by these bacterial strains. The grain yield of bacterized plants artificially inoculated with *P. graminea* was not affected, except that of the cultivar Arabi Abiad treated with Bs2508 and Bs2504. Triggering of resistance by treating seeds with the bacterial strains would be of great value in agriculture, especially in case of barley infection by *P. graminea* at an early stage of plant development.

Additional keywords: *Bacillus subtilis* Bs2500, Bs2504, Bs2508, Barley leaf stripe, *Pseudomonas putida* BTP1

Introduction

Biological control, i.e. the use of microbial antagonists to suppress plant diseases, has gained acceptance in recent years. Among the different microbial species tested for that purpose, several aerobic spore-forming bacteria possess features that make them good candidates for use as biological control agents in the field (Sharma and Johri, 2003). Plant growth-promoting rhizobacteria (PGPR) are defined as root-colonizing bacteria with the ability to establish on or in the plant root, to propagate and to survive, exerting a beneficial effect on plant growth and development (Choudhary and Johri, 2009). Many different biological control agents have been introduced into different planting materials and can protect plants against various diseases (Bakker *et al.*, 2007; Adam *et al.*, 2008; Choudhary and Johri, 2009; De Vleesschauwer and Höfte, 2009; Reglinski and Walters, 2009); in particular species belonging to the *Pseudomonas* and *Bacillus* genera have been used, relying on their different mechanisms to directly antagonize pathogen growth (Haas and Défago, 2005).

The systemic, seed-transmitted (seed-borne) hemi biotrophic fungus *Pyrenophora graminea* Ito & Kuribayashi [anamorph *Drechslera graminea* (Rabenh. ex. Schlech. Shoem.)] (Mathre, 1997) is the causal agent of leaf stripe disease in barley (*Hordeum vulgare* L.) which often leads to yield reductions (Porta-Puglia *et al.*, 1986; Arabi *et al.*, 2004). The fungus survives within the kernels as mycelium between the paranchymatical cells of the pericarp in the hull, and the seed coat but not in the embryo (Arru *et al.*, 2002). During seed germination, the fungal hyphae begin to grow intercellularly within the coleorhiza into the embryo structures, the roots and scutellar node. The pathogen behaves as a biotroph and degrades host-cell walls using hydrolytic enzymes without causing cellular necrosis (Hammouda, 1988; Haegi *et al.*, 2008). Once infection spreads into the young leaves, growth switches to a necrotrophic phase with the production of a host-specific glycosyl toxin (Haegi and

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Porta-Puglia, 1995) that causes longitudinally dark brown discoloration of leaves. In susceptible plants, the disease usually results in severe stunting, premature death and complete loss of grain (Tekauz and Chiko, 1980).

The vast majority of knowledge about PGPR has been gathered from studies on dicots such as cucumber, tobacco, and *Arabidopsis* (Ramamoorthy *et al.*, 2001). The knowledge about induced resistance in monocots remains elusive (Van Loon, 2007; Vlot *et al.*, 2008). The potential of PGPR to induce resistance in monocots depends on the host-PGPR combination and on the pathogen (De Vleesschauwer *et al.*, 2006). The efficacy of PGPR in monocots against necrotrophic pathogens has been demonstrated in a few cases (Van Wees *et al.*, 2008; Pinedra *et al.*, 2010).

To improve the field performance and consistency of biocontrol agents against *Pyrenophora graminea* in barley, as a monocot crop, a deep knowledge of the physiological mechanisms on which the biological control by the known PGPR bacterial strains *Pseudomonas putida* BTP1and *Bacillus subtilis* strains Bs2500, Bs2504 and Bs2508 rely is important. The capacity of these strains to induce resistance in several pathosystems has been proved previously (Ongena *et al.*, 2004; Ongena *et al.*, 2007; Adam *et al.*, 2008). The main goal of the present study was to examine the biological potential of the abovementioned four rhizobacterial strains, differing in lipopeptide production, against barley leaf stripe disease incidence and severity and also to determine their possible impact on growth and yield using three barley cultivars under field conditions.

Materials and Methods

Bacterial strains and growth conditions

The non-pathogenic rhizobacterial strain *Pseudomonas putida* BTP1, isolated from barley roots, was selected for use in this study as it is a strain with a pyoverdin-mediated iron system, which is regarded as an enhancer of the colonization and persistence of the strain in the rhizosphere (Ongena *et al.*, 2002; Ongena *et al.*, 2005). *P. putida* BTP1 and *Bacillus subtilis* Bs2508, Bs2504, and Bs2500 were kindly provided by Dr. Philippe Thonart (Wallon Center for Industrial Biology, University of Liège, Belgium). All bacterial strains were maintained on King's B medium agar plates (King *et al.*, 1954) at 4°C before experimental use, and stored at -80°C in cryotubes according to the manufactures' recommendations (Microbank; Prolab Diagnostic, Richmond Hill, Canada) for long term conservation. For utilization, *P. putida* BTP1 was grown on Casamino acids (CAA) medium (5 g/l CAA, 0.9 g/l K_2HPO_4, 0.25 g/l $MgSO_4$ and 15 g/l agar) (Ongena *et al.*, 2002) for 24 h at 30±1°C. *Bacillus subtilis* strains were grown on 868 medium (20 g/l glucose, 10 g/l peptone, 10 g/l yeast and 15 g/l agar) (Jacques *et al.*, 1999), and incubated for 24 h at 30±1°C in the dark. Subsequently bacterial cells were collected and resuspended in 10 mM $MgSO_4$ to a final density of 10^8 colony- forming units (CFU) per mL before use.

Fungal isolate and host genotypes

After an extensive screening for more than fifteen years in the field and in our laboratory, isolates of *P. graminea* have been obtained from barley leaves showing leaf stripe symptoms in different regions of Syria. The *P. graminea* Sy3 strain (*P.gSy3*) was selected for use in this study based on morphological and physiological criteria (virulence). In specific, this strain had been proven to be the most virulent isolate to all barley genotypes available so far (Arabi and Jawhar, 2012a; Arabi and Jawhar, 2012b).

Strain *P.gSy3* was cultured on potato dextrose agar (PDA, DIFCO, Detroit, MI, USA) with 13 mg/l kanamycin sulphate and incubated for 10 days at 22 ± 1°C in the dark to allow mycelia growth and sporulation. Two spring barley types [Arabi Abiad (landrace) and WI 2291(Yield improved cultivar)] and one winter type (Banteng) were chosen for their variable reaction to *P. graminea* ranging from being susceptible to being resistant to this pathogen (Table 1) (Arabi and Jawhar, 2012a; Arabi and Jawhar, 2012b).

Table 1. Genotypes and main features of the barley cultivars used in this study.

Genotype	Origin	Row type[y]	Growth habit	Proportion of diseased leaves	
				% Diseases leaves[x]	Disease development
WI2291	Australia	2	Spring barley	96.67	Up to flag leaf
Aarbi Abiad	Syria	2	Spring barley	91.33	Up to flag leaf
Banteng	Germany	6	Winter barley	1.33	first leaf

[x] Arabi and Jawhar , 2012(a)
[y] Arabi and Jawhar , 2012(b)

Seed health test

To determine the health status of the barley seeds used in this study, random seed samples (50 seeds) of each cultivar were taken from protected nursery germplasm, surface- sterilized in 5% sodium hypochlorite solution (NaOCl) for 5 min, rinsed three times (5 min each) in sterile distilled water and dried between sterilized filter paper (Arabi *et al.*, 2004). They were plated on Petri dishes containing PDA medium and incubated for 72 h at 23 ± 1°C in the dark.

Seed inoculation

Seeds were surface-sterilized as previously described for the seed health test. Inoculation was carried out using the modified method of Hammouda (1986). Six hundred seeds of each cultivar were placed on an active 8-day-old mycelial culture of *P.gSy3* growing on PDA medium in Petri dishes (50 seeds/ Petri dish) and incubated at 6°C for 14 days in the dark. As negative control, seeds were incubated on PDA medium without the fungus. To confirm artificial inoculation of the seeds by the fungus, seeds from the Petri dishes with the *P.gSy3* culture were randomly collected, surface- sterilized as described above, placed on PDA medium and incubated for 72 h, at 23 ± 1°C; the seeds were then examined under a microscope for the presence of *P. graminea*.

Field assay to assess resistance induced by rhizobacteria

One-hundred and fifty inoculated (with *P. graminea*) and the same number of non-inoculated seeds per cultivar (Arabi Abiad, WI2291 and Banteng) were soaked for 15 min in each bacterial strain suspension at a concentration of 10^8 CFU/ml prior to sowing in the field. The trials were conducted at a site approximately 20 km west of Damascus (33°. 29′ 37.27′′ N, 36° 04′ 57.66′′ E, 1000 m altitude), under natural rain-fed conditions [about 200-250 mm growing season rainfall conditions (10 December - 30 May)]. Soil temperature was below 9°C in the two seasons (2013-2014). The experiments were conducted using a randomized complete block design with three replicates. Individual plots were 50 x 50 cm with 1m border. Each plot consisted of three rows, 25 cm apart with approximately 17 seeds sown per row. The experiment was designed to allow for sampling of individual plants grown from seeds treated as follows: 1) infection with *P. graminea*. 2) infection with *P. graminea* and soaking with one of the rhizobacterial strains. 3) soaking with one of the rhizobacterial strains. 4) No infection with *P. graminea* and soaking in buffer free from rhizobacteria. Soil fertilizers were drilled before sowing at a rate of 50 Kg/ha urea (46%N) and 27 Kg/ha super phosphate (33% P_2O_5).

Disease rating

In every field plot, infected (showing leaf stripe symptoms) and healthy plants were counted at the heading stage (GS50) (Zadoks *et al.*, 1974). Plant resistance level was expressed as the incidence (I) of infection (number of plants with nonzero severity divided by the total number of plants in a plot) according to the scale described by Delogu *et al*. (1989). Severity (S) was recorded as the number of infected leaves per plant expressed as a percentage of the total number

of leaves per plant. The data for I and S were analysed using analysis of variance (Student-Newman-Keuls test), applying the STAT-ITCF program (Beaux *et al.*, 1988).

1000-kernel weight and yield determination

All infected and non-infected (negative control) plants of each plot were harvested at maturity. Grain yield and 1000-kernel weight (TKW) were determined on individual plants.

In vitro antagonistic test

0.1 ml of the suspension of one of the rhizobacterial strains under study (10^8 CFU/ml) was transferred onto the center of: CCA Petri dishes for *P. putida* BTP1 and 868 Petri dishes for *B. subtilis* Bs2504, Bs2508 and Bs2500 stains, using sterile pipettes, and spread cross-wise by sterile glass spreader. Then mycelial discs of 2 mm diameter of *P. graminea* were cut using a sterile cork borer and placed at 2.5 cm from the center of the above CCA or 868 medium Petri dishes (4 discs / plate). Mycelial discs on the same media without bacteria were used as control. The cultures were incubated at room temperature (25±1°C) in dark for 3-5 days and the diameter of fungal mycelium growth was measured. The experiments were repeated twice.

Results and Discussion

The rhizobacterial strains used in this study were *in vitro* tested for their antagonistic effects against the leaf stripe pathogen (*P. graminea* Sy3 strain). The four bacterial strains tested (*P. putida* BTP1 and *B. subtilis* Bs2500, Bs2504, and Bs2508) showed that there was no antagonistic effect against *P. graminea* compared with the control and were not able to inhibit pathogen growth. This result is supported by the work of Ongena *et al*. (1999) on *P. putida* BTP1, who found that this strain does not secrete any fungitoxic compounds *in vitro* on several media.

The effect of the four rhizobacterial strains on the response against *P. graminea* Sy3 of three barley cultivars grown under field conditions during two growing seasons (2013 and 2014) is presented in Table 2. Student-Newman-Keuls test on incidence and severity of barley leaf stripe disease values (expressed as percentage data) showed highly significant (*P<0.01*) main and interaction effects of cultivar and rhizobacterial strain, with no significant differences among the replicates. This indicates that both cultivars and rhizobacterial strains differ in resistance and ability to induce resistance, respectively. Growing season had no effect on disease severity (S), while had significant (*P<0.01*) effect on incidence (I) (Table2). Differences (*P<0.01*) in mean I and S values were detected among rhizobacterium and cultivar treatment, with values being consistently higher in the diseased controls, in both seasons.

Compared with the diseased control, all bacterial strains had a positive effect in reducing I and S (main effect, Table 2). The two spring barley cultivars, Arabi Abiad and WI2291, were highly susceptible to barley leaf stripe disease, whereas, the six rows winter barley Banteng was more resistant with mean values for S and I ranging between 18.6% and 23.8%. Results of the two seasons were highly correlated (r= 0.98, *P<0.001*), indicating a similar performance trend for the cultivars and bacterial strains (Table 2). The *P. putida* BTP1and Bs2508 strains were in general the best in reducing both I and S, with mean I values 23.1 and 28% and mean S values 31.8 and 35.1%, respectively. Compared with the diseased control, *P. putida* BTP1 showed decreases of 57.5 and 49.4% for I and S, respectively, for the two seasons and the three cultivars.

There was a barley genotype (cultivar) difference in the response to strain treatment. The susceptible landrace cultivar Arabi Abiad exhibited a significant (*P<0.01*) induction of resistance by Bs2508 and *P. putida* BTP1 treatment with disease incidence decreasing by 64.9 and 66%, respectively (growing season 2013). The same trend for the Bs2508 and BTP1 strains was observed in the grow-

Table 2. Mean leaf stripe disease incidence (I) and severity (S) (%) of three barley cultivars inoculated with *P. graminea* Sy3 soaked with *rhizobacteria* during two growing seasons (2013, 2014).

Treatment/ Cultivar	2013								2014								Main effect	
	Arabi	Abiad	Banteng		WI2291		Mean		Arabi	Abiad	Banteng		WI2291		Mean			
	I	S	I	S	I	S	I	S	I	S	I	S	I	S	I	S	I	S
Control	60.7a*	90a	27.3a	24.3a	62.7a	72.7a	50.2a	62.3a	72a	88a	25a	21.7a	78.4a	80.7a	58.5a	63.4a	54.4a	62.9a
BTP1	20.6c	36.7c	17.7a	16.7b	20.9b	41.7d	19.8d	31.7e	31.1c	38.7c	20.3a	16a	28d	41c	26.5c	31.9d	23.1d	31.8d
Bs2500	40.7b	58.3b	28.3a	25.7a	51.1a	62.3b	40b	48.8b	52.3b	49.7c	23.7a	18.3a	50.7b	56b	42.2b	41.3c	41.1b	45.1b
Bs2508	21.3c	44.7c	23.1a	18.3b	31b	42.3d	25.1c	35.1d	37.4bc	41.7c	18.3a	19a	36.7c	44.3c	30.8c	35d	28c	35.1c
Bs2504	45.5b	55b	22.9a	25.3a	52.3a	50.3c	40.2b	43.6c	49b	61b	23a	18a	48.8b	64b	40.3b	47.7b	40.22b	45.1b
LSD	8,4	9	-	4,3	11,8	5,3	5,1	3,2	12,7	9,1	-	-	6,6	8,2	4,9	3,5	2,2	2,3
Mean	*B 37.8	A 56.9	C 23.8	C 22.1	A 43.6	B 53.9			A 48.4	A 55.8	B 22.1	B 18.6	A 48.5	A 57.2				
Main effect	B 35.1(I)					44.3 (S)			A 39.6(I)					43.9(S)				

* Means preceded by different capital letters (line) and followed by different small letters (column) differ significantly at (P<0.01) according to Student-Newman-Keuls test.

ing season 2014 with incidence of disease reduction of 48 and 56.8% respectively. The same behavior was noted for this cultivar in reducing plant severity 50.3 and 59.2% in 2013 and 52.6 and 56% in 2014, respectively. Results obtained for WI2291 indicated the same trend as those for Arabi Abiad regarding both I and S in the two growing seasons (Table 2). The resistant winter barley Banteng did not exhibit any significant increase in its resistance based on incidence or severity of barley leaf stripe disease for the two seasons, with the exception of a weak decrease in severity when the cultivar was subjected to *P. putida* BTP1 and *B. subtilis* Bs 2508 treatment in 2013.

The resulting resistance in our assays can be long lasting with disease reduction ranging from 0 (Banteng/Bs2500) to 66% (Arabi Abiad/BTP1, Table 2), since induced resistance is a host genotype response, and its expression under field conditions is generally expected to be influenced by the environment. Walters *et al.* (2013) reported that understanding the impact of these influences on the expression of induced resistance is still poor. Host genotype is known to affect the expression of induced resistance (Resende *et al.*, 2002; Tucci *et al.*, 2011). Our results are in agreement with the results found by Walters *et al.* (2011b), that expression of induced resistance varied in spring barley varieties to *Rhynchosporium commune*. It may not be surprising that in our work the landrace Arabi Abiad was the most responsive cultivar in terms of induced disease resistance under all conditions. Arabi Abiad is characterized by lower yield level than the other spring cultivar (WI2291) and showed a high susceptibility in the control stage, meaning a high potential for an improvement of its resistance after rhizobacterial application. Along the same line, the lack of an induced resistance response in the winter cultivar Banteng should be attributed to its extremely high level of basal resistance in its genotype, which simply might not be improved any more. For a similar reason, cultivars expressing high basal resistance were less responsive to Benzothiadiazole than highly susceptible cultivars in soybean (Dann *et al.*, 1998) and in barley (Walters *et al.*, 2011a). Cordova-Campos *et al.* (2012) found that basal resistance to *Pseudomonas syringae* pathovars was significantly greater in wild accessions of bean *Phaseolus vulgaris* than in modern cultivars. In a recent work on barley, Molitor *et al.* (2011) demonstrated that following inoculation of powdery mildew infected plant with *Piriformospora indica*, there was a priming of powdery mildew defuse-associated genes at an early stage of the infection.

Most of previous work was applied in the field to plants either as foliar sprays or as root drench. Seed treatments can be particularly useful, since they can provide protection to very young plants during germination and shoot development, particularly in a systemic seed-borne disease such as barley leaf stripe, as during seed germination, the fungal hyphae begin intensive intercellular growth. In our work, the protection was significantly substantiated by the reduced I and S. Priming of induced resistance by treating seeds would be of great value in agriculture, especially for crops that are likely to face pathogen attack early in their development, such as that of *P. graminea*.

The cultivars planted during the two growing seasons of this study varied in resistance to leaf stripe disease. However, a resistant cultivar may in fact have different resistance responses to the spread of the fungus within the infected plants, hence a wide range of severity values may be observed across cultivars for any given incidence value. It appears that differences in weather conditions during the two growing seasons, did not result in any different patterns in the I and S relationship. As shown in Table 3, the grain yield was not affected by rhizobacterial strains during the two growing seasons (2013, 2014), except in the susceptible landrace cultivar Arabi Abiad, whose grain yield was increased significantly ($P > 0.01$) by 48.7 and 33.5% using Bs2504 and Bs2508 respectively. The 1000-Kernal weight of the three cultivars used in this study was not positively or negatively in-

Table 3. Effect of rhizobacteria strains on grain yield (g/plant) of three barley cultivars inoculated with *P. graminea Sy3* during two growing seasons (2013, 2014).

Cultivar/ Treatment	2013				2014				Main effect
	Arabi Abiad	Banteng	WI2291	Mean	Arabi Abiad	Banteng	WI2291	Mean	
Control**	4.60c*	3.45a	6.27a	4.77b	4.20a	4.32a	7.52a	5.35a	4.9b
BTP1	4.85c	5.26a	7.09a	5.74a	4.81a	4.66a	7.78a	5.75a	5.69ab
Bs2500	5.22bc	3.60a	7.21a	5.34ab	4.82a	4.58a	7.26a	5.55a	5.34ab
Bs2508	6.14ab	3.94a	6.45a	5.51ab	5.97a	5.66a	7.49a	6.06a	5.84a
Bs2504	6.84a	3.82a	6.96a	5.79a	5.97a	4.44a	7.09a	5.72a	5.8a
LSD	0,93	-	-	0,66	-	-	-	-	0,53
Mean	*B 5.48	C 4.02	A 6.80		B 5.09	B 4.53	A 7.44		
Main effect	A 5.43				A 5.69				

*Means preceded by different capital letters (line) and followed by different small letters (column) differ significantly at (P<0.01) according to Student-Newman-Keuls test.
**Infected with *P. graminea* Sy3.

fluenced by any of the rhizobacterial strains used (Table 4). Our results are in agreement with the work of Reglinski *et al*. (1994) on barley demonstrating that there was no effect of induced resistance on yield. The expression of resistance in barley to leaf stripe disease was not associated with an increase in grain yield and 1000-kernel weight. In general, there was a stability of these two traits. The data presented here suggest that either the plants possessed sufficient resources to support both growth and defense, or they use resources diverted from growth to defense. This phenomenon has been reported by several workers (Murray and Walters, 1992; Ziadi *et al.*, 2001; Prats *et al.*, 2002; Córdova-Campos *et al.*, 2012). A number of hypotheses have been put forth to explain how plants reallocate resources during the induction of plant defenses and how induced resistance benefits the overall fitness of the plant relatively to constitutive defense mechanisms (Ahmad *et al.*, 2010; Córdova-Campos *et al.*, 2012).

In this context, it is interesting to raise the hypotheses of balance between plant growth and defense. For that purpose, experiments were conducted under the same resource-limiting conditions (200-250 mm rainfall during the two growing seasons) using the same design as for induced resistance. The experiment included the same three barley cultivars and the four rhizobacterial strains, without applying any pathogen. Analysis of variance on grain yield showed significant effects among cultivar, rhizobacterial strain and their interaction (Table 5). Growing season had no effect on grain yield. Compared with the control (free of *P. graminea* and rhizobacterial strains), all treatments with rhizobacterial strains had a positive effect on grain yield (main effect, Table 5). The Bs2508 and BTP1 strains had in general a positive effect on yield. Compared with the control, Bs2508 showed an increase of 29.5% for the two growing seasons and the three cultivars. Arabi Abiad exhibited significant (*P<0.01*) increase of grain yield by using Bs2508, that reached 93.6% and 30.9% during 2013 and 2014 seasons, respectively. The winter barley cultivar Banteng did not exhibit any significant increase in yield during the two growing seasons.

The present study, on the one hand, showed that *P. putida* BTP1 and *B. subtilis* Bs2005, Bs2504, and Bs2508 strains, could not inhibit *in vitro P. graminea* growth (no direct antagonism between them). This observation is supported by the work of Ongena *et al*. (1999) on *P. putida* BTP1 in which the bacteria did not produce or secrete any fungitoxic compound. On the other hand, the

Table 4. Effect of rhizobacteria strains on 1000- kernel weight (g) of three barley cultivars inoculated with *P. graminea Sy3* during two growing seasons (2013, 2014).

Cultivar/ Treatment	2013				2014				Main effect
	Arabi Abiad	Banteng	WI2291	Mean	Arabi Abiad	Banteng	WI2291	Mean	
Control	49.00a*	27.00a	49.33a	41.78a	44.38a	24.65a	43.98a	37.67a	39.74a
BTP1	49.07a	28.33a	49.67a	42.33a	46.46a	24.97a	44.4a	38.51a	40.42a
Bs2500	47.67a	26.67a	54.00a	42.78a	46.49a	25.37a	43.31a	38.39a	40.58a
Bs2508	46.67a	26.67a	51.00a	41.41a	45.18a	24.06a	43.19a	37.4a	39.42a
Bs2504	47.83a	26.67a	50.00a	41.11a	43.95a	23.65a	42.09a	36.57a	38.84a
Mean	*B 47.8	C 27.07	A 50.80		A 45.24	C 24.5	B 43.33		
Main effect	A 41.89				B 37.71				

* Means preceded by different capital letters (line) and followed by different small letters (column) differ significantly at (*P<0.01*) according to Student-Newman-Keuls test.

Table 5. Effect of rhizobacteria strains on grain yield (g/plant) of three barley cultivars during two growing seasons (2013, 2014).

Cultivar/ Treatment	2013				2014				Main effect
	Arabi Abiad	Banteng	WI2291	Mean	Arabi Abiad	Banteng	WI2291	Mean	
Control**	4.10d*	4.12a	5.67b	4.64c	5.27b	3.70a	6.23b	5.07d	4.85c
BTP1	5.23c	4.43a	7.30a	5.66b	6.49a	3.96a	7.20a	5.88ab	5.77b
Bs2500	6.82a	3.84a	7.00a	5.89ab	5.20b	4.03a	7.07a	5.43c	5.66b
Bs2508	7.94a	4.27a	6.80a	6.33a	6.90a	4.47a	7.33a	6.23a	6.28a
Bs2504	6.67b	3.97a	6.97a	5.87ab	6.43a	3.93a	6.50b	5.62bc	5.74b
LSD	1,12	-	0,88	0,46	0,72	-	0,75	0,36	0,29
Mean	*B 6.15	C 4.13	A 6.75		B 6.06	C 4.02	A 6.87		
Main effect	A 5.68				A 5.65				

*Means preceded by different capital letters (line) and followed by different small letters (column) differ significantly at (P<0.01) according to Student-Newman-Keuls test.
**Free of *P. graminea* Sy3 and *rhizobacteria*.

fungal hyphae survive in the kernel between the paranchymatical cells in the hull, while cells of the bacterial strains were on seed surface, *i.e.* there was no contact between them and they were spatially separated. All bacterial strains used in this study have reduced the incidence and severity of barley leaf stripe disease caused by *P. graminea*, with effect more pronounced when *P. putida* BTP1 and Bs2508 were used. Adam *et al.* (2008) demonstrated that tomato bacterized-plants with *P. putida* BTP1 showed elicited systemic resistance, by means of lipoxygenase (LOX) pathway related defense. Ongena *et al*. (2005) provided evidence that an *N*-alkylated Benzylamine derivative (NABD), isolated from *P. putida* BTP1, elicits resistance in bean against *Botrytis cinerea*. Mariutto *et al*. (2014) reported that induced systemic resistance (ISR) stimulation in tomato by *P. putida* BTP1 was associated with induction of the first enzyme of the oxylipin pathway, the lipoxygenase (LOX). The oxylipin pathway was found to be differentially regulated (Mariutto *et al*. 2014). Thus, NABD and other elicitors produced by *P. putida*

BTP1 may be active on different plant species (monocots and dicots) for the control of various pathogens.

In our study, the Bs2504 and Bs2500 strains induced resistance in barley against *P. graminea* but not as high as that induced by Bs2508 strain. Our results are in agreement with the work of Ongena *et al*. (2007) on tomato and bean, who found that Bs2500 and Bs2504 produce surfactin and fengycin, respectively, whereas Bs2508 produces both of these compounds; they suggested that these compounds can be perceived by plant cells as signals to initiate defense mechanisms. In future work we will devote time to identify and quantify compounds essential for the ISR-eliciting activity of the above rhizobacterial strains.

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Επίδραση στελεχών ριζοβακτηρίων στην ανθεκτικότητα του κριθαριού έναντι του *Pyrenophora graminea* σε συνθήκες αγρού

A. Adam, M.I.E. Arabi, I. Idris και E. Al-Shehadah

Περίληψη Μελετήθηκε η επίδραση των βακτηριακών στελεχών *Pseudomonas putida* BTP1, *Bacillus subtilis* Bs2500, Bs2504, και Bs2508 στη συχνότητα εκδήλωσης (I) και τη σοβαρότητα (S) της ασθένειας «ραβδωτή κηλίδωση του κριθαριού» που προκαλείται από το μύκητα *Pyrenophora graminea* σε συνθήκες αγρού. Χρησιμοποιήθηκαν τρεις ποικιλίες κριθαριού οι οποίες διέφεραν ως προς την ανθεκτικότητα. Η ανθεκτικότητα που επιτεύχθηκε στην παρούσα μελέτη είχε μεγάλη διάρκεια. Τα στελέχη *P. putida* BTP1 και Bs2508 ήταν γενικά τα πιο αποτελεσματικά στο να περιορίσουν σημαντικά τόσο τη συχνότητα εκδήλωσης (I) όσο και τη σοβαρότητα (S) της ασθένειας στις τρεις ποικιλίες κριθαριού και στις δύο καλλιεργητικές περιόδους 2013/2014. Η ασθένεια μειώθηκε έως και 66% στην ποικιλία Arabi Abiad, η οποία δέχτηκε επέμβαση με το στέλεχος *P. putida* BTP1. Η ευαίσθητη τοπική ποικιλία Arabi Abiad εμφάνισε σημαντική αύξηση της ανθεκτικότητας υπό την επίδραση των στελεχών Bs2508 and BTP1. Ωστόσο, η ανθεκτική ποικιλία Banteng δεν έδειξε περαιτέρω σημαντική αύξηση της ανθεκτικότητας υπό την επίδραση αυτών των βακτηριακών στελεχών. Η απόδοση σε σπόρο των φυτών που δέχτηκαν τις επεμβάσεις με τα βακτηριακά στελέχη και μολύνθηκαν τεχνητώς με *P. graminea* δεν επηρεάστηκε, εκτός από την περίπτωση της ποικιλίας Arabi Abiad όταν δέχτηκε την επέμβαση με τα στελέχη Bs2508 and Bs2504. Η επαγωγή της ανθεκτικότητας μέσω επέμβασης στο σπόρο με βακτηριακά στελέχη θα μπορούσε να έχει σημαντική εφαρμογή στη γεωργία, κυρίως στην περίπτωση της προσβολής του κριθαριού από τον μύκητα *P. graminea* στα πρώιμα στάδια της ανάπτυξης των φυτών.

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SHORT COMMUNICATION

Can high pest pressure of the red palm weevil *Rhynchophorus ferrugineus* beat the defense of *Phoenix theophrasti?*

O. Melita, V. Gkounti, D. Kontodimas, D. Papachristos and F. Karamaouna*

Summary The Cretan date palm, *Phoenix theophrasti,* is a less susceptible and suitable host for the red palm weevil compared to the Canary palm, *P. canariensis*, even at high pest pressure. Nevertheless, *P. theophrasti* is not invulnerable to the red palm weevil, hence under continuous and high pest pressure young offshoots/palms can be deadly infested. The slow development of the insect in the Cretan date palm should probably allow a larger 'window of time' for an effective plant protection management against the pest.

Additional keywords: Cretan palm, pest density, suitability, susceptibility

Introduction

The red palm weevil *Rhynchophorus ferrugineus* (Olivier) was recorded for the first time in Greece in November 2005 in the island of Crete (Kontodimas *et al*., 2007). Since then, the pest was established all over the country, causing severe damage to ornamental palm trees occurring in urban and natural landscape areas. The widely distributed Canary palm, *Phoenix canariensis* Chabaud (Arecaceae), was proved to be highly susceptible, while other ornamental species have shown different types of resistance (Dembilio *et al*., 2009; Cangelosi *et al.,* 2016). The Cretan date palm, *Phoenix theophrasti* Greuter (Arecaceae), which occurs naturally in Crete and some Aegean islands, is threatened by the presence of the pest. However, incidents of infestation of *P. theophrasti* are not numerous, leading to the assumption that a level of resistance of the Cretan date palm to the weevil might exist. Dembilio *et al.* (2011) report that healthy 4 years old *P. theophrasti* palms were not infested by adult females after 9 days exposure in a population density of 3 adult females per plant and that a gummy secretion observed in infested palms indicates the existence of antibiosis in this species. Kontodimas *et al*. (2006) reported that development of *R. ferrugineus* adults and their emergence was possible at *P. theophrasti* seedlings after exposure to 6 females/palm (simultaneous presence of 6 males) in laboratory conditions 26°C and 16:8 L:D. The current study aimed at assessing the susceptibility of young plants (corresponding to young offshoots) of *P. theophrasti* to the red palm weevil in response to different population densities in semi-field conditions (compared to the susceptible *P. canariensis),* and their suitability for the development of adult weevils.

Materials and methods

The trials were carried out in semi- field conditions (glasshouse) at Benaki Phytopathological Institute (BPI), Kifissia, Greece. Eight screened metal mesh cages (3 x 1.5 x 2.3 m) were used for different treatments. Assays were performed on 3 years-old potted plants of *P. theophrasti* and *P. canariensis* (the stipe was approx. 15-20 cm high and 15-20 cm wide). *Phoenix canariensis* palms were used as a control susceptible species to the red palm weevil. Five palm trees of each

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palm species were located in each cage. The palms were exposed to different densities of female weevils, which had been captured in monitoring traps in urban parks of Attica Prefecture. After capture, weevils (male and female) were kept in a rearing Perspex cage (50 x 41 x 50 cm bearing two openings (14 x 28 cm) covered with 2 mm metal mesh for ventilation) under a diet of apples in constant conditions (27 ± 2°C, 60% R.H., 12:12 L:D). A sex ratio of 3:1 (female: male) was sustained to ascertain successful mating. Female individuals were tested for their fertility before the experiment. For this, the females were placed individually in plastic containers (100 cm^3) and were let to lay eggs on a thick slice of apple for 24 hours. Females that laid less than 2 eggs were discarded. After the fertility test, the insects were released in the cages, where they were left to roam freely for 9 days and then were removed. Two experiments were conducted:

i. *P. theophrasti* susceptibility response to pest-density. The palms were exposed to three (3) population densities of female weevils (3, 6 and 12 individuals/palm x 5 palms/cage x 1 cage per species and density) starting at the end of July-beginning of August until middle of September 2013 (21 to 45.7°C the first 20 days after release) to examine the susceptibility of the Cretan palm under different pest pressure compared to the susceptible *P. canariensis*.

ii. *P. theophrasti* suitability at high pest-density. Palms were exposed at the maximum density of those tested in the previous experiment (12 female individuals/palm x 5 palms/cage x 4 cages per species) starting at the beginning of May 2014 (13 to 44°C the first 20 days after release) to examine the suitability of Cretan palms at the age of young offshoots to support the development of the red palm weevil to adulthood.

In the pest-density response experiment, the palms were dissected 4 weeks after the withdrawal of the insects and the successfully hatched larvae were recorded per palm. Percentage successful infestation of the palms [palms bearing live individuals (larvae and pupae) out of total palms exposed] was estimated. The data were subjected to statistical analysis with the Kruskal-Wallis test. Larvae were taken to the laboratory, their head capsule width was recorded and their larval stage was determined according to Dembilio and Jacas (2011). In the suitability experiment, the palm leaves were shortened and the palms were covered individually with a mesh cage and checked daily for adult emergence.

Results and Discussion

The effect of pest density on successful infestation was not found significant (Kruskal-Wallis, H= 2.68; d.f= 1; *p= 0.102),* although an increasing infestation rate (based on oviposition holes and minute larval tunnels without larval development) in higher pest densities was noticed. Successful infestation was significantly less in the *P. theophrasti* palms (0.33 ± 0.27 individuals per palm) compared to that in the *P. canariensis* palms (2.80 ± 1.55 individuals per palm) [(Kruskal-Wallis, H= 4.10; d.f= 1; *p= 0.043* (adjusted for ties)]. Overall pest densities, 13.3% of the exposed *P. theophrasti* palms were successfully infested whereas successful infestation in *P. canariensis* was 33.3%. The number of larvae and pupae per palm ranged from 1 to 4 in *P. theophrasti* and 1 to 24 in *P. canariensis*. In total, 39 larvae developed in *P. canariensis* palms and 5 larvae hatched in *P. theophrasti*, in approximately 4-5 weeks from oviposition. Moreover, the larvae found in *P. theophrasti* did not exceed L7-L8 whereas most of the larvae in *P. canariensis* were L9-L13 [larval stage determination according to Dembilio and Jacas (2011)], indicating that development of the larvae was slower in *P. theophrasti* (Figure 1). A gummy secretion was observed at the oviposition holes and larvae tunnels like in the case of the Dembilio *et al.* (2011) study, the presence and consequent action of which support the assumption for an antibiosis defense mechanism against the pest.

In the high density effect experiment, all *P. canariensis* palms were successfully infested by the red palm weevil while only 25% of *P. theophrasti* palms were infested. In Canary palms, the infestation was evident 6.5-7.5 weeks after oviposition by the weevil (pulpy mass of plant tissue emerging from the basis of leaves and gradual drying and bending of outer leaves) whereas in the Cretan palms the symptoms were seen 13-14 weeks after oviposition (pulpy mass of plant tissue emerging from the palm stem due to larval feeding, Figure 2). A total of 595 adults emerged from 18 palms of *P. canariensis,* 338 individuals of which were females (56.8%) and 257 were males (43.2%). Adult emergence occurred between 10.5 to 15.5 weeks with the majority of them emerging 13 weeks after oviposition. In *P. theophrasti* palms one dead malformed adult, which failed to emerge, and one emerged adult female were recorded 14 months after exposure to the pest, supporting the results by Kontodimas *et al.* (2006) on the development of the red palm weevil in young seedlings of *P. theophrasti* within 4 months at constant 26 °C.

In conclusion, *P. theophrasti* is a less susceptible and suitable host for the red palm weevil compared to *P. canariensis*, even at high pest pressure. The defense of *P. theophrasti* against the pest seems to depend on antibiosis through a gummy secretion of the attacked plants at the oviposition holes and minute larval tunnels (Dembilio *et al.*, 2011). In case of successful infestation, the development of the insect in *P. theophrasti* is very slow in comparison to that in *P. canariensis*, possibly allowing a larger 'window of time' for an effective plant protection management. Nevertheless, our results indicate that although *P. theophrasti* exhibits a high level of resistance to the red palm weevil, it is not invulnerable; under continuous and high pest pressure, young offshoots/palms can be deadly infested. In the wild, infestation of older *P. theophrasti* offshoots by the red palm weevil was reported in Chania-Crete (Conservatory of the Mediterranean Agronomic Institute of Chania) in September 2014 (Prefecture of Chania, C. Fournaraki, personal communication; Kontodimas *et al.*, in press).

The presence of adult individuals has been recorded in the natural habitats of *P. theophrasti* in Crete i.e. the palm forest of Vai and adjacent nurseries (Prefecture of Lasithi, October 2014) and the palm forest of Preveli (Prefecture of Rethymno, November 2014) (F. Karamaouna and O. Melita, personal communication). The present findings should be taken into account for the update of the Action Management Plan towards the most effective protection of the Cretan date palm habitats from the red palm weevil.

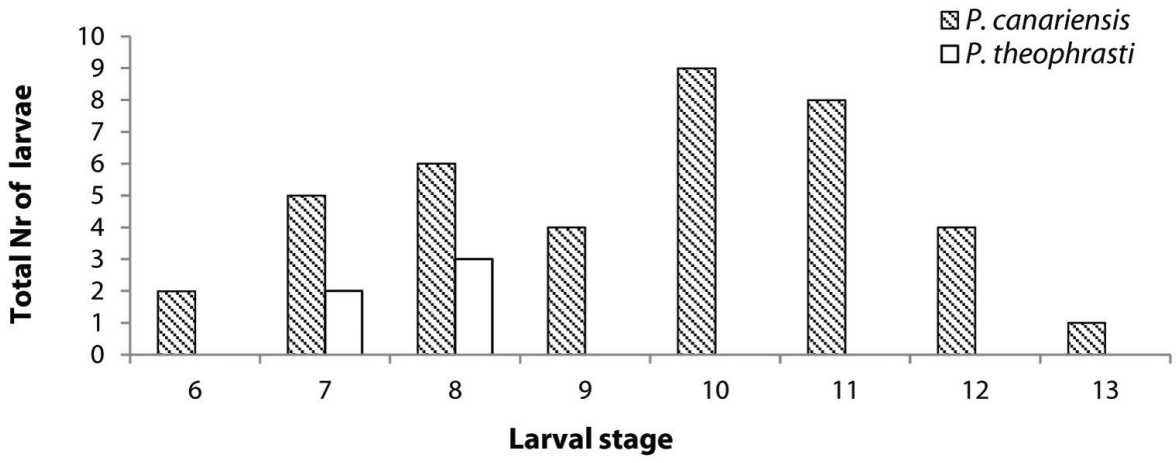

Figure 1. Distribution of larval stages of *Rhynchophorus ferrugineus* in *Phoenix theophrasti* and *Phoenix canariensis* palms in 4 week infested palms (15 plants/species) in semi-field conditions at air temperatures 21 to 45.7°C

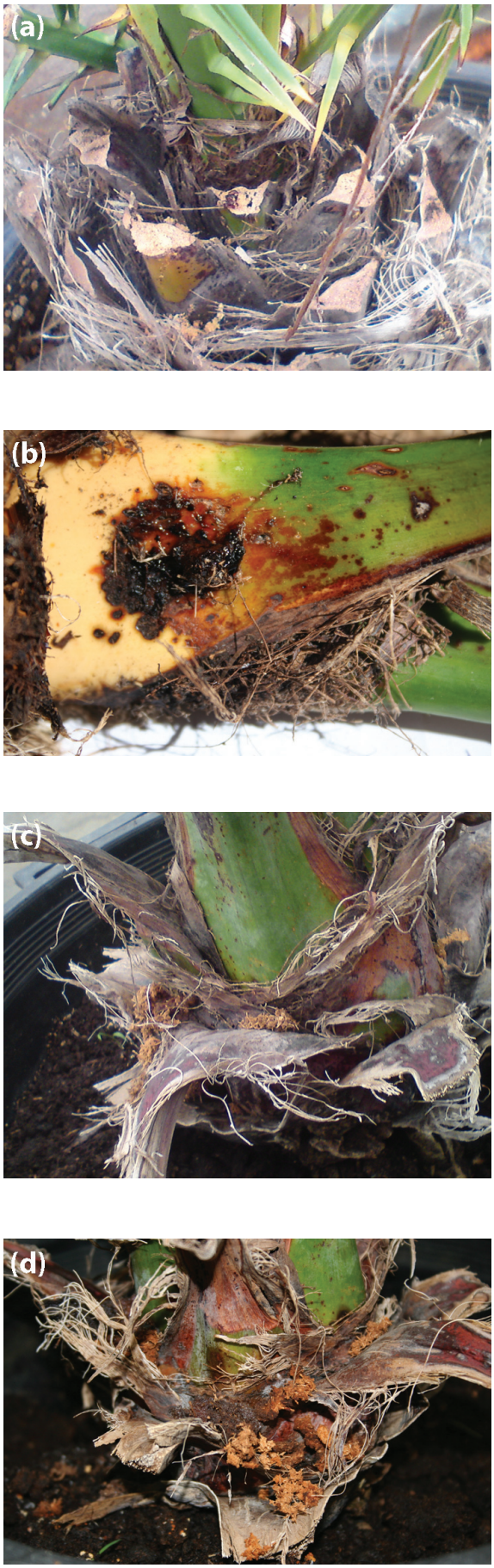

Figure 2. Infestation of *Phoenix theophrasti* by *Rhynchophorus ferrugineus*: gummy secretion on the leaf basis (a) and oviposition holes with gummy secretion (b), 4 weeks after exposure to high pest-density; pulpy mass of plant tissue, approximately 4 weeks after exposure to the pest (c, d) and 14 weeks after exposure to high pest-density (e); malformed adult, which failed to emerge, 14 months after exposure to high pest-density (f).

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ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Μπορεί η υψηλή πίεση πληθυσμού του ρυγχοφόρου των φοινικοειδών, *Rhynchophorus ferrugineus,* να υπερνικήσει την άμυνα του φοίνικα του Θεόφραστου *Phoenix theophrasti*;

Ο. Μελιτά, Β. Γκουντή, Δ. Κοντοδήμας, Δ. Παπαχρήστος και Φ. Καραμαούνα

Περίληψη Ο φοίνικας του Θεόφραστου, *Phoenix theophrasti,* έχει μικρότερη ευαισθησία και είναι λιγότερο κατάλληλος ξενιστής για το ρυγχοφόρο των φοινικοειδών σε σύγκριση με τον Κανάριο φοίνικα, *P. canariensis*, ακόμα και σε μεγάλη πίεση πληθυσμού. Εντούτοις ο φοίνικας του Θεόφραστου δεν είναι απρόσβλητος στο ρυγχοφόρο, επομένως κάτω από συνθήκες συνεχούς και υψηλής πίεσης πληθυσμού είναι δυνατή η προσβολή και νέκρωση νεαρών παραφυάδων/φυτών του. Η αργή ανάπτυξη του εντόμου στο φοίνικα του Θεόφραστου πιθανώς να επιτρέπει ένα μεγαλύτερο «παράθυρο» δράσης για την αποτελεσματική αντιμετώπισή του.

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