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Effect of *Aspidiotus rigidus* infestation on the volatile chemical profile of the host plant *Garcinia mangostana*

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Summary Plants respond to stress or damage by releasing volatile compounds, primarily for defense purposes. These volatiles function as signals for different interactions of the plant with its environment. In this study, the volatile chemical profile of healthy *Garcinia mangostana* L. (mangosteen) leaves was compared against leaves infested with the scale insect, *Aspidiotus rigidus* Reyne (Hemiptera: Diaspididae) through solid phase microextraction (SPME) coupled with gas chromatography- mass spectrometry (GC-MS). Analyses revealed that leaves emit the terpene kaur-16-ene in response to *A. rigidus* infestation. Kaur-16-ene is a precursor of gibberellin, a plant hormone for growth and development. The results suggest that the emission of kaur-16-ene in infested *G. mangostana* may play a role relevant in increasing the resistance of the plant towards infestations by herbivores.

Additional keywords: diterpenes, plant physiology, scale insect, semiochemistry

Introduction

Plants adopt various mechanisms to deter and counteract herbivore - induced damage. The emission of volatile chemicals upon attack is one of the effective ways plants deal with herbivory. These herbivore - induced plant volatiles (HIPV) serve various purposes such as a warning to surrounding plants of similar species, attract predators of the herbivore (De Moraes *et al.*,1998), and increase the fitness and vitality of the HIPV emitting plant (Schuman *et al.,* 2012). The identification of HIPVs is not only important for understanding complex plant - insect dynamic interactions, but it can also pave the way for the development of effective and sustainable pest control measures (Shrivastava *et al.,* 2010; Dicke *et al.,* 1990). Regarding the chemical profile of HIPVs, the green leaf volatiles (GLV) which are released by the plants as soon as they are attacked or wounded, are composed mainly of alcohols, aldehydes, and esters. In addition, the volatile signals that are commonly released for defense include secondary metabolites produced from different pathways like lipoxygenase (LOX), shikimic acid, and terpenoids (Dudareva *et al.,* 2006).

Recently, a massive infestation on plantations of coconut, *Cocos nucifera* (L.) (Arecaceae), was observed in the Philippines by the coconut scale insect, *Aspidiotus rigidus* Reyne (Hemiptera: Diaspididae). The infestation caused a great decline in the coconut palms of the area, affecting around 50-70% of the coconut farms in 2013 (Watson *et al*., 2015). Another economically important host of the scale is mangosteen, *Garcinia mangostana* L. (Clusiaceae). It is considered a priority crop in the Philippines wherein 13,352 metric tons of this fruit was produced during the 2007 – 2011 period (Bureau of Agricultural Statistics, 2012).

Aspidiotus rigidus settles on the lower surface of the leaf, blocking the stomata and preventing the plant to photosynthesize which may even result to death (Watson *et al*., 2015). Little is known about the inter-

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action of *A. rigidus* with the host plant and information about scale insect - induced volatile compounds is scarce. Considering that scale insects in general pose a serious threat to agricultural productivity (Miller *et al.,* 2005), understanding their interaction with the host plant is of paramount importance. In this study, the effect of *A. rigidus* infestation on the volatile chemical profile of the host plant *G. mangostana* was investigated. *Aspidiotus rigidus* is the only scale species infesting mangosteen (Watson *et al.,* 2015) and hence its selection as host plant in the experiments eliminates the possibility for infestation of the plant tissue by other similar scale insect species, e.g. *Aspidiotus desctructor* Signoret (Hemiptera: Diaspididae), infesting coconut. The results presented are expected to contribute to the understanding of the pest - host plant interaction, which may be useful in devising strategies to mitigate the damage caused by *A. rigidus*.

Materials and Methods

Leaf Sample Collection

Healthy and *A. rigidus* - infested mangosteen leaves were collected from De La Salle University - Science and Technology Complex in Laguna, Philippines from January to May 2016. Mature leaves weighing 1.5g to 4.5g were used for the analysis. For the infested leaves, leaves with similar degrees of infestation were harvested on the basis of visual estimation of the presence of scales on the underside of the leaves (number of scales). The collected leaves were acclimatized for 1 hour prior to analysis.

Extraction of Volatiles

To extract the volatiles, a Supelco 0.1 mm Polydimethylsiloxane Solid Phase Microextraction (SPME) fiber was used. The SPME fiber was baked prior to use at 250°C for 30 minutes following manufacturer's instructions. A 500 mL Pyrex® Erlenmeyer Flask covered with aluminum foil and parafilm was used as a headspace chamber. The flask was cleaned with technical grade acetone and oven baked at 100°C for 1 hour before analysis. Once cooled, one mangosteen leaf sample was placed inside. This was heated to 30°C to 40°C while the fiber was exposed for 25 minutes. Heating facilitates efficient extraction of the volatile compounds (Silva *et al.,* 2017). After extraction, the fiber was directly injected to the GCMS. Two blank runs were also conducted to ensure the integrity of the yielded data. The first blank involved running the GCMS using the programmed temperature without SPME injection. This was done to make sure that the column used for the testing is clean. The second blank involved the exposure of the SPME fiber to the oven baked flasks which did not contain the leaf samples. All analyses were done in eight replicates.

Chromatographic and Spectroscopic Analysis

Gas Chromatography coupled to Mass Spectrometry (GC-MS) analysis was performed on Agilent Technologies 7890A GC System and Agilent Technologies 5977A MSD. A HP-5 MS ultra-inlet capillary column (30 m x 250 mm x 0.25 mm) was used. The injection temperature was set to 250°C and operated on splitless mode. The oven was held at 50°C for 5 minutes then programmed at 10°C/10 minutes until the final temperature of 200°C. Helium was used as the carrier gas with constant flow of 1 mL/min. Detection was performed in Electron Impact (EI) mode. Spectra acquisition was performed in scanning mode (mass range m/z 50-550). Chromatograms and spectra were recorded by means of GC/MSD ChemStation Software and MassHunter Workstation with MSD Chemstation DA Software (Agilent Technologies). The identity of the compound was determined by National Institute of Standards and Technology (NIST) Mass Spectral Library 2.0.

Statistical Analysis

The Mann – Whitney U test for nonparametric analysis was conducted using the software Statistica, on the basis of the com-

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pound peak area. All analyses were done at a 5% significance level.

Results and Discussion

The results of the GC-MS analysis on the healthy and infested leaves are presented in Figure 1. Peak identifications were limited to compounds that exhibited match factors greater than 800, which signifies a high degree of confidence in the identification (Hubschmann, 2015; Stein, 1999). The compounds emitted by infested or healthy mangosteen leaves are summarized in Table 1. The profile of volatile compounds emitted by mangosteen leaves infested with *A. rigidus* was different to the one emitted by healthy leaves. Both quantitative and qualitative differences were found.

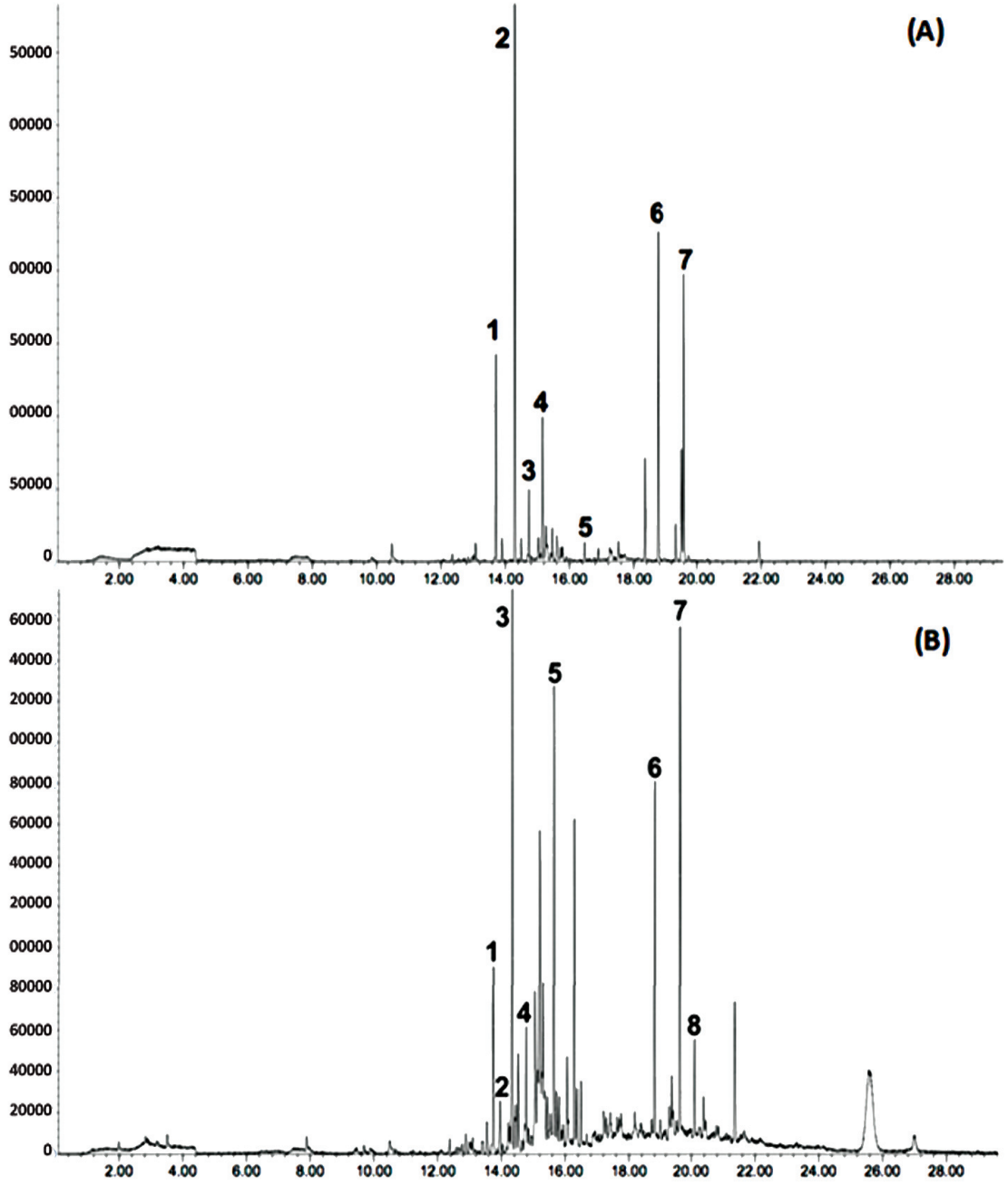

Figure 1. Chromatograms of healthy leaves of *Garcinia mangostana* (A) and infested leaves by *Aspidiotus rigidus* (B).

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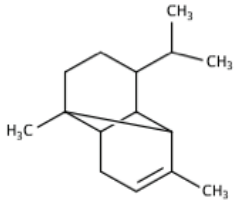
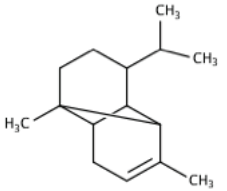
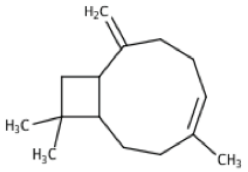
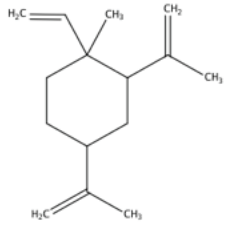
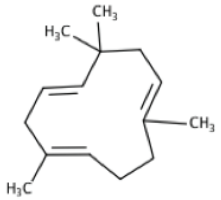
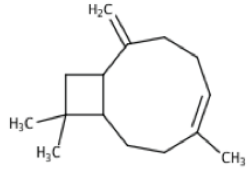
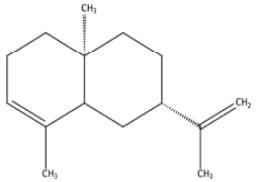
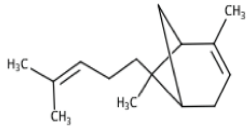
Table 1. Retention time and match factors of volatile compounds from healthy leaves of *Garcinia mangostana* (A) and infested leaves by *Aspidiotus rigidus* (B).

Healthy Mangosteen Leaves (A)					Infested Mangosteen Leaves (B)				
Label	Retention Time (min)	Identity of Compound	Match Factor	Structure	Label	Retention Time (min)	Identity of Compound	Match Factor	Structure
1	13.712	alfa-copaene	943		1	13.74	alfa-copaene	922	
2	14.296	caryophyllene	947		2	13.952	cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1a,2ß,4ß)]-	885	
3	14.742	humulene	906		3	14.324	caryophyllene	935	
4	15.166	naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-,[2R-(2a,4aa,8aß)]-	907		4	14.524	bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-	930	

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5	15.634	naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	915	
6	18.804	2-ethylhexyl salicylate	865	
7	19.634	Homosalate	919	
8	20.109	kaur-16-ene	809	

5	16.465	propanoic acid, 2-methyl-,1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester	825	
6	18.759	2-ethylhexyl salicylate	878	
7	19.589	homosalate	928	

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Caryophyllene, which was a common compound in healthy and infested leaves is a sesquiterpene found in oils produced by plants (Pinho-da-Silva *et al.,* 2012). β-caryophyllene is one of the most common volatile compounds secreted by plants that has the ability to limit the growth and development of insects infesting plants (Huang *et al.,* 2011). In addition, β-caryophyllene can attract naturally – occurring parasitoids of the phytophagous insects (Wang *et al.,* 2015).

The volatile compounds cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1a,2ß,4ß)], bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl), and kaur-16-ene-were emitted only by mangosteen leaves infested by *A. rigidus*. Notably, kaur-16-ene emission was significantly elevated in infested *vs* healthy leaves (Mann – Whitney U test, p-value = 0.000939). The emission of kaur-16-ene in infested leaves suggests that it is the physiological response of the plant to the infestation of *A. rigidus* which may play a role in the compensation of the plant to the damage.

Kaur-16-ene has been reported to be emitted by coniferous trees in Japan (Matsunaga *et al.,* 2012) but its role in herbivore – induced signaling is not known. Kaur-16-ene is recognized to be a precursor to the hormone gibberellic acid (GA) which affects the growth and development of plants (MacMillan 1997). GA helps the plants to compensate the damage caused by certain herbivores (Bari and Jones, 2009). Gibberellins, in general, function as promoters and regulators of growth and development (Otsuka *et al*., 2004). They also alter plant metabolism and induce physiological responses in plants (e.g. invertase activity and photosynthetic rate) favoring growth under stress (Iqbal *et al*., 2011). The increased fitness and vitality as a consequence of stress is widespread among plants, and is considered to be a form of adaptive plasticity (Anurag, 1998). Some examples of plants that can exhibit this form of defense adaptation include radish (Anurag, 1999) and tobacco (Kessler and Baldwin, 2004).

In conclusion, the infestation of the scale insect *A. rigidus* on the *G. mangostana* leads to the emission of the terpene kaur-16-ene, which possibly suggests the induction of hormone precursor compounds that will compensate for the damage. Overall, the results presented are expected to contribute to our understanding on the chemical ecological interaction of *A. rigidus* with the host plant and its natural enemies. Future research direction will aim at analyzing the potential of the HIPV kaur-16-ene to attract known parasitoids of *A. rigidus*.

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Επίδραση της προσβολής από το κοκκοειδές *Aspidiotus rigidus* στο χημικό προφίλ των πτητικών ουσιών του φυτού ξενιστή *Garcinia mangostana*

M.A.A. Tavera, J.C.A. Lago, V.K.D. Magalong, G.A.V. Vidamo, J.S.R. Carandang VI, D.M. Amalin και J.I.B. Janairo

Περίληψη Τα φυτά ανταποκρίνονται στο στρες ή στις βλάβες απελευθερώνοντας πτητικές ενώσεις, κυρίως για αμυντικούς σκοπούς. Αυτές οι πτητικές ενώσεις λειτουργούν ως σήματα για διαφορετικές αλληλεπιδράσεις του φυτού με το περιβάλλον του. Σε αυτή τη μελέτη, το χημικό προφίλ πτητικών ουσιών από υγιή φύλλα του φυτού *Garcinia mangostana* L. συγκρίθηκε με τα φύλλα που είχαν προσβληθεί από το κοκκοειδές *Aspidiotus rigidus* Reyne (Hemiptera: Diaspididae) με τη μέθοδο της μικροεκχύλισης σε στερεή φάση (SPME) σε συνδυασμό με αέρια χρωματογραφία- φασματομετρία μάζας (GC-MS).

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Οι αναλύσεις έδειξαν ότι τα φύλλα εκλύουν το τερπένιο kaur-16-ene σε απόκριση της προσβολής από το *A. rigidus*. Το kaur-16-ene είναι πρόδρομος της ορμόνης ανάπτυξης γιβερελλίνη. Τα αποτελέσματα υποδεικνύουν ότι η εκπομπή kaur-16-ene σε προσβεβλημένα φυτά *G. mangostana* μπορεί να παίζει ένα ρόλο στην αύξηση της ανθεκτικότητας του φυτού έναντι των προσβολών από φυτοφάγα έντομα.

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Resistance screening of lentil cultivars against the root-knot nematode *Meloidogyne incognita*

T. Ansari, M. Asif* and M.A. Siddiqui

Summary The root-knot nematode *Meloidogyne incognita* is a major soil parasite of lentil crops. Increasing restrictions of chemical nematicides have triggered a growing attention and interest in alternate root-knot nematode management. The present study was conducted to examine the level of resistance and/or susceptibility of five lentil cultivars (PL-456, KLS-218, Desi, DPL-62, Malika), grown in pots, against the root-knot nematode *M. incognita*. Root-knot nematode reproduction and host damage were assessed by recording the nematode infestation levels and reduction percentage of plant growth parameters. Nematode response and plant growth differentiated amongst the lentil cultivars. None of the cultivars was found immune or highly resistant. The cultivar Malika was found moderately resistant as it showed the lowest number of galls and egg masses/root as well as the lowest reduction of plant fresh weight (10.4%) and dry weight (6.9%). On the other hand, the cultivar Desi manifested the highest susceptibility exhibiting the highest number of galls and egg masses. There was a significantly negative correlation between the number of galls and plant growth parameters (plant fresh and dry weight and plant height).

Additional keywords: cultivars, lentil, *Meloidogyne incognita*, resistance, root-knot nematode

Introduction

Lentil (*Lens culinaris* Medik.) is one of the most important pulse crops and a major source of protein (30% weight, second protein content legume after soybeans) for the predominant vegetarian population of India. India ranks second (after Canada) in lentil production, with lentil cultivation occupying an area of 1,800,000 hectares and annual production reaching 1,100,000 metric tonnes of grain. The average yield is 6000kg/ha (FAO, 2014). Lentils are an important source for the Indian household income.

Meloidogyne spp. cause yield losses in pulses, which are estimated annually up to 20-35% (Gaur *et al*., 2001). In India, the average loss caused by root-knot nematodes on pulses may be 14.6%, and could go as high as 50-80% in some crops (Bhatti, 1992).

Root-knot nematodes are difficult to control because of their short life cycle, high population densities and reproductive potential (Sikora and Fernandez, 2005). The immense loss caused by root knot nematodes can be minimized by using various strategies such as soil treatment with chemicals, biocontrol agents (Vagelas and Gowen, 2012), cultural practices such as crop rotation, antagonistic plants (Hussain *et al.,* 2011), resistant cultivars (Tariq *et al*., 2016) and soil amendments (Asif *et al.,* 2017a, b; Ansari *et al.,* 2016), which are common methods of nematode control.

Synthetic nematicides can rapidly reduce nematode population. However, their indiscriminate and consistent use can be toxic to beneficial soil flora and fauna, and may induce emergence of resistant plant parasitic nematodes (Akhtar, 1991). Nevertheless, nematode infestation levels in soil could be reduced by using resistant cultivars with crop rotation strategy of non-hosts. According to Oostenbrink (1966), the cultivation of a resistant cultivar may suppress the nematode population by 10-50% of its harmful density. Susceptibility of different pulse crops has been reported by sev-

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eral researchers such as Pandey *et al.* (2016) and Montasser *et al.* (2017). Sasser (1954) found that the roots of resistant plants were not invaded as rapidly as susceptible ones. Such variability in tolerance might be related to their genetic makeup, coding for a resistance mechanism possessed by the particular cultivar (Anwar and McKenry, 2007). All cultivars referred as resistant to root-knot nematodes have comparatively better crop yield as compared to susceptible cultivars (Mukhtar *et al.,* 2014).

The present investigation was carried out as a resistance screening of five of the most commonly grown cultivars of lentil in India against *Meloidogyne incognita* (Kofoid and White) Chitwood under greenhouse conditions with a view to better exploit the use of most resistant cultivars for the suppression of root-knot nematodes in integrated management programmes.

Materials and Methods

Meloidogyne incognita was collected from naturally infested eggplant (*Solanum melongena* L.) fields near Punjipur village, Aligarh (U.P). *Meloidogyne incognita* was mass cultured from a single egg mass on a susceptible variety of tomato, S-22, in a greenhouse of the Section of Plant Pathology and Plant Nematology, Department of Botany, A.M.U., Aligarh. Second-stage juveniles (J2) were obtained from hatched eggs by incubating handpicked egg masses in sterile distilled water at 28 ± 1°C.

Seeds of five lentil cultivars namely, PL-456, KLS-218, Desi, DPL-62 and Malika were obtained from the Indian Institute of Pulses Research, Kanpur, India as these are most commonly grown and chief cultivars concerning with the productivity of lentil in India. Lentil seeds of all five cultivars were surface sterilized with 0.1% mercuric chloride and sown in clay pots of 15cm diameter containing 1kg sterilized sandy loam soil. Four lentil seeds of each cultivar were sown per pot, which was considered one replication. The pots were placed in a greenhouse at a completely randomized design (CRD). The experiment had four replications per cultivar and it was repeated twice in time. Three weeks after seed germination, thinning was performed, leaving one plant per pot. Two days after thinning, approximately 1500 second stage juveniles of *M. incognita* were pipetted around each plant by making holes in soil. A total of eight plants (four inoculated and four uninoculated) of each cultivar were used for this experiment. The uninoculated plants served as controls. Ninety days after inoculation, the plants were uprooted gently from the pots and different plant growth parameters were noted.

Varietal response against *M. incognita* infestation of different crops is accessed on the basis of growth parameters and nematode reproduction parameters (Hayat, 2011). Hence, in the present study data were recorded for plant growth viz., shoot height and root length, dry and fresh shoot and root weight, number of pods, number of nodules per root system, weight of 100 seeds and nematode reproduction parameters like number of egg masses, number of eggs/root system, root-knot index and final nematode population. Physiological parameters like chlorophyll content (MacKinney, 1941) and carotenoid content were also estimated. The percent increase and reduction in the growth parameters over the control were calculated by using the formula

$$\% \text{ reduction or increase} = \left(\frac{\text{Uninoculated - Inoculated}}{\text{Uninoculated}}\right) \times 100$$

(Irshad *et al*., 2012; Mukhtar *et al*., 2014).

Analysis of Variance was conducted and the Duncan's Multiple Range Test was employed for the comparison of means between the cultivars according to Critical Difference (C.D.) at P=0.05 level. To detect relation between the parameters, Pearson correlation and multiple linear regression was performed. The data were analysed using SPSS 12.00 software (SPSS Inc., Chicago, IL, USA).

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Results

None of the cultivars was found immune according to the rating scale of Table 1 based on number of galls on the root system (Table 2). The resistance rating of the cultivars was: Malika (moderately resistant), KLS-218 (moderately susceptible), PL-456 (moderately susceptible), DPL-62 (susceptible), Desi (susceptible). The same trend was observed for the egg masses and the nematode population per root system (Table 2).

The cultivars Malika, KLS-218, PL-456 had a comparable shoot height before infestation by *M. incognita*, which was significantly higher than the one of the DPL-62 and Desi. After infestation by the nematode, Malika continued to have significantly higher shoot compared to the other cultivars. These heights differentiated in the following descending order: KLS-218, PL-456, DPL-62 and Desi (Table 3).

Prior to the infestation by the nematode, the root length of the cultivars Malika, KLS-218, PL-456 was significantly higher than the one of the cultivars DPL-62 and Desi. After infestation, Malika had the least reduction in root length as compared to the other cultivars (Table 3).

The cultivar Malika had significantly heavier shoot fresh and dry weight than the other cultivars both before and after nematode infestation. The cultivars had the following decreasing order in shoot fresh and dry weights: KLS-218, PL-456, DPL-62 and Desi. A similar trend was also observed in

Table 1: Rating scale for the assessment of level of resistance of plant cultivars against root-knot nematodes, based on number of galls (Sasser and Taylor, 1978).

Root knot Index	No. of galls/root system	Resistance rating
0	0	Immune
1	1-2	Highly Resistant
2	3-10	Moderately Resistant
3	11-30	Moderately Susceptible
4	31-100	Susceptible
5	>100	Highly Susceptible

Table 2. Reproduction of the root-knot nematode *Meloidogyne incognita* on five lentil cultivars and resistance rating of the nematode.

Cultivars		No. of egg masses/ root	No. of eggs/ egg mass	Nematode population	Number of galls	Reaction
Desi	Control	-	-	-	-	Susceptible
	Inoculated	112±0.43[a]	196±0.40[a]	2000±0.42[a]	43±0.84[a]	
DPL-62	Control	-	-	-	-	Susceptible
	Inoculated	89±0.48[b]	154±0.45[b]	1692±0.32[b]	35±0.76[b]	
PL- 456	Control	-	-	-	-	Moderately Susceptible
	Inoculated	58±0.72[c]	97±0.46[c]	1590±0.21b[c]	24±0.72[c]	
KLS- 218	Control	-	-	-	-	Moderately Susceptible
	Inoculated	26±0.88[d]	69±0.68[d]	1440±0.25c[d]	16±0.88[d]	
Malika	Control	-	-	-	-	Moderately Resistant
	Inoculated	8±1.20[e]	25±1.15[e]	1161±0.19[e]	6±1.00[e]	

Values are mean of eight replicates.
Values in each column followed by the same letters are not significantly different according to Duncan's Multiple Range Test ($P \leq 0.05$).

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Table 3. Effect of infestation by the root-knot nematode *Meloidogyne incognita* on growth parameters of five lentil cultivars.

Cultivars						Fresh weight (g)				Dry weight (g)			
		Shoot height	Root length	Total plant height	% Reduction of plant height over control	Shoot	Root	Total	% Reduction of plant fresh weight over control	Shoot	Root	Total	% Reduction of plant dry weight over control
Desi	Control	21.8±0.48^{f}	12.9±0.74^{ef}	41.7±0.44^{fg}	35.4%	4.8±0.43^{fg}	1.9±0.48^{ef}	6.7±1.02^{fg}	32.8%	1.2 ±0.43^{fg}	0.7±0.47^{bc}	1.9±0.49^{fg}	31.5%
	Inoculated	17.5±0.76^{g}	9.4±1.08^{h}	26.9±0.56^{j}		2.9±0.41^{i}	1.6±0.42^{g}	4.5±1.29^{h}		1.0 ±0.48^{g}	0.5±0.42^{cd}	1.3 ±0.44^{g}	
DPL-62	Control	31.8±0.68^{bc}	14.9±0.75^{cd}	46.7±0.57^{cd}	29.7%	6.5±0.28^{de}	2.1±0.45^{de}	8.6 ±0.76^{ef}	29.0%	1.7 ±0.50^{ef}	0.7±0.68^{bc}	2.4 ±0.30^{ef}	25.0%
	Inoculated	21.1±0.74^{f}	11.7±0.81^{fg}	32.8±0.52^{i}		3.9±0.37^{gh}	2.2±0.38^{de}	6.1±1.21^{gh}		1.2±0.53^{fg}	0.6±0.32^{cd}	1.8 ±0.48^{fg}	
PL- 456	Control	34.3±0.57^{a}	16.9±0.98^{b}	51.2±0.52^{ab}	22.4%	8.3±0.19^{bc}	2.8±0.39^{bc}	11.1±0.67^{cd}	24.3%	2.3 ±0.37^{cd}	1.0±0.38^{a}	3.3 ±0.31^{cd}	21.2%
	Inoculated	25.4±0.63^{e}	14.3±0.97^{de}	39.7±0.43^{gh}		5.8±0.30^{ef}	2.6 ±0.40^{cd}	8.4±1.06^{ef}		1.7±0.50^{fe}	0.9±0.52^{ab}	2.6±0.40^{ef}	
KLS- 218	Control	34.9±0.56^{a}	18.6±0.61^{a}	53.5±0.36^{a}	17.0%	9.2±0.18^{b}	3.1±0.36^{ab}	12.3±0.66^{bc}	15.4%	2.6 ±0.43^{bc}	1.1±0.36^{a}	3.7±0.24^{bc}	18.9%
	Inoculated	28.4±0.60^{d}	16.0±0.87^{bc}	44.4±0.49^{de}		7.1±0.20^{cd}	2.9±0.39^{bc}	10.4±0.86^{de}		2.1±0.32^{de}	0.9±0.40^{ab}	3.0±0.27^{de}	
Malika	Control	32.4±0.75^{ab}	15.2±0.86^{cd}	47.6±0.43^{c}	8.4%	11.8±0.63^{a}	3.6±0.31^{a}	14.4±0.61^{a}	10.4%	3.1±0.36^{a}	1.2±0.27^{a}	4.3 ±0.22^{a}	6.9%
	Inoculated	31.8±0.74^{bc}	11.8±0.82^{fg}	43.6±0.54^{ef}		9.5±0.21^{b}	3.4±0.27^{ab}	12.9±0.77^{ab}		2.9±0.35^{ab}	1.1±0.29^{a}	4.0±0.20^{ab}	

Values are mean of eight replicates.
Values in each column followed by the same letters are not significantly different according to Duncan's Multiple Range Test ($P \leq 0.05$).

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the root fresh and dry weight (Table 3).

When the higher number of nodules/root system was compared between cultivars, Malika had significantly higher number of nodules/root system. Inoculation of *M. incognita* caused reduction in nodulation in all lentil cultivars. Malika continued to have significantly the highest number of nodules after nematode inoculation while Desi had the least number of nodules compared to the other cultivars (Table 4).

Yield parameters in terms of number of pods/plant and weight of seeds showed significant reductions after inoculation with nematodes in all lentil cultivars when compared to their respective controls. Among all cultivars, Malika had significantly more pods/plant and higher weight of seeds followed by KLS-218, PL-456, DPL-62 and Desi (Table 4).

Before infestation by *M. incognita* the cultivars Malika and KLS-218 had comparable chlorophyll and carotenoid content, which was significantly higher than PL-456, DPL-62 and Desi. Chlorophyll and carotenoid content of leaves was significantly reduced by nematode infestation in all lentil cultivars (Table 4).

Regression analysis showed a significant positive relation between the number of galls and reduction in plant fresh weight (R^2=0.974) (Fig. 1a), plant dry weight (R^2=0.930) (Fig. 1b), plant height (R^2=0.997) (Fig. 1c), number of pods/plant (R^2=0.988) (Fig. 1d), number of nodules/root system (R^2=0.918) (Fig. 1e).

Pearson correlation coefficients (r) indicate a significantly negative relation between nematode infestation parameters and all the plant growth parameters (Table 5): Total plant length (r= -0.948, P=0.05), Total plant fresh weight (r= -0.999, P=0.01), Total plant dry weight (r= -0.995, P=0.01), No. of pods (r= -0.961, P=0.01), No. of nodules/root system (r= -0.974, P=0.01), Chlorophyll content (r= -0.964, P=0.01), Carotenoid content (r= -0.895, P=0.05). Correlation between weight of seeds and nematode infestation in terms of eggs, eggs/egg masses, nematode population and galls had a negative relation, but not significant.

Discussion

M. incognita failed to reproduce and multiply on the cultivar Malika and exhibited the lowest reduction in growth, responding as moderately resistant against *M. incognita* infestation. The cultivar Desi was the most susceptible to the root-knot nematodes. The cultivars KLS-218 and PL-456 showed a moderately susceptible response in terms of reproduction and an intermediate reduction in growth parameters, which was less severe than that observed for the susceptible cultivar. The cultivars PL-456 and DPL-62 were found moderately susceptible and susceptible, respectively, but showed less severe damage by the nematode as compared to the most susceptible cultivar.

Most of the plant growth characters of the lentil cultivars were significantly negative correlated with the number of galls of *M. incognita*. Moreover it can be suggested that eggs, eggs/egg masses and nematode population also had a direct effect on the reduction of plant growth. *Meloidogyne* spp. induces galling in the roots and giant cells formation in the stellar region, which destroys the xylem tissues and ultimately reduces the absorption and movement of water and nutrients (Abad *et al*., 2003). This limitation of nutrient elements in the plant is probably the first effect that the nematode infestation has on the physiology and metabolism of its host (Lu *et al*., 2014). These effects increase with the duration of infestation (Melakeberhan *et al.,* 1987).

A reduction in total chlorophyll, as in the case of our experiment, has also been reported in French bean and rice infested by *M. javanica* (Melakeberhan *et al*., 1986; Swain and Prasad, 1988). Reduction in chlorophyll content has also been reported to lead to the disturbance of nodule function (Chahal *et al*., 1983).

In conclusion, the current study demonstrated that five of the most broadly cultivated lentil cultivars in India are susceptible to moderately susceptible to *M. incognita* with the exception of the cultivar Malika, which was found moderately resistant. The culti-

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Table 4. Effect of infestation by the root-knot nematode *Meloidogyne incognita* on yield and physiological parameters of five lentil cultivars.

Cultivars		No. of pods per plant	No. of nodules per root system	Weight of 100 seeds (g)	Chlorophyll content (mg/g)	Carotenoid content (mg/g)
Desi	Control	28±0.88^{g}	22±0.75^{h}	1.2±0.20^{de}	1.86±0.041^{ef}	0.800±0.005^{cd}
	Inoculated	16±0.93^{h}	13±0.96^{i}	0.9±0.19^{ef}	1.31±0.023^{g}	0.624±0.004^{i}
DPL-62	Control	59±0.73^{d}	34±0.80^{f}	1.4±0.14^{de}	2.57±0.022^{ab}	0.842±0.005^{ab}
	Inoculated	40±0.72^{f}	23±0.98^{h}	1.0±0.28^{ef}	1.85±0.026^{ef}	0.719±0.009^{g}
PL- 456	Control	53±0.64^{de}	42±0.72^{e}	1.8±0.21^{c}	2.42±0.017^{bc}	0.791±0.004^{ef}
	Inoculated	40±0.73^{f}	29±0.87^{g}	1.4±0.15^{de}	1.96±0.025^{e}	0.691±0.006^{h}
KLS- 218	Control	74±0.54^{c}	68±0.56^{ab}	1.6±0.14^{cd}	2.61±0.016^{ab}	0.860±0.007^{a}
	Inoculated	60±0.71^{d}	52±0.50^{d}	1.3±0.09^{de}	2.14±0.025^{d}	0.796±0.005^{de}
Malika	Control	92±0.48^{a}	72±0.54^{a}	3.4±0.11^{a}	2.78±0.018^{a}	0.820±0.006^{bc}
	Inoculated	83±0.51^{b}	65±0.57^{bc}	3.1±0.11^{b}	2.56±0.022^{ab}	0.801±0.006^{cd}

Values are mean of eight replicates.
Values in each column followed by the same letters are not significantly different according to Duncan's Multiple Range Test ($P \leq 0.05$).

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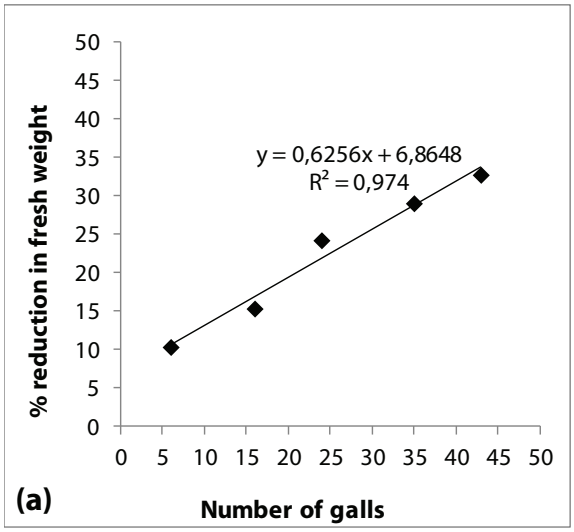

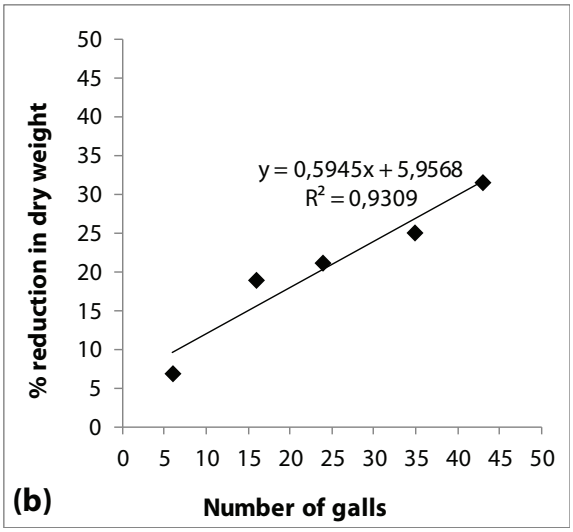

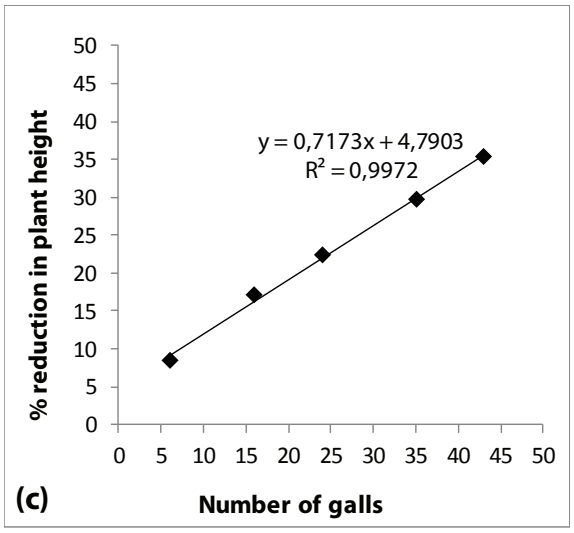

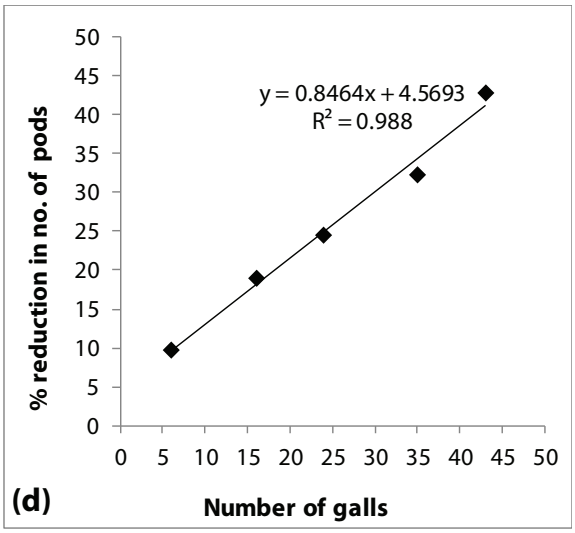

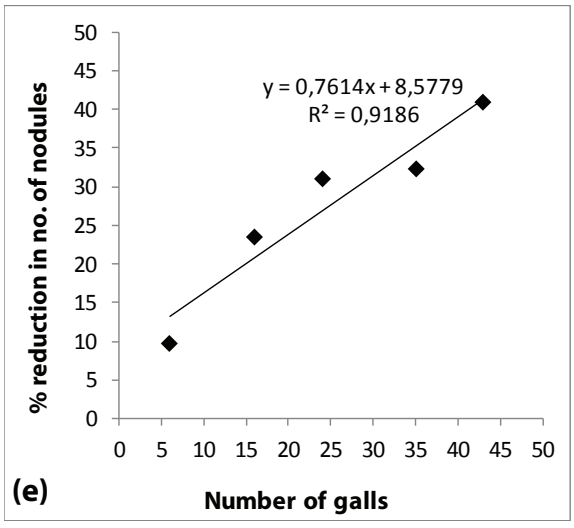

Fig.1. Relationship between number of galls by the root-knot nematode *Meloidogyne incognita* and growth parameters of five lentil cultivars.

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Table 5. Matrix of Pearson's correlation coefficients between plant growth parameters and nematode infestation variables of lentil cultivars.

Variables	TTL	TTF	TTD	NPD	NOD	WTS	CHL	CAR	EGMS	EGS	NMP	GALLS
TTL	1											
TTF	.800**	1										
TTD	.770**	.996**	1									
NPD	.713*	.951**	.943**	1								
NOD	.738*	.968**	.964**	.958**	1							
WTS	.456	.847**	.860**	.841**	.793**	1						
CHL	.876**	.902**	.880**	.926**	.856**	.728*	1					
CAR	.889**	.704*	.659*	.729*	.703*	.442ns	.862**	1				
EGMS	-.966**	-.993**	-.981**	-.952*	-.977**	-.789ns	-.950*	-.914*	1			
EGS	-.960**	-.993**	-.991**	-.950*	-.958*	-.822ns	-.966**	-.884*	.992**	1		
NMP	-.914*	-.979**	-.980**	-.989**	-.961**	-.856ns	-.997**	-.922*	.968**	.978**	1	
GALLS	-.948*	-.999**	-.995**	-.961**	-.974**	-.839ns	-.964**	-.895*	.995**	.998**	.980**	1

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed)
ns: correlation is not significant..
TTL=Total Plant Length, TTF=Total Plant Fresh Weight, TTD=Total Plant Dry Weight, NPD=No. of Pods, NOD=No. of Nodules/Root system, WTS=Weight of 100 Seeds, CHL=Chlorophyll Content, CAR=Carotenoid Content, EGMS=No. of Eggmasses/Root, EGS=No. of Eggs/Eggmass, NMP=Nematode population, GALLS=Number of Galls

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vars KLS-218 and PL-456 were moderately susceptible whereas DPL-62 and Desi were susceptible. There was an intense reduction of the plant growth parameters in susceptible cultivars as compared to less susceptible ones. Growing of the moderately resistant cultivar Malika in severely infested fields with *M. incognita* may assist to minimize root-knot nematode multiplication. Further studies are necessary in search of possibly higher resistance levels to root-knot nematodes in other commercially available lentil cultivars and the development of resistant cultivars.

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Διερεύνηση ανθεκτικότητας ποικιλιών φακής κατά του κομβονηματώδη *Meloidogyne incognita*

T. Ansari, M. Asif και M.A. Siddiqui

Περίληψη Ο κομβονηματώδης *Meloidogyne incognita* είναι ένα σημαντικό παράσιτο εδάφους για την καλλιέργεια της φακής. Οι αυξανόμενοι περιορισμοί των συνθετικών νηματωδοκτόνων έχουν αυξήσει την προσοχή και το ενδιαφέρον για εναλλακτικά μέσα διαχείρισης των κομβονηματωδών. Στην παρούσα μελέτη εξετάστηκε το επίπεδο αντοχής και / ή ευαισθησίας πέντε ποικιλιών φακής (PL-456, KLS-218, Desi, DPL-62, Malika), σε φυτά σε γλάστρες, έναντι του κομβονηματώδη *M. incognita*. Η αναπαραγωγή του κομβονηματώδη και η ζημιά στον ξενιστή αξιολογήθηκαν με βάση τα επίπεδα προσβολής από το νηματώδη και το ποσοστό μείωσης διαφόρων παραμέτρων ανάπτυξης των φυτών. Η απόκριση των νηματωδών και η ανάπτυξη των φυτών διαφοροποιήθηκαν μεταξύ των ποικιλιών φακής που μελετήθηκαν. Καμία από τις ποικιλίες δεν βρέθηκε απρόσβλητη ή πολύ ανθεκτική. Η ποικιλία Malika βρέθηκε μέτρια ανθεκτική, καθώς παρουσίασε το μικρότερο αριθμό όγκων και ωόσακων/ρίζα και τη χαμηλότερη μείωση του νωπού (10,4%) και του ξηρού (6,9%) βάρους των φυτών. Η ποικιλία Desi παρουσίασε τη μεγαλύτερη ευαισθησία, με το μεγαλύτερο αριθμό όγκων και ωόσακων. Διαπιστώθηκε σημαντικά αρνητική συσχέτιση μεταξύ του αριθμού των όγκων και των παραμέτρων ανάπτυξης των φυτών (νωπό και ξηρό βάρος φυτού και ύψος φυτού).

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Identification and determination of characteristics of endophytes from rice plants and their role in biocontrol of bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae*

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Summary Endophytic bacteria of rice plants (*Oryza sativa* L.) from eight different cultivars were screened for their ability in inducing disease symptoms, plant growth promotion and antagonistic activity against *Xanthomonas oryzae* pv. *oryzae*. Out of the 63 whole isolates, five were plant pathogens. Based on phenotypic characteristics and 16S rDNA sequence analysis, these were identified as *Pseudomonas oryzihabitans*, *P. fulva*, *Pantoea ananatis*, *Pantoea* sp., *Cellulomonas* sp. Four out of the 63 isolates behaved as potentially good plant growth-promoting and biocontrol agents. These were identified as *Bacillus* sp., *B. subtilis*, *Pseudomonas putida* and *Enterobacter* sp. This is the first report of pathogenic and endophytic bacteria from rice grown in field conditions in North of Iran.

Additional keywords: endophytic bacteria, *Oryza sativa* L., plant pathogens, PGPR, 16S rRNA gene

Introduction

Beneficial endophytic microorganisms are primarily comprised of fungi and bacteria that colonize internal plant tissues without causing visible damage to their hosts. (Schulz and Boyle, 2006; Rodriguez *et al*., 2008; Rodriguez *et al*., 2009; Reinhold-Hurek and Hurek, 2011). These local and systemic colonizers of the internal tissue can be isolated from sterile surface or internal parts of plants (Hallmann *et al*., 1997) from both monocotyledonous and dicotyledonous plants (Ryan *et al*., 2008).

Endophytes enter the plant through the natural vents and wounds, or by secretion of hydrolytic enzymes (Hallmann *et al*., 1997). They subsequently establish closer interaction with the host by increasing access to nutrients such as nitrogen, phosphorus and iron, or by inducing plant defense mechanisms (production of anti-pathogen agents, suppression of pathogens through competition for the colonization of places and food) and can potentially positively affect plant health and protection (Kang *et al*., 2007; Reinhold-Hurek and Hurek, 2011; Santoyo *et al*., 2016; Moronta-Barrios *et al*., 2017).

Several reports have highlighted the benefits of using endophytic bacteria as biocontrol agents against fungal and bacterial rice pathogens. *Burkholderia* sp. KJ006, for example, was used against *Burkholderia glumae* (Cho *et al*., 2007) and *Rhizobium leguminosarum* bv. *phaseoli* against *Rhizoctonia solani* (Mishra *et al*., 2006). Similarly, other endophytic bacteria have been used to protect rice cultivars from major fungal infections including *Penibacillus* spp., *Microbacterium* spp., *Bacillus* spp. and *Klebsiella* spp. against *Fusarium oxysporum* and *Rhizoctonia solani* (Ji *et al*., 2014). Recently certain potential biological control agents such as *Bacillus amyloliquefaciens*, *B. methylotrophicus* and *B. subtilis* were isolated from rice plants showing the highest antimicrobial activities against the two major rice pathogens, *Rhizoctonia solani* and *Burkholderia glumae*, which cause sheath blight and bacterial panicle blight, respectively (Shrestha *et al*., 2016). For these and other reasons, nowadays endophytes are increasingly receiving attention as potential agents in the biologi-

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cal control of plant pathogens as well as for the improvement of plant growth.

Rice is the staple food of more than half of the world's population. It is considered the oldest and the most important crop throughout the world, especially in Asia. Considerable agricultural areas are under cultivation of rice in the world.

Bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a devastating pathogen in rice, especially in Asian countries. It reduces rice production by 20% in natural conditions and by 70% in epidemic situations, being one of the factors that limit the global production of rice (Ou,1985). For the control of the pathogen, plant disease resistance (R) genes are used to generate new disease-resistant cultivars (Peng *et al*., 2015) and isolation of potent endophytes as new sources for biological control of plant pathogens is under investigation.

The aim of the present study was to isolate and identify certain endophytic bacteria from commonly used rice cultivars in northern Iran, in order to evaluate their effect as biocontrol agents against rice bacterial blight caused by *Xoo*.

Materials and methods

Isolation of endophytic bacteria from rice plants

Samples were collected from eight rice cultivars cultivated in regions under rice plantation in the north of Iran. They were washed with tap water to separate soil particles from the root surface. Disinfection followed by using 2% hypochlorite sodium for 10 minutes and 70% alcohol for 1 minute. The plant samples were then rinsed four times with 0.02 M phosphate potassium buffer (pH=7). To ensure the effectiveness of disinfectants, a 0.1 ml buffer from the final wash was added to 9.9 ml Tryptic soy agar (TSA) as control. Any growth in the control plates within 48 hours, these were eliminated. The root, stem and leaf samples were then cut into small pieces via a sterile blade. One gram of sample tissue with 9.9 ml phosphate buffer was squashed in a sterile mortar. The solution was diluted serially in the buffer and cultured on a TSA medium in a Petri dish. Single colonies were selected and purified on fresh TSA plates (McInroy and Kloepper, 1995).

Preliminary diagnosis of bacterial pathogenic isolates

To determine the pathogenic properties of 63 isolates, appropriate suspensions of yellow colonies and fluorescent bacteria were prepared and the inocula were infiltrated into tobacco leaves to fulfill HR development. To confirm pathogenicity tests on rice plants cv. Hashemi, two pathogenicity tests were conducted simultaneously (Zou *et al*., 2006; Shrestha *et al*., 2016; Jabeen *et al*., 2011).

In the first experiment, the leaves were cut off and surface-sterilized with sodium hypochlorite solution for 1 minute and then washed with ddH_2O. The sterilized leaves were then placed on a three-layer paper towel in Petri dishes and inoculated with suspensions of representative isolates with 10^8 cfu/ml concentration using the pin prick method. All treated leaves including water treated control leaves were inoculated at 25°C. The test was conducted in three replications for each treatment.

In the second experiment, one-month-old rice plants were inoculated with bacteria at a concentration of 10^8 cfu/ml from fresh cultures by the leaf-clipping method (Kauffman *et al*., 1973). The treated plants were transferred to the greenhouse at 25-30°C with a 12- hr exposure period. They were observed every day within one month for any disease symptoms. The control plants were inoculated with sterile phosphate buffer. Experiments were performed in three replicates for each treatment.

Phenotypic characteristics of the pathogenic isolates

Pathogenic isolates were studied by a series of key phenotypic tests, including physiological and biochemical tests, in terms of the determination of genus and species

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(Schaad *et al*., 2001).

In vitro assay for antagonistic properties of isolates

To carry out the tests for antagonistic properties of isolates, *Xoo*-strain (MT) was used. From a 24-48 hour culture of the strain on a TSA medium, a suspension was prepared in sterile distilled water and its optical density (OD_{600}) was adjusted 1. Isolates with colonies other than yellow colours, all positive fluorescent bacteria and saprophytes with no HR reaction, were spot-inocuated on TSA medium and incubated for three days at 25°C. Then, *Xoo* suspension at 10^8 cfu/ml concentration was sprayed uniformly on TSA agar medium. The plates were incubated at 25°C, and inhibition zones were measured. This was done in triplicates.

Germination and vigour index of inoculated rice seeds with endophytic bacteria

The test was conducted on the basis of the standard method of roll towel (ISTA, 1993). At first, 20 surface sterilized seeds were treated in 3×10^8 cfu/ml concentration of each isolate and each batch was placed separately between two plies of a wet tissue and the seeds were a slightly compressed. The tissues containing the treated seeds were then placed separately in plastic bags which were rolled and kept in a growth chamber for 14 days. Three replications were considered for each treatment. The percentage of seed germination, and the mean root and shoot length of each seedling were indices evaluated for the growth-inducing activity of isolates. To calculate the vigour index of the seedling, the following method was used (Baki and Anderson, 1973):

Vigour index = % germination × seedling length
Seedling length = (shoot length + root length)

Evaluation of plant growth promotion of endophytic bacteria on rice in greenhouse conditions

An assessment of the growth improvement activity of 17 selected isolates from the Vigour index test was done according to Chithrashree *et al*. (2011). Seeds treated with fresh suspensions of antagonistic isolates (3×10^8 cfu/ml) and untreated control seeds were separately planted in pots filled with sterile soil and sand in equal volumes. The pots were irrigated daily and each pot received 25 ml of Hoagland 1/3 (V/V) solution once a week. Within 30 days after planting, the plant's growth promotion activities such as plant height and fresh weight of seedlings were measured. Each treatment consisted of four replicates (Chithrashree *et al*., 2011).

Evaluation of the effect of endophytic-antagonistic bacteria against *Xoo* in greenhouse conditions

The rice seeds were kept in sterile distilled water for 48 hours after surface disinfection with 2% hypochlorite sodium for 30 seconds. Then, the extra water of the seeds was removed and air-dried in the shade for half an hour. Six seeds were planted in vases containing sterile soil. Each cellular suspension of antagonistic bacteria was used separately in greenhouse during plantation. The one-month plants with suspension of *Xoo* (10^8 cells/ml) were inoculated using the leaf clipping method (Kauffman *et al*., 1973). The test was done in the form of a complete random design in three replications. The control plants were inoculated with sterile distilled water. The length of lesions on leaves of each plant was measured separately after 45 days of inoculation with the pathogen, based on a five-point scale: 0=no sign of diseases and lesion length less than 0.2 cm, 1=lesion length between 0.2 and 1.5 cm, 3=lesion length between 1.5 and 3 cm, 5=between 3 and 5 cm, 7=between 5 and 10 cm, 9=above 10 cm. The percentage disease index was calculated by the following formula (Fang *et al*., 1999):

$$\text{Disease index (\%)} = \{(1\times N_1 + 3\times N_3 + 5\times N_5 + 7\times N_7 + 9\times N_9) / 9Nt\} \times 100$$

where $N_{1\text{-}9}$ is the number of the leaf index and Nt is the total number of tested leaves.

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In this test, growth factors such as fresh weight of root and shoots were measured.

Bacterial identification using 16S rRNA gene sequencing

DNA Extraction

To extract DNA of isolates, the boiling method was used. Bacterial cultures were obtained in 5 ml Nutrient broth (Nb) medium. After keeping it in 37°C incubator for 20 hours, 1ml from each culture was transfered into sterile Eppendorf tube, and centrifuged in 13000 rpm for 5 minutes. The supernatants were discharged and pellets were resuspended by vortexing with 300 µl lysis buffer containing 0.1 N NaOH and 0.5% SDS (Elboutahiri *et al*., 2009). The tubes were placed in a boiling bain-marie for 10-13 minutes and immediately were placed on the ice for one minute. The tubes were centrifuged in 13000 rpm for 5 minutes and the supernatants contain suspended DNA was transferred into new tubes for PCR reaction.

PCR amplification

The amplification of 16S rDNA was carried out in a reaction with a final volume of 25 µl containing 2.5 µl of 10x PCR buffer, 1 µl of $MgCl_2$, 0.5 µl of dNTP (2.5 mM) (Bio-Rad), 1 µl (100 ng/µl) of the P1 primer (5'-CGGGATCCAGAGTTTGATCCTGGTCAGAACGAACGCT -3'), 1 µl (100 ng/µl) of the P6 primer (5'-CGGGATCCTACGGCTACCTTGTTACGACTTCACCCC -3') (Palacio-Bielsa *et al*. 2009), 0.3 µl of Taq DNA polymerase, 2 µl of total DNA and 16.7 µl of sterile distilled H_2O. The PCR reaction conditions were as follows: 94°C for 2 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min and extension at 72°C for 3 min, before a final elongation at 72°C for 20 min.

Statistical analysis

The results of the experiments were analyzed independently. The treatment means were compared by Duncan's multiple range test. The statistical software SAS version 92.1 was used for the statistical analyses.

Results

Isolation, identification and characterization of endophytic bacteria

Sixty three (63) culturable bacterial endophytes were isolated from internal tissues of the rice plants collected from different parts of North of Iran. Yellow and fluorescent colonies HR positive were tested for HR and pathogenicity tests on rice plants (Table 1).

Pathogenicity test

Of the 63 endophytic bacterial isolates, 6 isolates had positive HR reaction on tobacco leaves. Five isolates namely, OS6, OS14, OS18, OS22 and OS36, caused disease symptoms on inoculated rice plants. Some of these were considered as pathogens with low disease potential. All pathogenic strains in the detached leaf assay showed a typical elongated water soaked lesion which developed into a brownish black necrosis. These were recorded 48-72 h post-inoculation (hpi). In the pot experiment all plants inoculated with pathogenic endophyte strains showed progressive water-soaking lesions in the inoculated point after 2-4 days. The lesions became necrotic and produced a necrotic stripe on the leaf that spread to the full length of the leaf. The isolates with low disease potential produced confined dark brown spots on the inoculated region. Koch's postulates were fulfilled for all pathogenic strains.

Phenotypic properties of the pathogenic isolates

The tested endophytes isolates were divided into two groups; yellow colonies up to fluorescent pigmentation on king's B medium and the non-fluorescent, and the white colonies group. As presented in Table 2, five strains were tentatively identified based on morphological and various biochemical characteristics. Two of the strains namely, OS6 and OS36, were designated as *Pseudomonas* spp. Two other strains (OS14 and OS18) were identified as *Pantoea* spp., and the strain OS22 could not be assigned

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Table 1. Overall characteristics of endophytic bacteria isolated from rice cultivars.

Cultivar	Tissue origin	Pathogenicity in rice	Strain number	Colony color on TSA
Hashemi	10^{-3} Leaf	-+	OS4, OS18	yellow
	10^{-4} Leaf	++-	OS6, OS14, OS46	yellow
	10^{-5} Leaf	--	OS15, OS16	yellow
	10^{-3} Root	-	OS2, OS19	yellow
Tarom amrollahi	10^{-5} Leaf	-	OS7	colorless
	10^{-6} Leaf	-	OS17	yellow
Kouhsar	10^{-2} Leaf	+	OS22	yellow
	10^{-4} Leaf	-	OS38	yellow
	10^{-2} Root	-	OS20	colorless
Binam	10^{-3} Leaf	+	OS36	yellow
	10^{-4} Leaf	-	OS25	yellow
	10^{-2} Root	-	OS28	white
	10^{-4} Root	-	OS49	creamy
Amol	10^{-2} Stem	-	OS31	colorless
	10^{-2} Root	-	OS29	yellow
	10^{-3} Root	-	OS30	yellow
Fajr	10^{-3} Root	--	OS32, OS34	yellow
	10^{-4} Leaf	-	OS33	yellow
Khazar	10^{-3} Root	-	OS42	yellow
	10^{-3} Root	-	OS43	brown
Tarom hashemi	10^{-3} Root	-	OS53	colorless
	10^{-3} Leaf	-	OS60	yellow
Tarom mahalli	10^{-4} Root	--	OS61, OS62	yellow
	10^{-5} Root	-	OS63	yellow

Table 2. The phenotypic characteristics of five main endophytic bacteria with low disease potential isolated from rice plants.

Test	OS6	OS14	OS18	OS22	OS36
Yellow pigment	+	+	+	+	+
Gram staining	-	-	-	+	-
Oxidase test	-	-	-	+	-
Fermentative	No	Yes	Yes	No	No
Fluorescence on King's B medium	-	-	-	-	-
Starch hydrolysis	-	-	-	-	-
Catalase hydrolysis	+	+	+	+	+
Litmus milk	ALK	ND	ND	-	ALK
Levan from sucrose	+	-	-	-	+
H_2S from cysteine	-	-	-	-	-
Xanthomondin	-	-	-	-	-
Mucoid growth on YDC	-	+	+	+	-
Growth on 0.1% TTC	+	+	+	+	+
Growth at 40°C	-	+	+		-
HR on Tobacco	+	+	+	+	+
Pathogenicity on rice	+	+	+	+	+

+= positive; -= negative; O= oxidative; v= variable; ALK= alkaline; NC= no change; ND= not determined.

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to a known species (Table 2).

In vitro assay for antagonistic activities of isolates to *X. oryzae* pv. *oryzae*

In the initial screening of the antagonistic activity of 39 bacterial isolates against *Xoo* strain, 21 bacterial isolates exhibited the most potent antagonistic activity against the *Xoo*. The maximum inhibitory activity was recorded for OS59 with an inhibition zone of 32.67 mm, whereas the isolates OS58, OS43, OS20 and OS40 could produce inhibition zones of 32.33 mm, 26.65 mm, 18 mm and 16.7mm, respectively (Fig.1). The selected isolates were consequently used for further experimentation.

Germination and vigour index of inoculated rice seeds with endophytic bacteria

In total, 17 bacterial strains selected from the in vitro assays, namely, OS3, OS10, OS12, OS20, OS21, OS23, OS28, OS31, OS40, OS43, OS44, OS49, OS52, OS53, OS55, OS58 and OS59, were tested. The treatment of seeds with some of these strains significantly increased the seed germination and seedling vigour in comparison to the untreated control. Of these, 6 isolates namely, OS40, OS23, OS43, OS52, OS31 and OS53, had statistically significant effects compared to the control (Table 3). Further tests were carried out to evaluate their potential in promoting plant growth.

Effect of endophytic bacteria on growth promotion of rice in greenhouse conditions

Under greenhouse conditions, of the 17 isolates tested, 41.176% of the isolates caused a significant difference in growth parameters compared to the control plants. Seed treatments with OS3, OS23, OS31, OS40, OS53, OS58 and OS59 isolates increased seedling height and fresh weight in comparison with the control. The highest heights of 23.7, 22.81, 21.9 cm were obtained from seeds treated with the OS58, OS40 and OS31 isolates. The application of the OS53, OS31, and OS59 isolates also resulted in the highest seedling weights of 0.893, 0.806 and 0.6662 grams (Fig. 2).

Evaluation of the effect of endophytic-antagonistic bacteria against *Xoo* in greenhouse condition

Seventeen (17) representative antagonistic bacteria with potential plant growth promotion activities were assessed for the control of bacterial blight disease 60 days after the treatment of rice seeds with endophytic bacteria. As shown in Fig. 3, the plants with bacterial inoculants of OS52, OS40, OS23 and OS53, reduced the incidence of the disease by 28.5%, 33.25%, 34.25% and 37.25%, respectively (Fig. 3). Also in presence of the pathogen *Xoo*, root and shoot fresh weight of the rice plants inoculated with the mentioned isolates showed significantly increased values of these parameters compared to the control treatment (Fig. 4). This indicates their function as biological control agents of *Xoo* causing bacterial blight in rice.

PCR test for identification of representative bacteria

To identify the four most efficient antagonistic endophytes (OS52, OS40, OS23 and OS53), the extracted DNA of these isolates were amplified using universal 16S rDNA primers (P1 and P6) and a PCR product size of 1500 bp was obtained. *The sequencing* samples were sent to Macrogene Inc. (Seoul, South Korea). The sequence results were compared with those available in the NCBI GenBank. It was shown that the isolates OS23, OS40, OS52 and OS53 with 97%, 90%, 95% and 98% identities belonged to *Bacillus* sp., *B. subtilis*, *Enterobacter* sp. and *Pseudomonas putida*, respectively (Fig. 5). However, 2 species were indistinguishable by *16S rRNA* gene sequences.

Discussion

Eight cultivars of rice were screened for beneficial endophytic bacteria in the provinces of Mazandaran and Gilan in northern Iran during two seasons in the year 2016. Sixty three endophytes were isolated from differ-

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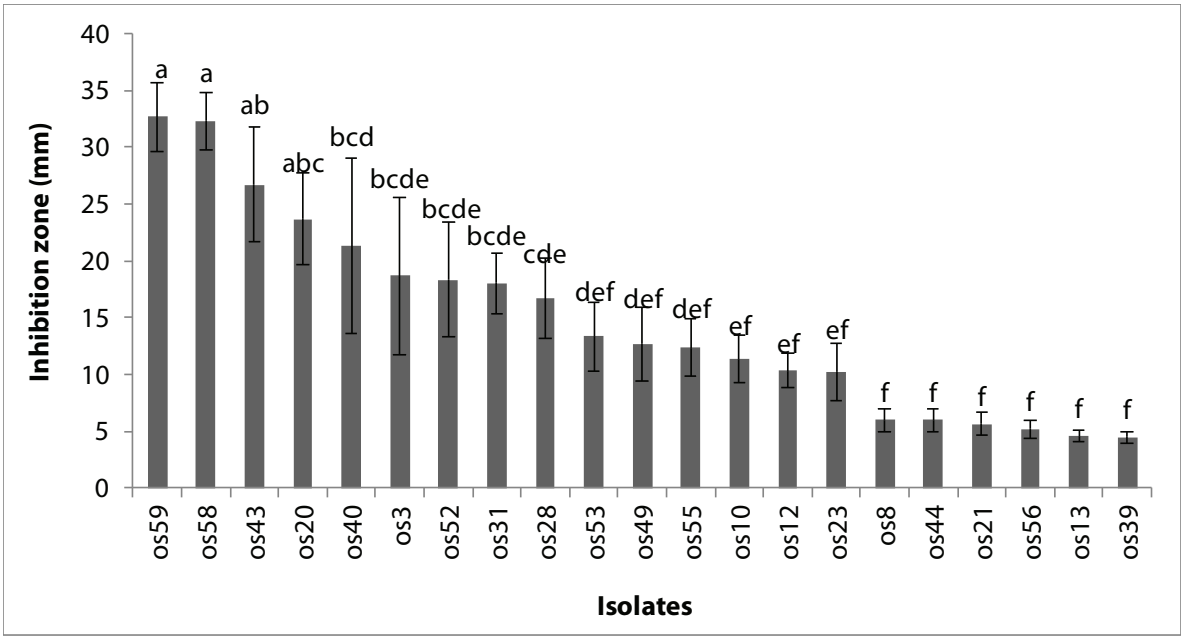

Figure 1. Comparative antagonistic potential of 21 endophytic bacteria against *Xanthomonas oryzae* pv. *oryzae* in rice.
*Data are mean of three replications; in a column, means followed by a common letter are not significantly different at 5% level by Duncan's Multiple Range Test.

Table 3. Plant growth promoting activity of endophytic bacterial isolates in rice plants.

Isolates	MRL(cm)	MSL(cm)	Germination (%)	VI*
OS3	2.72 ± 0.60^{bcd}	5.57 ± 0.51^{ef}	100	8.16 ± 0.44^{fg}
OS10	2.59 ± 0.13^{cde}	4.69 ± 0.45^{g}	96	7.12 ± 1.00^{h}
OS12	2.40 ± 0.26^{de}	5.78 ± 0.62^{cdef}	98	8.01 ± 0.77^{fgh}
OS20	3.12 ± 0.21^{b}	5.41 ± 0.25^{f}	100	7.55 ± 0.22^{gh}
OS21	2.17 ± 0.40^{de}	4.00 ± 0.27^{gh}	100	4.90 ± 0.36^{i}
OS23	4.19 ± 0.32^{a}	6.45 ± 0.47^{abcd}	100	11.01 ± 0.77^{ab}
OS28	2.4067 ± 0.1^{de}	3.37 ± 0.21^{h}	94	5.43 ± 0.21^{i}
OS31	2.52 ± 0.12^{de}	6.18 ± 0.13^{abcde}	100	9.64 ± 0.32^{bcd}
OS40	4.31 ± 0.25^{a}	6.51 ± 0.58^{abc}	100	11.28 ± 0.56^{a}
OS43	4.01 ± 0.10^{a}	6.73 ± 0.39^{a}	100	10.51 ± 0.38^{ab}
OS44	2.03 ± 0.17^{e}	3.63 ± 0.27^{h}	96	5.43 ± 0.44^{i}
OS49	2.56 ± 0.16^{cde}	6.13 ± 0.25^{abcdef}	100	8.66 ± 0.11^{def}
OS52	4.03 ± 0.25^{a}	5.95 ± 0.58^{bcdef}	100	10.03 ± 0.18^{bc}
OS53	3.15 ± 0.37^{b}	6.64 ± 0.41^{ab}	100	9.45 ± 0.66^{cd}
OS55	3.07 ± 0.26^{bc}	6.57 ± 0.53^{ab}	100	9.20 ± 0.27^{cde}
OS58	3.82 ± 0.26^{a}	5.70 ± 0.40^{def}	100	7.78 ± 0.42^{fgh}
OS59	2.55 ± 0.40^{cde}	5.40 ± 0.35^{f}	100	8.36 ± 0.94^{efg}
Co	2.02 ± 0.33^{e}	4.00 ± 0.21^{gh}	100	5.65 ± 0.64^{i}

MRL= Mean root length, MSL= Mean shoot length, VI= Vigour Index .
* Data are mean of three replications; in a column, means followed by a common letter are not significantly different at 5% level by Duncan's Multiple Range Test.

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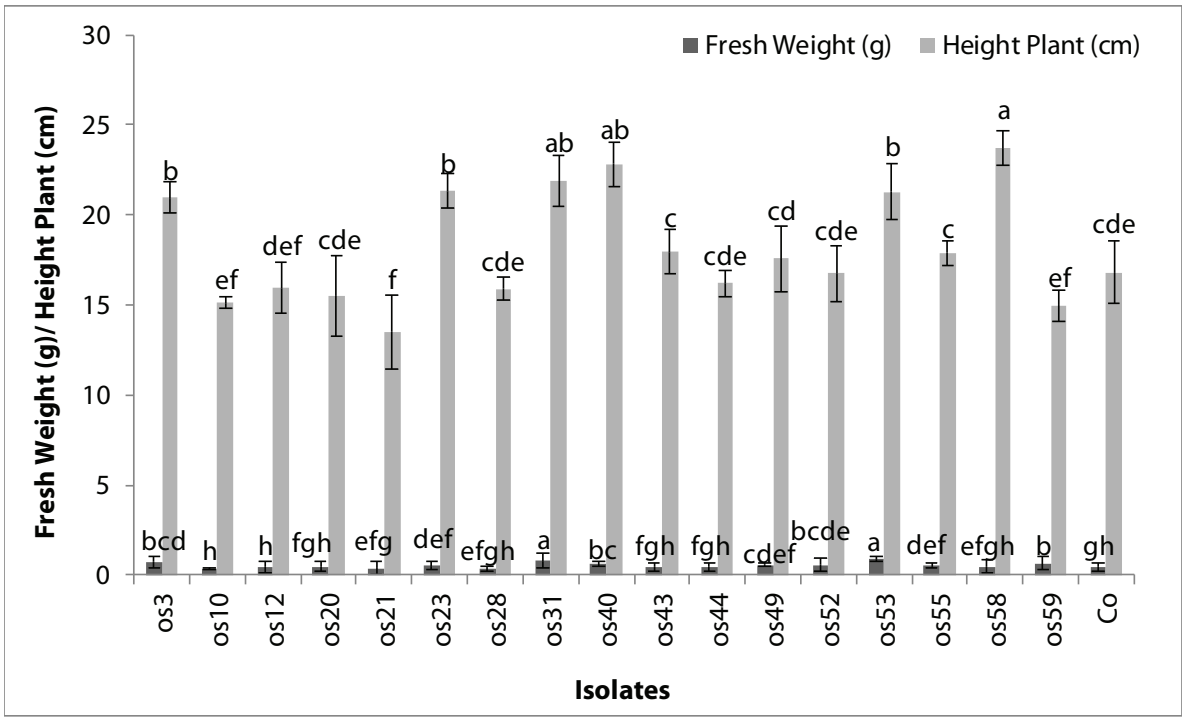

Figure 2. Effect of endophytic bacteria on growth promotion of rice seedlings 30 days after application in greenhouse conditions.
*Data are mean of four replications; in a column, means followed by a common letter are not significantly different at 5% level by Duncan's Multiple Range Test.

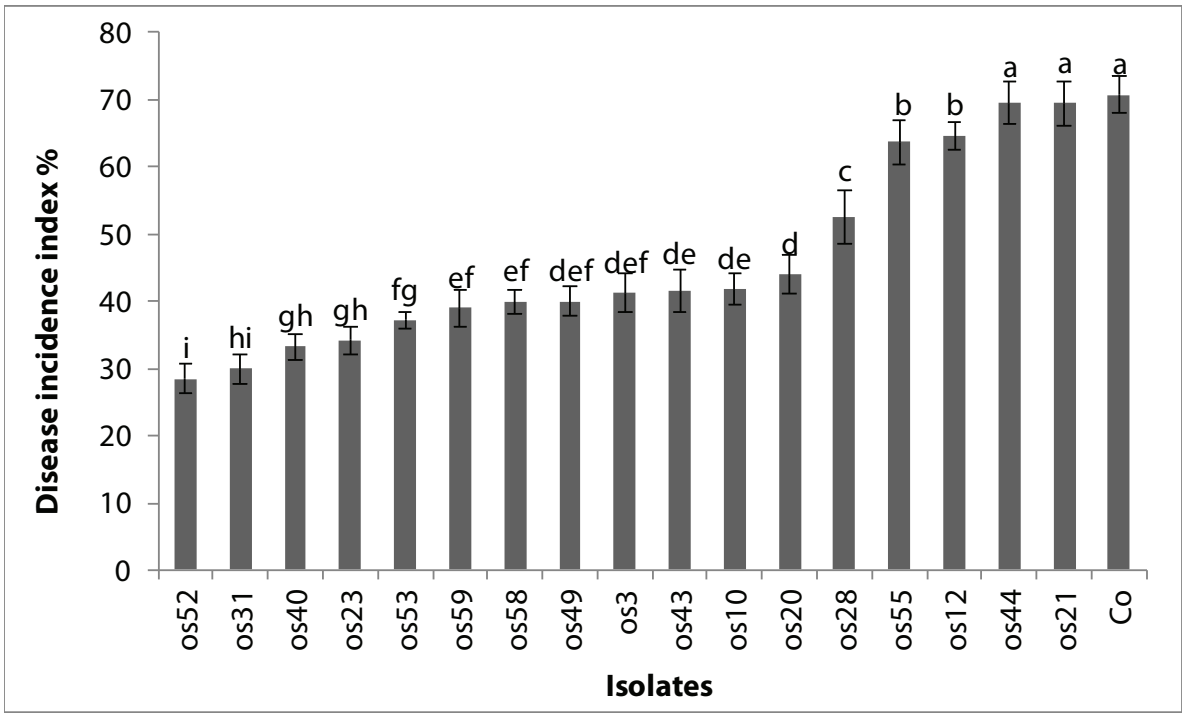

Figure 3. Control of bacterial blight of rice by endophytic bacteria under greenhouse conditions 60 days after treatment.
*Data are mean of four replications; in a column, means followed by a common letter are not significantly different at 5% level by Duncan's Multiple Range Test.

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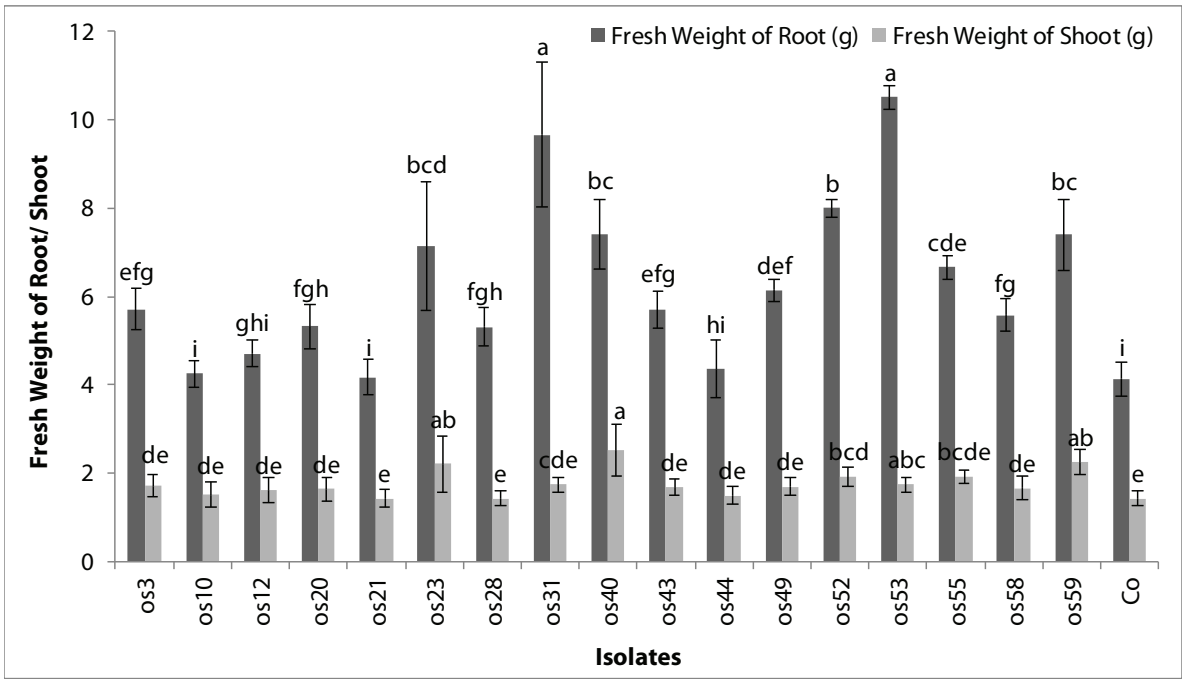

Figure 4. Different plant growth promoting activity of the representative endophytic bacteria on rice in greenhouse conditions and in presence of the pathogen *Xanthomonas oryzae* pv. *oryzae* in rice.
*Data are mean of four replications; in a column, means followed by a common letter are not significantly different at 5% level by Duncan's Multiple Range Test.

ent parts of the plants including the leaves, stem and roots.

Based on pathogenicity tests, five of the isolates were considered as potential pathogens of rice, which after 16s rDNA sequencing were identified as *Pseudomonas oryzihabitans* (99%), *Pantoea ananatis* (96%), *Pantoea* sp. (96%), *Cellulomonas* sp. (97%) and *Pseudomonas fulva* (97%). It is worth mentioning that neither of these bacteria have been previously reported as rice pathogens in Iran. The remaining isolates were considered non-pathogenic isolates.

Of the pathogenic isolates found on rice, some have already been reported on rice plants. *Pantoea ananatis* from Cambodia, and *Pantoea* spp. and *Pantoea stewartii* from the Philippines have been identified as pathogens with different pathogenic potential (Cother *et al*., 2010; Mano *et al*., 2006; Mano *et al*., 2007; Okunishi *et al*., 2005; Cottyn *et al*., 2001; Cottyn *et al*., 2009). *Pseudomonas oryzihabitans, Pseudomonas* spp. and *Cellulomonas flavigena* have been reported as seed saprophytes in irrigated areas with low pathogenic potential trait from the Philippines (Cottyn *et al*., 2001; Cottyn *et al*., 2009). Moreover, *Pseudomonas fulva* has been identified as a pathogen in pepper from China and as a diazotrophic endophyte in rice seeds (Qiang *et al*., 2017; Verma *et al*., 2001).

According to Cottyn *et al*. (2009), it can be assumed that under certain conditions of rice cultivation, these endophytes can eventually appear as pathogens. Generally, some endophytes may turn into pathogens depending on several factors such as: the host and endophyte growth stage, plant defensive responses, environmental changes such as CO_2 accumulation or O_2 depletion, the production of specific metabolites in another host, the presence of other microorganisms interacting with it (Lund and Wyatt, 1972; van Peer *et al*., 1990; Sturz *et al*., 1997; Schulz and Boyle, 2005; Rosenblueth and Martínez-Romero, 2006). Nevertheless, in artificial inoculation, factors such as inoculation procedure and high inoculum concentrations may increase pathogenicity (Cottyn *et al*., 2009).

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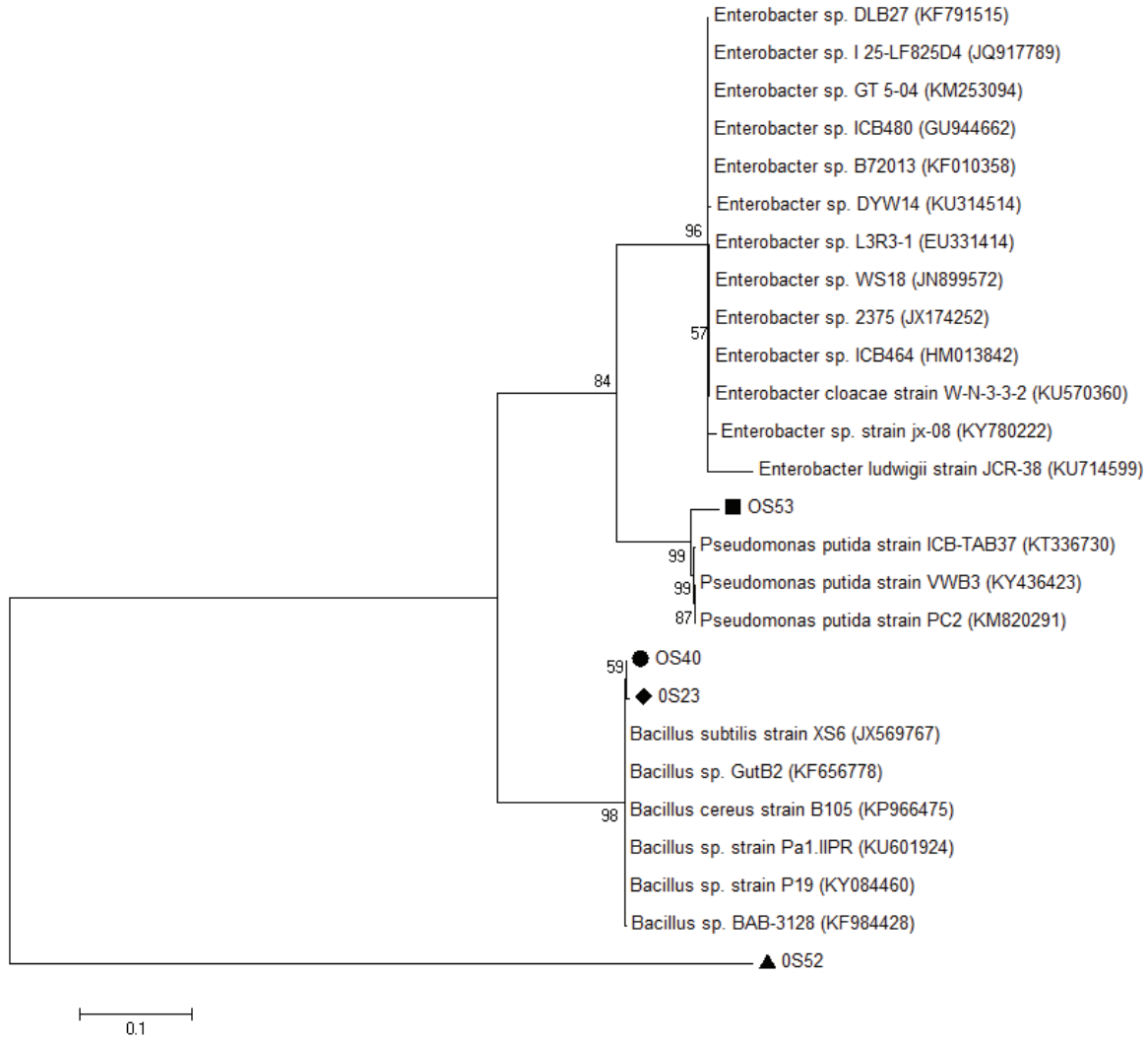

Figure 5. Phylogenetic tree based on the 16S rRNA gene of 4 beneficial endophytic bacteria and related bacterial species. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The numbers at the nodes are boot strap values based on 1000 replications. The analysis involved 26 nucleotide sequences. Evolutionary analyses were conducted in MEG 7 (Tamura *et al.*, 2013; Kumar *et al.*, 2016).

To select the best antagonists, screening of isolates was based on their *in vitro* antagonistic activity to *Xoo*, their effect on seed germination rate, seedling growth promotion and the reduction of disease intensity in greenhouse conditions. Four isolates, OS52, OS40, OS23 and OS53, which were identified by nucleotide sequence analysis as *Enterobacter* sp., *B. subtilis*, *Bacillus* sp. and *Pseudomonas putida*, respectively, could increase plant growth and decrease *Xoo* infection under greenhouse conditions. To the best of our knowledge, this is the first report of isolation of endophytes from rice in Iran. Seed treatment with these antagonists caused disease to cease in over 60% of plants. These species have been found as endophytes in rice among many other species including *Coryneform* spp., *Pantoea* spp., *Burkholderia glumae, Xanthomonas* spp., *Staphylococcus* spp., *Paenibacillus polymyxa, Methylobacterium* spp., *Curtobacterium* spp., *Azospirillum amazonense, Caulobacter crescentus, Kocuria palustris, Micrococcus luteus, Microbacterium* spp., *Klebsiella* spp., *Azospirillum* spp., *Herbaspirillum* spp., *Rhizobium* spp., *Sphingomonas* spp. and *Brevibacillus* spp. (Elbeltagy *et al.*, 2000; Okunishi *et al.*, 2005; Mano *et*

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al., 2007; Mano and Morisaki, 2008; Cottyn *et al*., 2009; Kaga *et al*., 2009; Ji *et al*., 2014; Nhu and Diep, 2014).

Several endophytic bacteria such as *Enterobacter* sp. CNPSo 2480, *Bacillus* sp. CNPSo 2481, *Pseudomonas rhodesiae*, *Pantoea ananatis*, *Pseudomonas* sp. PsJN, *H. rubrisubalbicans*, *G. diazotrophicus*, *A.amazonense* and *Burkholderia* sp. induce growth and increase in plants like corn, pepper, grape and sugarcane (Barka *et al*., 2002; Oliveira *et al*., 2003; Kang *et al*., 2007; Szilagyi-Zecchin *et al*., 2014). In addition, endophytic bacteria have been reported to be involved with other mechanisms such as competition for place and food, and antibiosis (Sturz *et al*., 2000; Reiter *et al*., 2002; Compant *et al*., 2005; Aravind *et al*., 2009; Ji *et al*., 2013). Especially in rice, Ji *et al*. (2013) found that treatment of rice seeds with the diazotrophic endophytes *Paenibacillus, Microbacterium*, *Bacillus* and *Klebsiella*, induced systemic resistance against *Fusarium oxysporum* and *Rhizoctonia solani* in addition to the growth induction of the rice plant.

In the present study, *B. subtilis* seemed to work well against *Xoo* as well as the other *Bacillus* spp., which need to be investigated further. Recent studies by Chung *et al*. (2015) and Hossain *et al*. (2016) showed that *Bacillus oryzicola*YC7007, a new endophytic bacterium isolated from rice roots, improves plant growth and controls bacterial blight, cluster blight and bakanae disease via the production of antibiotic and induction of systemic resistance. The application of an endophytic strain *Bacillus* spp. of tomato and potato with Silwet polysilicon surfactant caused colonization of cocoa leaves (for more than 68 days) and reduction of black pod rot disease *via* the induction of systemic resistance (Melnick *et al*., 2008).

The *Enterobacter* sp. isolate also has a potential beneficial effect on the control of bacterial blight. Of *Enterobacter* spp., *Enterobacter radicincitans* in cereals (Witzel *et al*., 2012), *Enterobacter* sp. SP1 in sugar cane (Zhu *et al*., 2012) and *Enterobacter cloacae* MSR1 in alfalfa (Khalifa *et al*., 2016) have shown an ability to increase production in plants. The production of plant hormones by the *Enterobacter* sp. 638 isolated from the stem of a type of poplar hybrid has led to a 40% increase in poplar growth (Taghavi *et al*., 2010). *Enterobacter cloacae* and *Enterobacter aerogenes* strains have been reported to control *Fusarium oxysporum* f. sp. *spinaciae* in spinach (Tsuda *et al*., 2001) and *Setosphaeria turcica*, the Northern corn leaf blight fungus, in maize (D'Alessandro *et al*., 2014). Biological control action of *E. cloacae* has also been reported by Roberts *et al*. (1994). Enterobacter isolates increased nodule occupancy of bradyrhizobial strains at nitrogen fixation on legumes (Gulpa *et al*., 1998).

The increase in the growth of rice plants by the activity of *P. putida* coincides with previous findings on endophytic *Pseudomonas* spp. e.g. nitrogen fixation by *Pseudomonas stuzeri* A1501 endophyte promoted plant growth (Yan *et al*., 2008). Sheoran *et al*. (2015) proved that the endophytic *P. putida* BP25, isolated from the black pepper root endosphere, could by internal colonization in Ginger and Arabidopsis plants induce production of volatile compounds that inhibit fungal pathogens and the plant parasitic nematode *Radopholus similis* (Sheoran *et al*., 2015).

Despite the interesting profile of *Enterobacter* sp. and *P. putida* as antagonists of *Xoo* on rice, these bacteria are potentially dangerous and harmful to human health (Brenner *et al*., 1986; Bouallegue *et al*., 2004; Berg *et al*., 2005; Flores-Tena *et al*., 2007; Molina *et al*., 2011; Thomas *et al*., 2013; Molina *et al*., 2014). Hence the hazardous properties of the bacteria and human safety issues should also be taken into account when considering their practical use as biological control agents in plant protection.

In conclusion, this is the first record of the pathogens *P. oryzihabitans*, *P. ananatis*, *Pantoea* sp., *Cellulomonas* sp. and *P. fulva* and the endophytic bacteria/ antagonists *Enterobacter* sp., *B. subtilis*, *Bacillus* sp. and *P. putida* against *Xoo* in rice plants from the northern parts of Iran, where rice is mostly cultivated. The beneficial bacteria showed promising efficacy and plant growth pro-

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motion activities against *Xoo* in rice both *in vitro* and in greenhouse conditions. Further research is required in the perspective of application of the present findings to the field.

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Ταυτοποίηση και προσδιορισμός χαρακτηριστικών ενδοφύτων σε φυτά ρυζιού και ο ρόλος τους στη βιολογική αντιμετώπιση του βακτηρίου *Xanthomonas oryzae* pv. *oryzae*

H. Yousefi, N. Hassanzadeh, K. Behboudi και F. Beiki Firouzjahi

Περίληψη Εξετάστηκαν απομονώσεις ενδοφυτικών βακτηρίων από φυτά ρυζιού (*Oryza sativa* L.) οκτώ διαφορετικών ποικιλιών ως προς την ικανότητά τους να επάγουν συμπτώματα ασθένειας στα φυτά, να ενισχύουν την ανάπτυξη των φυτών και την ανταγωνιστική δράση τους έναντι του βακτηρίου *Xanthomonas oryzae* pv. *oryzae*. Από τα 63 στελέχη, πέντε ήταν φυτοπαθογόνα. Με βάση τα φαινοτυπικά χαρακτηριστικά και την ανάλυση αλληλουχίας 16S rDNA, τα στελέχη αυτά ταυτοποιήθηκαν ως *Pseudomonas oryzihabitans*, *P. fulva, Pantoea ananatis, Pantoea* sp., *Cellulomonas* sp. Τέσσερις από τις 63 απομονώσεις έδειξαν ότι είναι δυνητικά καλοί παράγοντες επαγωγής της ανάπτυξης των φυτών και βιολογικής αντιμετώπισης. Τα στελέχη αυτά αναγνωρίστηκαν ως *Bacillus* sp., *B. subtilis*, *Pseudomonas putida* και *Enterobacter* sp. Αυτή είναι η πρώτη αναφορά παθογόνων ενδοφυτικών βακτηρίων σε καλλιεργούμενο ρύζι στο Βόρειο Ιράν.

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SHORT COMMUNICATION

Germination responses of *Ballota hirsuta* seeds under conditions of temperature, salinity and water stress

M. Dadach[1]* and Z. Mehdadi[2]

Summary Temperature, salinity and water deficit can be major environmental constraints which reduce distribution of indigenous plants in the Mediterranean region. Laboratory experiments were carried out to assess the effect of temperature, sodium chloride (NaCl) and polyethylene glycol 6000 (PEG) on germination of *Ballota hirsuta* seeds. The germination responses of seeds were determined over a wide range of temperature (5 to 35°C), salinity (0 to 136 mM NaCl) and water stress (0 to -1 MPa PEG). Germination percentage was optimum at 20°C (78%), and showed a decline at lower (5°C, 25%) or higher (30°C, 18%) temperature values and total inhibition of germination at 35°C. Under salinity and water stress conditions, there was a significant deterioration in most germination parameters such as lower final germination percentage, increased mean germination times and lower germination rates.

Additional keywords: *Ballota hirsuta*, NaCl, PEG, salt stress, seeds, water deficit

Introduction

Ballota hirsuta L. (Lamiaceae) is a Mediterranean wild shrub that is mainly used in traditional medicine (Kechar *et al.,* 2016). Aqueous plant extract of *B. hirsuta* is known to inhibit growth in larvae of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) (Pascual-Villalobos and Robledo, 1999). *Ballota hirsuta* is currently under biotic and abiotic pressures such as overexploitation, global warming, soil salinity and drought (Underwood *et al*., 2009). Knowledge on the effects of the environmental conditions on seed germination would assist to carry out reintroduction and conservation programs of existing populations in Tessala mount, Algeria.

Several environmental factors such as temperature, light, pH, and soil moisture are known to affect seed germination (El-Keblawy and Al-Rawai, 2006). Amongst those, temperature is the most prominent factor regulating germination and establishment of plants (Koger *et al*., 2004). Use of cardinal temperatures makes it possible to estimate geographical limitations for seed germination and select the most suitable time for plant establishment (Ramin, 1997).

Soil salinity is a major problem limiting plant distribution and productivity. Therefore, tolerance to salinity during germination is critical for the establishment of plants growing in arid and semi-arid regions (Khan and Gulzar, 2003). In such salinity conditions, seed germination would be successful only after high rainfall precipitation as soil salinity is usually reduced due to leaching (Redondo-Gomez *et al*., 2007).

Water deficit is an important constraint disrupting plant production worldwide (Kaya *et al*., 2006). Under drought stress, seed germination is inhibited due to low water potential that results in a decline in water uptake (Farooq *et al*., 2009). A common methodology to measure the water stress effects on seed germination is the application of polyethylene glycol (PEG) as an osmotic medium (Michael and Kaufman, 1973).

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The objective of this study was to determine the effect of temperature, osmotic stress, and salt stress on seed germination patterns of *B. hirsuta*.

Materials and methods

Seed collection site and germination experiments

Mature seeds were collected from plants of *B. hirsuta* growing in Tessala mounts located in North-Western Algeria. The climate in this region is semi-arid with typical Mediterranean characteristics (average annual rainfall is between 290 and 420 mm and average monthly temperatures are between 9.4-26.6°C).

Intact seeds were surface sterilized by sodium hypochlorite solution (0.58%) for 5 minutes, clean washed with distilled water and left to dry out. Germination studies were conducted on seeds placed on two disks of filter papers Whatman No. 1 in Petri dishes. The experiments were conducted in darkness. For the test of temperature effect, seeds were incubated at cardinal temperatures of 5, 10, 15, 20, 25, 30 and 35°C, moistened with distilled water. Tests on salinity and water deficit stress were conducted under optimum seed germination temperature conditions (20°C). In particular, seed germination was tested at saline concentrations of 0, 2, 4, 6 and 8 g/l NaCl, which were transformed to molarity (M), and at PEG solutions of 0, -0.03, -0.07, -0.2, -0.5 and -1 MPa. Three replicates of 20 seeds per treatment were used. Germinated seeds were counted every two days up until the final germination.

Methods of germination expression and statistical analysis

The following seed germination parameters were determined: Final germination percentage (FGP), initial germination day (IGD), final germination day (FGD), mean time of germination (MTG) and germination rate (GR).

MTG was calculated as follows:

$MTG = \Sigma i\ (n_i \times d_i)/N$ (Redondo-Gomez *et al.*, 2007).

Where:

n_i : number of seeds germinated at day i,

d_i : incubation period in days,

N : total number of germinated seeds.

GR is defined as days needed to reach 50% of final germination percentage (Farooq *et al.*, 2005) and it was calculated as follows:

$$GR = \frac{[(N/2)-N_1) \times (T_2-T_1)]}{N_2-N_1} + T_1$$

Where:

N : total number of germinated seeds,

N_1 : number of germinated seeds slightly less than $N/2$,

N_2 : number of germinated seeds slightly higher than $N/2$,

T_1 : incubation period corresponding to N_1,

T_2 : incubation period corresponding to N_2.

Analysis of variance (ANOVA) was carried out to test effects of the main factors. Duncan test was used to estimate least significant difference between means. Data were analyzed using SPSS for windows, version 20.

Results

Regarding the effect of temperature on seed germination, seeds of *B. hirsuta* can germinate at temperatures between 5°C and 30°C, however germination percentages are low (25% and 18%, respectively). The optimal temperature was 20°C (78% germination) (Table 1). At the optimum temperature for germination, the shortest mean time of germination (MTG = 10.8 days) and initial germination day (IGD = 5.4 days) were recorded, although not statistically significantly different to those of higher temperatures. At high temperatures (30°C), the shortest final germination day (FGD = 13 days) was measured. The rate of germination, decreased at temperatures over 20°C.

Salt stress significantly inhibited germination of *B. hirsuta* seeds (Table 2). The highest germination percentage (FGP = 72%)

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was measured in distilled water. The germination percentage decreased significantly as salinity increased (at 102 mM NaCl, 7% germination) with total inhibition of germination at 136 mM NaCl. The initial germination day and final germination day were significantly delayed at 102 mM NaCl (0 mM, 6 days vs 102 mM, 10.4 days for IGD, and 0 mM, 17.6 days vs. 102 mM, 11.5 days for FGD). Germination rate declined significantly when salinity increased to 68 mM NaCl (0 mM, 11 days vs. 68 mM, 8.84 days).

Water stress significantly inhibited germination of *B. hirsuta* seeds (Table 3). Control showed the maximum germination percentage (FGP = 72 %) (although statistically significantly higher only compared to that at -0.5 MPa PEG), the shortest mean time of germination (MTG = 10.8 days) and the quickest initial and final germination day (IGD = 5.6 days, FGD = 16.6 days) (although not statistically significantly different to the MTG and IGD values of the -0.03 MPa PEG). The germination percentage decreased significantly when water stress increased at -0.5 MPa PEG (FGP = 23% germination) with total inhibition of germination at -1.0 MPa PEG. Initial germination day was delayed when PEG was higher than -0.07 MPa and germination rate increased at PEG values higher than -0.07 MPa, compared to the control (0 MPa, 9.64 days vs -0.2 MPa, 14.44 days).

Table 1. Germination parameters of *Ballota hirsuta* seeds in response to temperature (mean ± SE, n = 3): initial germination day IGD (days); final germination day FGD (days); final germination percentage FGP (%); mean time of germination MTG (days); germination rate GR (days).

Temperatures (°C)	IGD (days)	FGD (days)	FGP (%)	MTG (days)	GR (days)
5	12.6 ± 1.2^{a}	18.7 ± 1.2^{a}	25 ± 4^{a}	17.6 ± 1.8^{a}	15.14 ± 0.9^{a}
10	11.4 ± 0.4^{a}	18.4 ± 0.4^{a}	53 ± 8^{b}	17.2 ± 3.2^{ab}	14.38 ± 0.6^{a}
15	7.6 ± 0.8^{b}	18.2 ± 1.2^{a}	68 ± 5^{bc}	14.8 ± 1.4^{abc}	14.75 ± 1.1^{a}
20	5.4 ± 0.4^{c}	16.0 ± 0.8^{b}	78 ± 4^{c}	10.8 ± 1.2^{c}	10.30 ± 1.4^{bc}
25	5.8 ± 0.5^{bc}	18.6 ± 0.6^{a}	57 ± 7^{b}	12.2 ± 1.2^{bc}	11.22 ± 1.6^{b}
30	6.4 ± 0.6^{bc}	13.0 ± 0.8^{b}	18 ± 4^{a}	12.5 ± 0.8^{bc}	8.50 ± 0.0^{c}
35	-	-	-	-	-
F-value	58.31	6.82	71.70	6.70	19.63

[1] Different lower case letters (column) show significant differences between the means (P ≤ 0.05) according to Duncan's Multiple Range test.

Table 2. Germination parameters of *Ballota hirsuta* seeds, incubated at 20°C, in response to salinity (mean ± SE, n = 3): initial germination day IGD (days); final germination day FGD (days); final germination percentage FGP (%); mean time of germination MTG (days); germination rate GR (days).

NaCl (mM)	Parameters				
	IGD (days)	FGD (days)	FGP (%)	MTG (days)	GR (days)
0	6.0 ± 0.8^{a}	17.6 ± 1.8^{ab}	72 ± 5^{a}	11.6 ± 0.6^{a}	11.0 ± 0.9^{ab}
34	7.4 ± 0.4^{a}	19.0 ± 2.6^{a}	40 ± 8^{b}	13.2 ± 1.5^{a}	12.5 ± 2.8^{a}
68	7.6 ± 0.8^{a}	14.6 ± 1.4^{bc}	25 ± 7^{bc}	10.4 ± 0.8^{a}	8.84 ± 1.1^{b}
102	10.4 ± 1.4^{b}	11.5 ± 1.6^{c}	7 ± 3^{cd}	9.6 ± 1.2^{a}	9.67 ± 1.0^{ab}
136	-	-	-	-	-
F- value	19.77	15.66	38.19	4.11	2.83

[1] Different lower case letters (column) show significant differences between the means (P ≤ 0.05) according to Duncan's Multiple Range test.

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Table 3. Germination parameters of *Ballota hirsuta* seeds, incubated at 20 °C, in response to water stress (mean ± SE, n = 3): initial germination day IGD (days); final germination day FGD (days); final germination percentage FGP (%); mean time of germination MTG (days); germination rate GR (days).

PEG (MPa)	IGD (days)	FGD (days)	FGP (%)	MTG (days)	GR (days)
0	5.6 ± 0.5^{a}	16.6 ± 2.2^{a}	72 ± 9^{a}	10.8 ± 0.8^{a}	9.64 ± 0.3^{a}
-0.03	6.6 ± 0.8^{ab}	20.6 ± 1.4^{bc}	68 ± 10^{a}	12.6 ± 1.2^{ab}	11.92 ± 1.3^{ab}
-0.07	7.8 ± 1.2^{bc}	22.8 ± 0.8^{c}	62 ± 8^{a}	13.4 ± 1.5^{bc}	12.92 ± 2.3^{bc}
-0.2	8.6 ± 0.8^{c}	24.2 ± 1.2^{c}	55 ± 4^{a}	15.8 ± 2.2^{cd}	14.44 ± 1.2^{c}
-0.5	9.0 ± 1.5^{c}	17.8 ± 0.6^{ab}	23 ± 5^{b}	16.2 ± 1.8^{d}	11.37 ± 0.3^{ab}
-1	-	-	-	-	-
F- value	12.35	17.21	26.50	15.50	5.65

[1] Different lower case letters (column) show significant differences between the means ($P \leq 0.05$) according to Duncan's Multiple Range test.

Discussion

Germination patterns of *B. hirsuta* are similar to that of some other medicinal plants that could maintain a relatively high germination percentage over a wide range of temperatures (Bannayan *et al*., 2006). In our study, it was shown that the best germination patterns (maximum final germination percentage (FPG), the shortest mean time of germination (MTG) and initial germination day (IGD)) were measured at temperature range between 10 to 25°C with the optimum temperature at 20°C. This specific thermal optimum also characterizes other Mediterranean species of arid and semi-arid regions belonging to the Lamiaceae family such as *Lavandula dentata*, *Teucrium gnaphalodes*, *Thymbra capitata* and *Thymus hyemalis* (Estrelles *et al*., 1999; Kadis and Georghiou, 2010). Additionally, Corme (1993) reported that seeds of *Salvia officinalis* and *Salvia sclarea* (Lamiaceae) could germinate satisfactorily in a wide range of temperatures 10-25°C and 10-30°C, respectively.

Salt stress significantly inhibited germination of *B. hirsuta* seeds and showed a deterioration of values regarding the final germination percentage and the onset of germination as compared to distilled water. This is in agreement with studies demonstrating that distilled water is the most suitable medium for germination of seeds (Huang *et al*., 2003; Deng *et al*., 2014). Our results showed that *B. hirsuta* seed germination was more affected by salinity as compared with other species in the literature e.g. *Campis radicans*, 20% germination at 160 mM NaCl (Chachalis and Reddy, 2000). The negative response of salinity on seed germination might be due to a potential reduction in cellular water or a decrease in hydration of proteins and the enzymatic activity involved in the germination process (Noguchi and Macias, 2005).

Water stress also significantly inhibited germination of *B. hirsuta* seeds by affecting negatively the initial and final day of germination and increasing the mean time of germination. The percentage of seed germination was dramatically decreased at -0.5 MPa PEG. Our results were similar with previous reports on the germination of other species belonging to Lamiaceae family including those of Abbad *et al*. (2011) who reported a decrease in germination capacity of *Thymus maroccanus* and *Thymus broussonetii* seeds at -0.53 MPa. Krichen *et al*. (2014) reported that the germination of *Stipa tinaciccima*, a plant distributed in semi-arid climate areas (similar to *B. hirsuta*), was also very sensitive to a range of water potential from 0 to -0.8 MPa. Plants adapted to arid conditions such as *B. hirsuta*, might have the ability to absorb sufficient water amounts during seed germination from their surroundings even when there is a water restriction.

Ballota hirsuta germinates over a broad

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range of temperatures and environmental conditions corresponding to the spring season in the Mediterranean region. Seed germination of *B. hirsuta* was negatively affected by salinity and water stress indicating that these conditions would restrict its distribution in the natural habitats and its potential utilization as a medicinal plant. This information should also be taken into account in programs of reintroduction of the plant in Algeria that should be implemented in non-saline soil and in zones with high rainfall precipitation.

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ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Βλαστικότητα σπόρων του *Ballota hirsuta* L. υπό οριακές συνθήκες θερμοκρασίας, αλατότητας και νερού

M. Dadach και Z. Mehdadi

Περίληψη Η θερμοκρασία, η αλατότητα και η έλλειψη νερού μπορεί να είναι σημαντικοί περιβαλλοντικοί περιορισμοί στην εξάπλωση των αυτοφυών φυτών στην περιοχή της Μεσογείου. Στην παρούσα μελέτη διεξήχθησαν εργαστηριακά πειράματα για την εκτίμηση των επιδράσεων της θερμοκρασίας, του χλωριούχου νατρίου (NaCl) και της πολυαιθυλενογλυκόλης 6000 (PEG) στη βλαστικότητα των σπόρων του ιθαγενούς Μεσογειακού θάμνου *Ballota hirsuta*. Η βλαστικότητα των σπόρων προσδιορίστηκε σε ένα ευρύ φάσμα θερμοκρασιών (5 έως 35°C), αλατότητας (0 έως 136 mM NaCl) και υδατικής στέρησης (0 έως -1 MPa PEG). Η βλάστηση των σπόρων ήταν βέλτιστη στους 20°C (78%), μειώθηκε σε χαμηλότερες (5°C, 25%) ή υψηλότερες θερμοκρασίες (30°C, 18%) ενώ στους 35°C σημειώθηκε αναστολή της βλάστησης. Κάτω από συνθήκες υψηλής αλατότητας και υδατικού στρες, παρατηρήθηκε σημαντική μείωση στις περισσότερες παραμέτρους βλάστησης δηλαδή χαμηλότερο τελικό ποσοστό βλάστησης, αυξημένος μέσος χρόνος βλάστησης και μικρότερος ρυθμός βλάστησης.

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SHORT COMMUNICATION

Direct determination of glyphosate and aminomethyl phosphonic acid in honeybees

K.M. Kasiotis*, Z.D. Tzouganaki and K. Machera*

Summary A straightforward LC-ESI-MS/MS method was developed and validated for the detection and quantitation of the herbicide glyphosate (GLY) and its metabolite aminomethyl phosphonic acid (AMPA) in honeybees. The method was validated, fulfilling the SANTE 11945/2015 guideline criteria, demonstrating acceptable mean recoveries at LOQ and 10×LOQ varying from 75-87% for both compounds. LOQ was determined at 0.2 and 0.5 μg/g $_{\text{bee body weight (bw)}}$ for GLY and AMPA respectively. Analysis of 14 honeybee samples displayed only one positive sample, containing GLY marginally above LOQ and traces of AMPA.

*Additional Keywords***:** AMPA, glyphosate, honeybees, LC-ESI-MS/MS, validation

Introduction

Glyphosate (GLY) is a non-selective herbicide with a broad range of applications against weeds that place it among the most important active substances of plant protection products (PPPs) in the market and the world's top-selling herbicide (Benbrook, 2016). Its broad use has augmented the interest of research community on its possible impact on non-target organisms such as bees. Although glyphosate exhibits moderate to low toxicity in honeybees (LD_{50}≥1000 μg/g $_{\text{bee bw}}$)(Glyphosate-LD50) its frequent applications should not be underestimated. Therefore, possible sublethal effects, and the potential synergistic effects of co-present compounds and other factors on bees, such as the parasitic mite *Varroa*, the microsporidian *Nosema apis*, or other pollutants, should be considered with caution.

With this in view, field-realistic doses of GLY were implicated in reducing sensitivity to nectar reward, and impair associative learning in honeybees (Herbert *et al*., 2014). In addition, sublethal doses of GLY have been reported to impact cognitive abilities of bees, hence their navigation (Balbuena *et al*., 2015). Metabolism of GLY in the environmental compartments and *in vivo* is extensively reported. AMPA is a major metabolite that in the environment is more persistent than GLY (Mamy *et al*., 2008).

GLY is a very polar and amphoteric compound, therefore can easily be engaged in hydrophilic or hydrophobic ion pairing reactions in the context of ionic equilibria. If these interactions are not favorable, poor chromatographic performance is anticipated and obtained. Due to glyphosate's chemical structure, its chemical analysis (incorporating its main metabolite AMPA) usually occurs by derivatization using appropriate agents, such as 9-fluorenylmethyl chloroformate (FMOC-Cl) and subsequent analysis using LC-ESI-MS/MS. The latter has been applied by several researchers with success and is considered as a routine procedure to quantitate GLY and AMPA in several commodities [indicatively see (Hanke *et al.,* 2008; Schrubbers *et al*., 2016)]. However, its main downside is that is laborious and time intensive.

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One of the first reports on GLY and AMPA direct determination in soybean employed the use of a reversed phase C8 column, using LC-MS/MS (Martins *et al*., 2009). In the same matrix, Chamkasem and Harmon reported GLY and AMPA detection using cation-anion mixed mode chromatographic column (Chamkasem and Harmon, 2016). Yoshioka *et al.* (2011) published related analytical work in serum utilizing hydrophilic interaction chromatography (HILIC). Another application of HILIC was reported in rice, maize, and soybean (Botero-Coy *et al*., 2013), whereas in plant-derived food similar analytical approach exhibited low-matrix effect (Ding *et al*., 2016). Recently, Jensen *et al.* (2016) published a pioneering analytical method for the direct determination of both molecules in milk and urine using a Cation-H guard column. This method proved sufficient in determining both analytes with substantial sensitivity in the respective matrices.

In this short communication, and to our knowledge, we present the first report on the direct analysis of glyphosate and AMPA residues in honeybees. Separation of analytes was grounded on the work by Jensen *et al.* (2016). Its importance is reinforced by the circumvention of the derivatization step, used in a previous work for bee larvae (Thompson *et al*., 2014) and the unambiguous prevalence of GLY and AMPA in foraging environments.

Materials and Methods

Certified chemical standards of GLY and AMPA were obtained from Dr Ehrenstorfer (Germany). Standard solutions were prepared in ultrapure water (SG Ultra-pure water system) inside polypropylene volumetric flasks and kept at 4°C away from light. Acetonitrile and formic acid of LC-MS grade were obtained from Fisher Scientific. Bees used as control sample were collected from Agricultural University of Athens experimental apiaries that are not subjected to chemical treatments.

Sample preparation

In 1g of honeybees, water was added (5mL) and homogenized for 5 min (Ultra-Turrax). Consequently, the mixture was magnetically stirred for 1 h, and subjected to ultrasonication for 30 min. Then, the mixture was centrifuged at 4500 rpm for 15 min (10°C), the supernatant was decanted, and 50 μL of buffering agent (1000mM of NH_4-COOH/HCOOH) were added and the mixture shaken for 10 seconds. Additional centrifugation (same conditions) and filtering of the supernatant provided the final extract that was injected into the LC-ESI-MS/MS system.

Chromatographic conditions

Chromatographic separation was performed following the conditions described by Jensen et al. (2016), incorporating the use of the Bio-Rad Cation-H guard column. The only modification was the addition of formic acid (0.05%) in acetonitrile phase (channel B). Retention times obtained were similar to this publication with AMPA eluting slightly earlier (6.7 min instead of 7.0 min).

Analytical Method Validation

Validation of the analytical method was based on the SANTE guideline (SANTE/11945/2015, 2015). Validation parameters considered were linearity, matrix effect, LOQ, specificity, trueness, precision, and robustness. Linear range varied from 0.1-5 and 0.2-5 μg/mL for GLY and AMPA respectively, containing at least five calibration levels. Up to three MRM transitions were monitored for the active substances (Table 1 and Figure 1a).

Results and Discussion

Analytical method validation criteria fulfilled requirements of the SANTE/11945/2015 document. More specifically, linearity was acceptable with residuals < ±20% (and correlation coefficients values, r^2=0.996 for both GLY and AMPA). LOQ for GLY and AMPA was established at 0.2 and 0.5 μg/g $_{\text{bee bw}}$, respec-

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tively. Trueness was assessed by the recovery study of both analytes at two concentration levels (LOQ and 10×LOQ), using 5 replicates per level. The results verified sufficient extraction of analytes from bees (recoveries ranging from 75-87%, for GLY and AMPA at both LOQ and 10×LOQ, RSD% 6-9.2%). Negligible matrix effect was evidenced for GLY and AMPA, varying from -1.1 to 1.6% respectively.

Consequently, analysis of 14 honeybees' samples after death incidents (year 2017) using the Cation-H column did not reveal residues of GLY and AMPA above the LOQs, except one sample that contained GLY slightly above LOQ (0.21±0.03 μg/g $_{\text{bee bw}}$) and traces of AMPA (Figure 1b). In this context, it should be pointed out that there is room for further improvement of the LOQs of the method. The latter might assist the disclosure of more positive samples if honeybees' colonies are nearby areas growing attractive crops to bees, where applications of GLY have taken place.

Even though field studies have not yet revealed synergistic effects of GLY with other classes of pesticides [such as the neonicotinoids, for imidacloprid see (Zhu *et al*., 2017)], GLY should be incorporated in monitoring schemes due to its wide and frequent use. The latter is reinforced by the ubiquity of pollutants that can interplay with GLY. In this context, a combination of GLY with cadmium, promoted lipid peroxidation in bees (Jumarie *et al*., 2017). Last but not least, Liao *et al*. (2017) reported that bees display a contradictory preference for floral tissues that contain GLY in sugar water at 10ppb. Therefore, GLY is not an obstacle for bees to visit floral nectar that contains it. Overall, GLY is an abundant active substance that should be screened and quantified in apiculture commodities. The presented methodology is a step forward in this direction and can be a starting point to build upon incorporating highly polar and cumbersome molecules such as GLY in monitoring schemes.

Conclusions

A direct LC-ESI-MS/MS analytical method was developed and validated for the detection and quantitation of GLY and AMPA in honeybees. The method does not require derivatization of GLY and AMPA and was applied in a limited number of honeybee samples collected after death incidents. In one sample GLY was quantified slightly above LOQ and traces of AMPA were detected. Next steps, including further fostering of sensitivity and the addition of internal standards, are ongoing.

Authors would like to thank Professor Harizanis of the Agricultural University of Athens for the provision of honeybees control samples.

Table 1. Chromatographic parameters and MRM transitions for the herbicide glyphosate GLY and its metabolite aminomethyl phosphonic acid (AMPA) in honeybees.

Active substances	Retention time (min)	Q1 (amu)	Q2 (amu)	Dwell time	Fragmentor voltage	CE (eV)
GLY	1.5	168	150[q]	30	100	5
			124[c]	30	100	8
			63[c]	15	150	30
AMPA	6.7	110	63[q]	15	150	26
			79[c]	15	150	36

q: quantitation, c: confirmation

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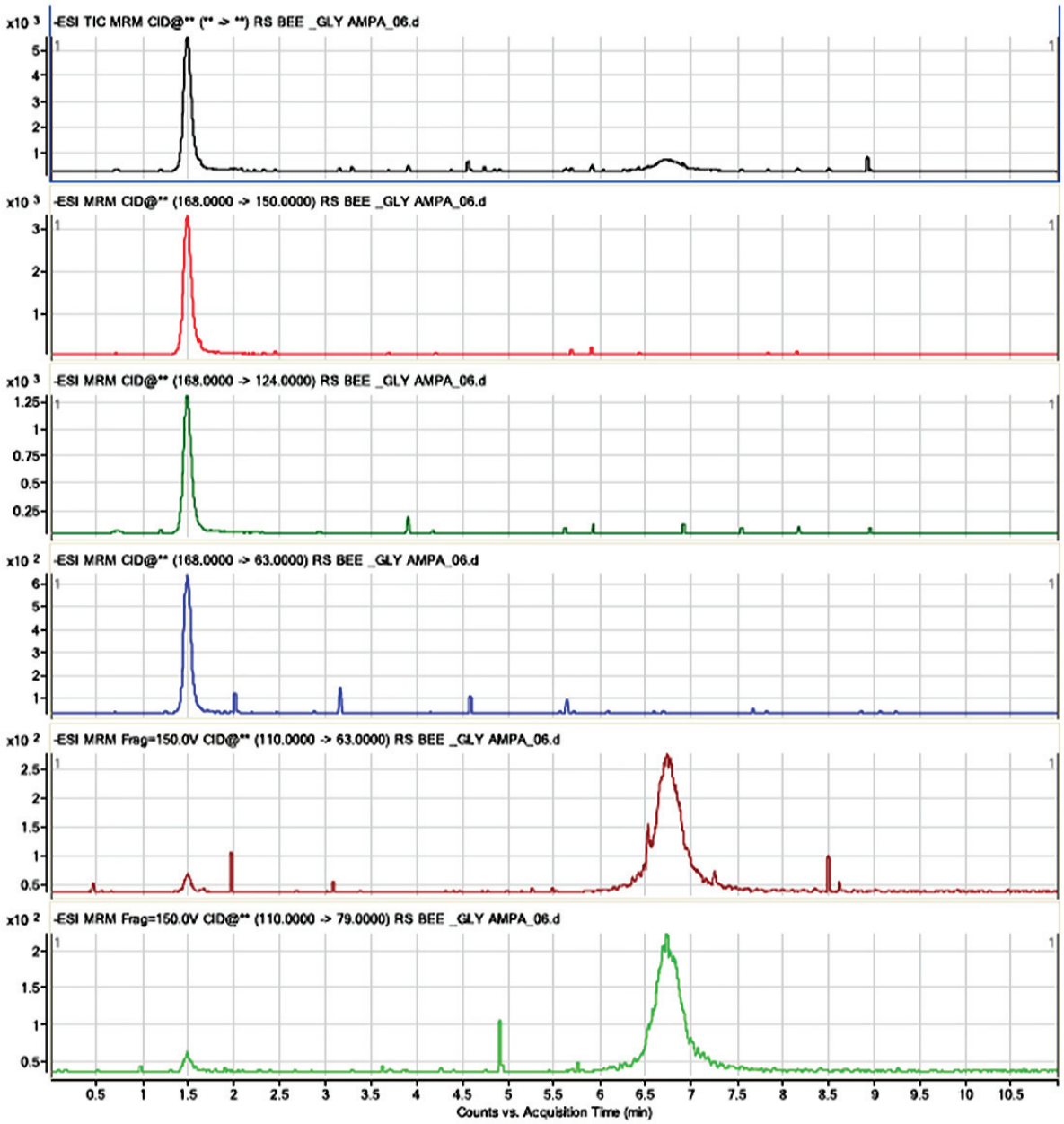

Figure 1a. MRM chromatograms of spiked at 400 ng/g $_{\text{bee bw}}$ control honeybee sample.

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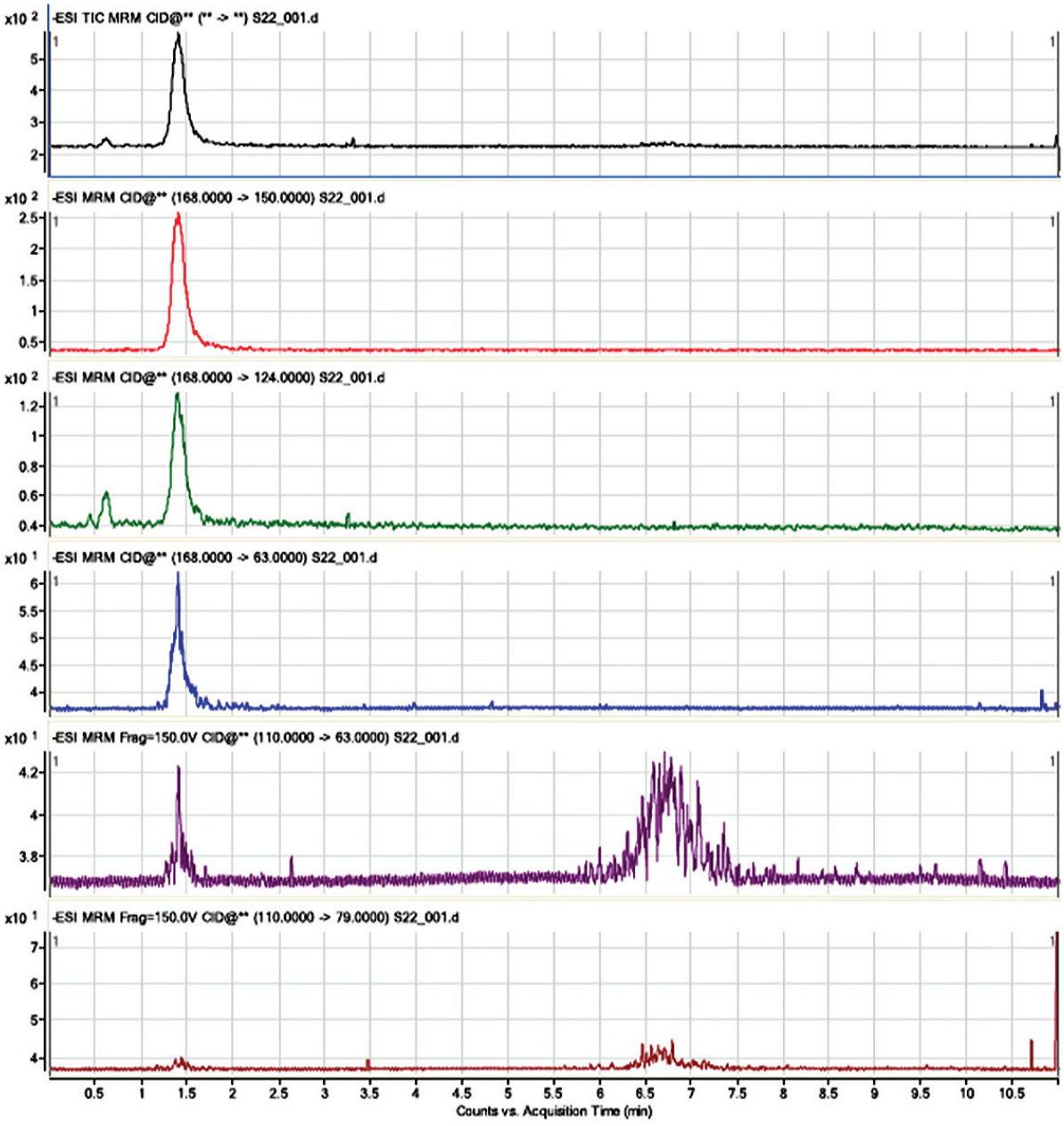

Figure 1b. MRM chromatograms of a honeybee sample containing the herbicide glyphosate (GLY) and traces of its metabolite aminomethyl phosphonic acid (AMPA)*.

*quantitation and confirmation transitions for both GLY and AMPA are presented

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ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Άμεση LC-ESI-MS/MS μέθοδος για τον προσδιορισμό του ζιζανιοκτόνου glyphosate και του μεταβολίτη του αμινομεθυλφωσφονικού οξέος στις μέλισσες

Κ.Μ. Κασιώτης, Ζ.Δ. Τζουγανάκη και Κ. Μαχαίρα

Περίληψη Μια απλή και άμεση LC-ESI-MS/MS μέθοδος αναπτύχθηκε και επικυρώθηκε για την ανίχνευση και ποσοτικοποίηση του ευρέως χρησιμοποιούμενου ζιζανιοκτόνου glyphosate (GLY) και του μεταβολίτη του αμινομεθυλφωσφονικού οξέος (AMPA) στις μέλισσες. Η μέθοδος επικυρώθηκε ικανοποιώντας τα κριτήρια της κατευθυντήριας οδηγίας SANTE 11945/2015, επιδεικνύοντας αποδεκτή μέση ανάκτηση σε δύο επίπεδα συγκέντρωσης, στο όριο ποσοτικοποίησης (LOQ) και στο 10×LOQ, που κυμαίνονται από 75-87% και για τους δύο αναλύτες με αποδεκτές τιμές % σχετικής τυπικής απόκλισης (RSD%). Το όριο ποσοτικοποίησης LOQ προσδιορίστηκε στα 0,2 και 0,5 μg/g $_{\text{σωματικού βάρους μέλισσας}}$ για το GLY και το AMPA αντίστοιχα. Η ανάλυση 14 δειγμάτων μελισσών εμφάνισε μόνο ένα θετικό δείγμα, που περιείχε GLY σε συγκέντρωση ελάχιστα ανώτερη του LOQ και ίχνη AMPA.

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