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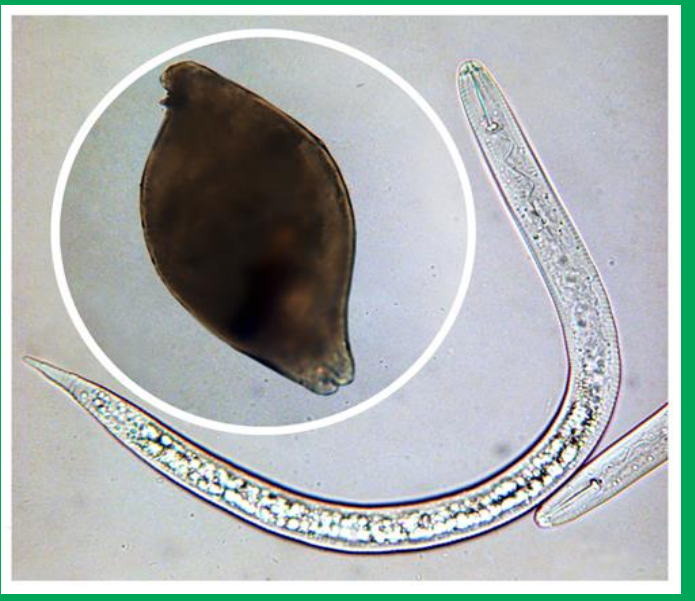

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WEED SCIENCE

PESTICIDE SCIENCE

Photo of the cover page: *Heterodera mediterranea* (Courtesy *Francesca De Luca*)

Guest Editorial

Insect strategy for expanding and occupying areas in agricultural and forest ecosystems

Agricultural and forestry ecosystems are mainly dependent on the diversity of insects that play an important role in the stability and maintenance of their dynamics. However, these insects are surprisingly diverse, both biologically and behaviorally, with an immense variability of the relationships between the host tree and various insects. This subject has been the focus of various researches exploring the ecological mechanisms responsible for the host tree selection.

Several insect species may share the area and the food at the same time in agricultural and forest landscapes and their dispersion depends on the climatic factors. In this context, the interspecific competition between the species, predation, and parasitism conditions the spatiotemporal distribution of the species and structures the communities. Species distribution is a dynamic phenomenon fluctuating between extinction and re-colonization of local populations according to the environmental conditions. The fragility of the ecosystems is a factor particularly favorable to the multiplication of pests depending on the instability conditions of the ecosystem. The forest landscape largely influences the activity and the dispersal of several pests. In the context of climate change, the increase of temperature and its annual seasonal fluctuations, as well as the amounts of rainfall recorded over time, influence the movement of insect populations in their natural environments. Among the pests, periodic outbreaks of various groups of insects, particularly the herbivores, are considered a permanent threat in agricultural and forest ecosystems. The intensification of agricultural practices and the clearing of forest areas have become triggering factors for pests' outbreaks. The periodic gradations of some insects and their expansion

observed in different bioclimatic stages attest the strategy of occupation and evolution.

Various species have become environmental indicators and biological models of the climate change. Furthermore, the expansion of the geographical range of phytophagous and xylophagous species has been directly associated with the increasing climate change. Despite the large data on the ecology, biogeography, physiology and phylogeny of various species, it is difficult to make general predictions of pests' gradations and their geographical distribution. The threats and risks of pests' infestation increase significantly with higher latitude and altitude. Based on recent research, the agroforestry ecosystems are susceptible to invasion of various pests with the range of insects extending strategically according to the environmental conditions.

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Performance of Barley Lines Selected under Drought Stressed Conditions and Ultra-Low Density

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ABSTRACT

Ben Ghanem, H., El Felah, M., Najar, A., Kehel, Z., Amri, A., Rezgui, S., and Tsivelikas, A.L. 2018. Performance of barley lines selected under drought stressed conditions and ultra-low density. Tunisian Journal of Plant Protection 13 (1): 1-25.

Rainfall and temperature are unpredictable in Mediterranean environments, which result in irregular environmental conditions for crop growth and a critical source of uncertainty for farmers. In this study, selected barley lines for grain yield stability under drought stressed conditions and ultra-low plant density (Honeycomb design), were evaluated for agronomic performance in semi-arid areas (Kef and Mornag) compared to the source material. Results showed a significant effect of genotype and genotype×environment (G×E) interaction which indicate the existence of differences among genotypes for plasticity. Biological and grain yield ranged from 3.72 to 7.13 t/ha and 1.46 to 2.66 t/ha across environments with higher values in Kef compared to Mornag. Five high yielding selected lines outyield the original populations (IH17 and IH4-H4 from Imen, AH10-H2 and AH10-H3 from Ardhaoui and MH18 from Manel). The first cycle low yielding lines showed a performance that ranked below the source material. Second cycle high yielding lines did not differ from the first cycle high yielding ones. In conclusion, selection under ultra-low density has been proven an efficient tool to select for lines with high agronomic performance and improved adaptation under the Tunisian dry conditions.

Keywords: Barley, selection, performance, semi-arid, ultra-low plant density

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Among all cereal crops, barley (*Hordeum vulgare*) comes after maize (*Zea mays*), rice (*Oryza sativa*) and wheat (*Triticum* spp.) in terms of total production (Schulte et al. 2009). About two-thirds of global barley crop is used for animal feed, while the remaining third serves for the

malting, brewing and distilling industries (Schulte et al. 2009). In several areas of the globe, such as in the semi-arid regions of North Africa, in Middle East, in the highlands of Nepal, Tibet and Ethiopia, in the Andean countries of South America and also the Himalayas, it is considered as a food crop (Lakshmi et al. 2016). Furthermore, barley has the widest range of production environments in the world (Horlsey et al. 2009). Barley is adaptable to stress conditions with early flowering, seed development and maturation occur in an optimum time period (Gürel et al. 2016). These attributes render barley as a well-suited crop for cultivation from boreal to equatorial regions (Schulte et al. 2009). The natural tolerance of barley to the different kind of adversities has led to an increasing interest for the identification of stress responsive genes using over expression of some of these resistance/tolerance genes by genetic transformation (Contreras-Moreira et al. 2017; Gürel et al. 2016). Barley production in 70% of the cultivated land in Tunisia depends on rainfall conditions, where drought stress occurs most often.

Breeding for abiotic stresses is a high priority for many barley breeders. A non-exhaustive list of these stresses includes drought and flooding, high and low temperatures, mineral deficiencies and toxicities, poor soil tilth and many others (Horsley et al. 2009). Frequently, more than one kind of stress occur in combination, such as the case of drought and heat, acting in a synergistic manner rather than in a simple additive way (Liu et al. 2015) or in succession (e.g. flooding followed by drought). This trend would maintain environments requiring the enhancement of multiple mechanisms (Mickelbart et al. 2015). However, among all of abiotic adversities, drought is already prominent at several major agricultural areas throughout the world (Luck et al. 2015). The effects are predicted to worsen due to growing water demand, shrinking water supply, and increased seasonal variability (Barnabas et al. 2008; Luck et al., 2015).

In Mediterranean regions, where barley is sown in large scale (Ceccarelli 1994; Ryan et al. 2009), drought occurs several times during the life cycle of crops, especially in the terminal growth stages (Turner 2004). The agronomic traits of grain yield could be strongly influenced (Araus et al. 2002; Fischer and Murner 1978; Saini and Westgate 1999). Breeding for drought tolerance has generated improved cultivars for drought prone environments, but progress has been slow (Nguyen 2000). Functional genomic technologies are used to gain a better understanding of how plants respond to drought and to different abiotic stresses (Langridge et al. 2006). However, establishment of innovative and effective breeding strategies at the field level to tackle biotic adversities is still an imperative process that needs to be employed.

An appropriate strategy to achieve this goal is the exploitation of genetic diversity not yet incorporated into the elite cultivars (Dwivedi et al. 2016). As in other crops, current barley cultivars exhibit a narrower genetic basis than wild progenitors (*Hordeum vulgare* ssp. *spontaneum*) and landraces, which are the primary source of useful genes for breeding programs (Dawson et al. 2015). Moreover, there are plenty of cases well documented that in low-input environments, landraces can perform equally or even better than modern cultivars (Ceccarelli et al. 1998; Pswarayi et al. 2008; Yahiaoui et al. 2014). Therefore, harnessing unexploitable genetic diversity of barley crop can be one of the pillars towards resilience to abiotic adversities.

Physiological responses of plants to drought stress are complex and vary with plant species and the degree or time of the exposure to drought (Bodner et al. 2015). One method to assess tolerance to stresses is by determining plant chlorophyll fluorescence (ChlF) (Sayed 2003). The physiological state of the photosynthetic apparatus is highly sensitive to different stresses, thus ChlF is considered to be a very reliable method for assessing the plant tolerance to different stresses (Ren et al. 2018). High chlorophyll content under stress conditions is also a good index as physiological trait. This trait indicates a low degree of photo inhibition of the photosynthetic apparatus (Talebi 2011). A canopy temperature measurement is another index effective method to assess for stress tolerance. This could be seen as relationship between leaf temperature and transpirational cooling (Jackson 1982).

Tailored to the above tools for developing stress tolerant cultivars, honeycomb methodology has been suggested as a breeding procedure to develop cultivars that entirely confront with the challenges of modern agriculture (Fasoula and Tokatlidis 2012). The primary principles that distinguish this method from the other conventional breeding schemes and experimentation designs include the evaluation and selection under ultra-low plant densities, and the systematic entry arrangement to cope with the soil heterogeneity. Selection in the absence of competition maximizes the phenotypic expression of genetic differences among individuals. This design is facilitating the detection of desirable genotypes (Fasoula and Fasoula 2002; Tokatlidis et al. 2010), while eliminates the confounding effects induced by the negative relationship between yielding and competitive ability (Chatzoglou and Tokatlidis 2012; Kyriakou and Fasoulas 1985; Ninou et al. 2014). In the honeycomb layouts (Fasoulas and Fasoula 1995), entries are always allocated evenly across the experimental area, in such a way that every plant of a given entry is consistently surrounded by plants of the remaining entries forming a complete circular replicate; this systematic instead of a randomized entry arrangement ensures the objective comparison of the entries (Papadopoulos and Tokatlidis 2011).

Improvement of drought tolerant barley genotypes is crucial task for breeders. Thus, the objective of the present study was to (i) investigate stability and drought tolerance of barley lines derived from three commercially released cultivars and two Tunisian landraces, using single-plant selection at ultra-low density under Tunisian drought stress conditions for two consecutive cycles, according to the honeycomb methodology; (ii) determine the efficiency of physiological traits to group barley lines into drought susceptible and drought tolerant; and (iii) investigate the relationships between physiological traits and yield parameters.

MATERIALS AND METHODS

Plant material and field experimentation.

A total of 50 first and second cycle selection lines along with the 5 original populations used as checks, were evaluated in this study. The original populations comprised three commercially released cultivars in Tunisia, i.e. Imen, Manel and Rihane and two Tunisian landraces, i.e. Ardhaoui and Djebali. Lines were selected for two consecutive years by applying intra-cultivar single plant selection for high and low yield, under ultra-low density, according to the honeycomb field layout (Fasoulas and Fasoula 1995). Selection was carried out at Kef experimental station of the National Agricultural Research Institute of Tunisia

(INRAT) during 2013/14 and 2014/15 cropping seasons, under rainfed conditions. Besides the five checks used, the entries included 12 first cycle lines (8 selected for high yield and 4 selected for low yield) and 38 second cycle lines (30 selected for high yield and 8 selected for low yield, the latter subjected for high grain yield selection during the first cycle). A detailed description on the selection process regarding these lines is previously given by Ben Ghanem et al. (2018).

Evaluation of agronomic performance traits and records of physiological parameters was carried out at two research stations in Tunisia, at Kef (36° 14' N; 8° 27' E; 518m) and Mornag (36° 37' N; 10° 17' E; 54m) during 2015/16 cropping season. These two research stations represent two distinct production environments for Tunisia. Mornag is characterized by clay soil and average annual precipitation of 450 mm. Kef is characterized by clay loam soil and barley being the most common rainfed crop for the region. Annual rainfall in Kef and Mornag stations during 2015/16 growing season was 325 and 295 mm, respectively.

A non-replicated augmented design field trial was established, in each of the stations, with five incomplete blocks and 15 entries per block. Plot size was composed of four rows of 2.5 m long each with 0.25 m spacing between rows, resulting in a plot area of 2.5 m^2. Trials planted late November with a seed rate of 120 kg/ha and harvested beginning of June. Distance between plots kept at 2 m; best management practices in terms of chemical applications were applied and weed control performed by hand. All four rows of the plots were harvested.

Data collection for agronomic and physiological traits.

During the growing season a number of agronomic and physiological traits were recorded. For both locations biological yield (BY: t/ha) and grain yield (GY: t/ha) per plot was measured at maturity and, Harvest index (HI) was calculated as the quotient between grain and biological yields. Plant height (PH) was measured at maturity from five randomly selected plants within each plot and recorded as the distance in centimeters from soil level to the tip of spikes excluding the awns. Spike length (SL) recorded in centimeters as the average of ten representative spikes of each plot from the base up to the tip of the spike. Each of these spikes then threshed individually and the average grain weight (SGW) per spike expressed in grams for each of the entries was also recorded. Powdery mildew (PM) reaction was also scored at the seedling stage at both locations based on the prevalence of the disease and entries characterized as resistant (R), moderately resistant (MR), moderately susceptible (MS), susceptible (S) and very susceptible (VS) (Saari and Prescot 1975).

A number of physiological parameters were also recorded for both locations. SPAD values at the middle of tillering stage (SPAD_TL) were measured on fully expanded leaves of three representative plants of each plot using a MINOLTA SPAD 502 Plus chlorophyll meter. Leaf canopy temperature (LCT) recorded as the average of five representative positions within each plot using an infrared scantemp 440 thermometer. Chlorophyll fluorescence F_0, F_m, F_v, F_m/F_v and F_v/F_o parameters were measured to test the differences of photosystem II (PSII) (Baker 2008) at the fully expanded flag leaves of three representative plants of each plot at heading time stage using an OPTI-SCIENCE 0530+ hand held portable fluorometer. At Kef station, some additional parameters were also recorded, such as days to heading (HD), (the number

of days needed from sowing to the time that 50% of the plot reaches Zadoks stage 59 (Zadoks et al. 1974)), number of total and fertile tillers per plant (T/P and FT/P) and SPAD values of five randomly selected plants from each entry/plot at the heading stage (SPAD_FL) (SPAD values per plant is the average from three fully expanded flag leaves).

Data analysis.

Raw data values for agronomic and physiological traits were analyzed using combined analysis of variance (ANOVA) for two locations, augmented design with locations and entries as fixed factors and blocks as random. Best linear unbiased estimates (BLUEs) were derived and appropriate standard errors of means (i.e. between checks, between lines of the same block, between lines of different blocks and between lines and checks) were used to determine significant differences.

To identify best performing lines, a biplot graph was generated using as the reference axes the BLUEs values for grain yield in each location. Those lines that outperformed the best checks jointly in both trials were considered as high yielders for both of the locations (Fig. 1).

Principal component analysis based on pairwise correlations among all agronomic and physiological traits was used to identify the parameters that are contributing mostly to the assessment of total variation and discrimination among lines.

A box plot graph was also generated based on grouping the lines by cycle (first or second cycle) and direction of selection (high or low yield), including also the checks (i.e. original populations) as a separate group to assess simultaneously for grain yield performance at both locations (Fig. 2).

Statistical analysis was performed with JMP statistical package ver. 13.0.0.

RESULTS

Agronomic performance of selected lines.

Combined analysis of variance (ANOVA) for agronomic performance traits revealed significant differences among lines for the biological and grain yield, as well as for the harvest index. No differences among lines were detected for plant height, spike length, and spike grain weight (Table 1). A significant effect of the location was found for biological and grain yield with both of the traits to record higher values in Kef rather than Mornag (average 7.13 t/ha in Kef versus 3.72 t/ha in Mornag for biological yield, and 2.66 t/ha in Kef versus 1.46 t/ha in Mornag for grain yield) (Tables 1 and 3). Significant location effect was also found for plant height (average 72.17 cm in Kef versus 54.19 cm in Mornag) and spike grain weight (average 2.33 g in Kef versus 2.14 g in Mornag) (Tables 1 and 3). A significant G × E (in this case indicated as Entries by Locations (E × L) interaction was observed for the grain yield and harvest index, while no interaction was traced for biological yield, plant height, spike length and spike grain weight (Table 1). Significant entry effects were also highlighted for days to heading, number of tillers and number of fertile tillers per plant, measured at Kef station (Table 2).

Table 1. Combined analysis of variance for biological yield (BY), grain yield (GY), harvest index (HI), plant height (PH), spike length (SL) and spike grain weight (SGW), measured in both locations, (Kef and Mornag)

Source of variation	DF	Mean square					
		BY	GY	HI	PH	SL	SGW
Entries (adj.)	54	3.287*	1.125**	0.01139**	132.44	0.311	0.152
Location (unadj.)	1	358.783**	43.702**	0.00348	10376.85**	0.026	1.074*
Entries x Location	54	1.919	0.469*	0.00394*	99.99	0.138	0.060
Block (unadj.)	4	2.171	0.113	0.00077	208.37	0.049	0.061
Residuals	36	1.109	0.172	0.00092	86.12	0.095	0.115

*Significant differences for α = 0.05; **Significant differences for α = 0.01

Table 2. Analysis of variance for traits measured in Kef location: days to heading (DH), number of total and fertile tillers per plant (T/P and FT/P) and SPAD values flag leaves (SPAD_FL)

Source of variation	DF	Mean square			
		DH	SPAD_FL	T/P	FT/P
Entries (adj.)	54	19.20**	13.12	0.730**	0.276*
• **Checks**	4	65.34**	8.37	0.654*	0.124
• **Checks + Checks.VS.aug.**	50	15.51**	13.33	0.723**	0.285*
Block (unadj.)	4	0.54	12.04	0.973**	0.035
Residuals	16	4.94	13.70	0.195	0.117

*Significant differences for α = 0.05; **Significant differences for α = 0.01

For all the agronomic performance traits, a number of superior selected lines were identified at both locations, outperforming the best check. In particular, eleven lines at Kef station outperformed the best check Imen, in terms of biological yield. All of these lines were selected for high yield during the first and second cycle (Table 3). Five of these lines were derived from Djebali (DH12, DH2-H1, DH2-H3, DH2-H4, and DH2-H5), two from Ardhaoui (AH10 and AH10-H1), two from Imen (IH17 and IH4-H1) and one from Manel (MH18-H2) (Table 3). For the same trait in Mornag, Manel was the best performing check while six lines recorded significantly higher BLUEs values (Table 3). These lines were selected as high yield lines, during the first and the second cycles. Three of them coming from Imen (IH17, IH4-H1, and IH4-H4), two from Ardhaoui (AH9-H3 and AH10-H2) and one from Djebali (DH2-H2) (Table 3).

BLUEs values for grain yield at Kef ranged from 0.746 (DH12-L0) up to 5.950 t/ha (IH4-4H) (Table 3). Sixteen of the lines evaluated outperformed the best check Imen; unless two of them, the rest fourteen lines had been selected as high yielding during the first and the second cycles of selection. The two remaining ones had been selected as high yielding lines for the first cycle and as low yielding lines during the second. Nine of these lines had been derived from Imen (IH4, IH17, IH4-H1, IH4-H3, IH4-H4, IH17-H1, IH16-H1, IH16-L0, and IH17-L0), three

from Ardhaoui (AH10, AH10-H2, and AH10-H3), two from Manel (MH18 and MH18-H2) and two from Djebali (DH2-H3 and DH2-H5) (Table 3). BLUEs values for grain yield at Mornag ranged from 0.204 (DH-L0) up to 2.858 t/ha (AH10-2H) (Table 3). As happened in Kef, also in Mornag, Imen was the best performing check in terms of grain (Table 3). A total of seven lines, selected from the first and the second cycle as high yielding outperformed significantly the best check in Mornag (Table 2). Four of these lines were coming from Ardhaoui (AH9-H1, AH9-H3, AH10-2, and AH10-H3), two form Imen (IH17 and IH4-H4) and one from Manel (MH18) (Table 3).

To identify simultaneously the high yielding lines at both locations, the biplot graph of Fig. 1 was generated using the BLUEs values of grain yield (in t/ha) at Mornag (x-axis) and the BLUEs values of grain yield (in t/ha) at Kef (y-axis). High yielding lines for each location were considered those outperforming significantly the best check in each location (plotted at the right side of the reference line traced vertically on the x-axis), for Mornag at the cutting point 2.203 t/ha and above the horizontal reference line traced from y-axis for Kef at the cutting point 3.179 t/ha (Fig. 1). Thus, by applying the joint process, five lines all selected as high yielding either at the first either at the second cycle of selection were identified to outperform the best check in both locations (namely IH17 and IH4-H4 from Imen, AH10-H2 and AH10-H3 from Ardhaoui and MH18 from Manel) (Fig. 1). On the other hand, most of the lines selected for low yield, confirmed a low performance at both locations in terms of grain yield, showing a trend to be plotted at the bottom left side of the biplot graph (Fig. 1).

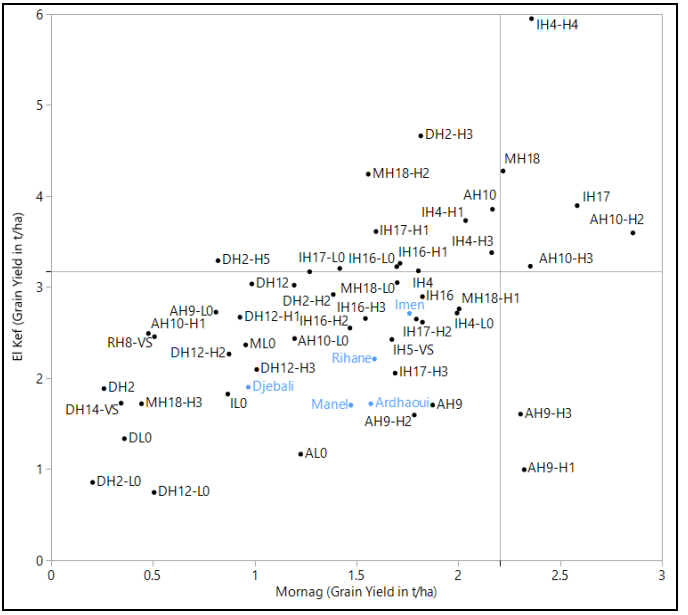

Fig. 1. Biplot graph for grain yield expressed as the best linear unbiased estimates (BLUE) between the two sites of experimentation.

Entries above reference lines indicate significant higher BLUEs values than best check in each of the locations. The entries in the upper right corner indicated superior grain yield for both sites.

First cycle high yielding selected lines outperformed in terms of grain yield performance their original populations (i.e. the checks); while the first cycle low yielding lines showed a performance lower than the checks (Fig. 2). Second cycle high yielding lines did not differ from the first cycle high yielding ones. On the contrary, the second cycle low yielding lines outperformed the ones of the first cycle, as well as the checks, since they have been subjected during the first cycle under selection for high yield (Fig. 2).

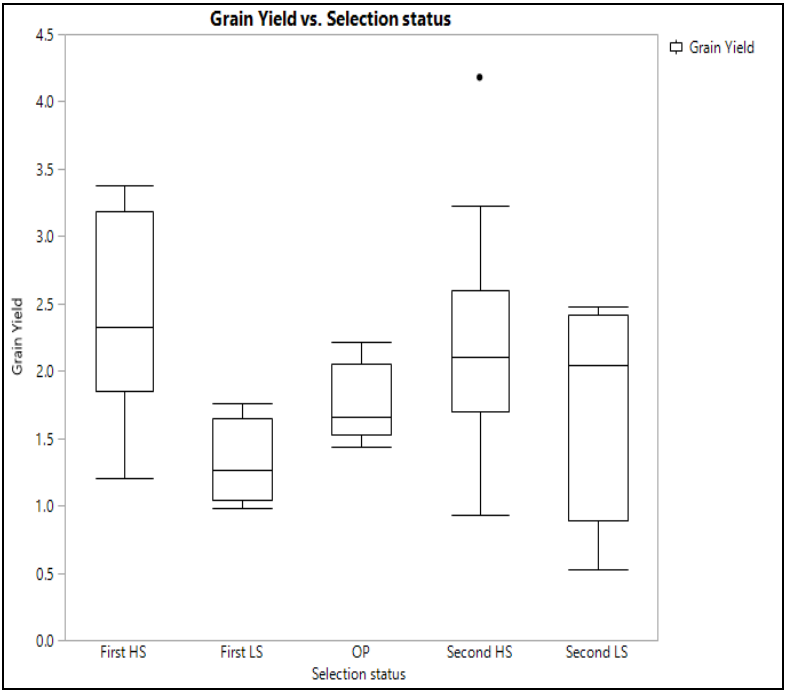

Fig. 2. Box plot graph for grain yield expressed as the best linear unbiased estimates (BLUE) across both sites, based on the selection status of the entries. First HS: First cycle of selection high yielding lines; First LS: First cycle of selection low yielding lines; OP: Original populations; Second HS: Second cycle of selection high yielding lines; Second LS: Second cycle of selection low yielding lines.

Regarding the harvest index, Imen was among all the checks the one with the highest BLUE value at Kef (Table 3). Six lines selected as high yielding at first or second cycles and one selected as low yielding from the second cycle showed higher values for harvest index than the best check. Four of these lines were coming from Imen (IH17, IH4-H4, IH17-H1, and IH17-L0), one from Ardhaoui (AH10-H2) and one from Manel (MH18) (Table 3). For Mornag, Rihane was the check that showed the higher harvest index (Table 3). A total of twelve lines were found to outperform Rihane for harvest index in Mornag (Table 3). All of these lines have been selected for high yield at the first or second cycle, while one has been selected for high yield at the first and low yield at the second cycle of selection. Out of these lines, the nine had been derived from Imen (IH4, IH16, IH17, IH4-H3, IH16-H1, IH16-H2, IH17-H2, IH17-H3, and IH16-L0) and the rest three from Ardhaoui (AH10, AH9-H1, and AH10-H2) (Table 3).

Plant height at Kef ranged from 48.72 (MH18-1H) up to 89.72 cm (AH10-1H) with an average of 72.17 cm (Table 3). Djebali was the tallest check among the five tested (Table 3). On the other hand, Imen was the shortest standing check, ranked as one of the shortest entries within the whole trial in Kef (Table 3). Plant height at Mornag ranged from 38.14 (DH2-L0) up to 68.24 cm (MH18) with an average of 54.19 cm (Table 3). Djebali was the tallest check and Imen the shortest one (Table 3).

Spike length at Kef ranged from 5.76 (DH12-3H) up to 8.96 cm (DH2-1H) with an average of 7.23 cm (Table 3). Manel was the check with the longest spike, while Rihane the check with the shortest one (Table 3). Similar range in terms of spike length was observed also at Mornag with the lower value being 5.99 cm (RH8-VS) and the upper one 8.98 cm (IH5-VS) and average at 7.19 cm (Table 3). At Kef, Manel was again the check with the longest spike, while this time Ardhaoui showed the shortest spike among all the checks of the trial (Table 3).

A wide range in terms of days to heading was recorded at Kef, starting from almost 84 (AH10) days after planting up to 97 days, with an average for the entries of 89 days (Table 4). Djebali, followed by Manel, were the two checks demanding 95 days after planting to reach heading time, while all the three low yielding selected lines derived from Djebali, from the first and the second cycles of selection (DH12-L0, DL0, and DH2-L0) were ranked as the latest entries among all within the trial (Table 4). On the other hand, Rihane was the earliest check, requiring 87 days from planting time to reach heading (Table 4). However, in this case, many of the selected lines ranked as earlier than Rihane, though the differences were not significant (Table 4).

BLUEs values for the number of total tillers per plant ranged from 1.51(DH12-2H) up to 5.85 (AH-10-2) with an average of 3.61 tillers per plant (Table 4). Ardhaoui has the highest number of total tillers per plant. Nine lines were identified showing significantly higher number of total tillers per plant than the best check (Table 4). Out of them, five lines had been selected for high yield during the first or second cycle of selection (IH4-H1 from Imen, DH2-H3 and DH2-H4 from Djebali, AH10-H2 from Ardhaoui, and MH18-H2 from Manel), and the rest four ones had been selected for low yield at the respective cycles (AL0 and AH9-L0 from Ardhaoui, ML0 from Manel, and IL0 from Imen) (Table 4). Among the five checks also tested, Manel and Djebali were the ones recording the lowest value for the number of total tillers per plant (Table 4).

Table 3. Entries' BLUEs values for agronomic performance traits (biological yield (BY), grain yield (GY), harvest index (HI), plant height (PH), spike length (SL) and spike grain weight (SGW)) for both locations (Kef and Mornag)

Entries	Block		BY (t/ha)		GY (t/ha)		HI		PH (cm)		SL (cm)		SGW (g)	
	Kef	Mornag	Kef	Mornag	Kef	Mornag	Kef	Mornag	Kef	Mornag	Kef	Mornag	Kef	Mornag
IH4-4H	1	5	7.438	6.347	5.950	2.360	0.824	0.400	50.12	54.24	7.07	6.59	2.33	1.82
DH2-3H	5	3	11.844	4.791	4.662	1.816	0.404	0.420	83.72	60.74	7.44	7.34	3.04	2.53
MH18	2	5	5.824	5.127	4.276	2.220	0.704	0.400	76.12	68.24	6.34	6.57	2.54	2.16
MH18-2H	5	1	10.914	4.243	4.242	1.558	0.404	0.400	98.72	62.14	6.83	6.86	3.08	2.85
IH17	2	2	8.294	5.591	3.896	2.584	0.504	0.500	74.12	49.74	6.75	7.32	2.11	2.13
AH10	3	1	9.618	4.493	3.856	2.168	0.384	0.500	76.72	64.14	6.25	7.39	1.98	2.46
IH4-1H	5	3	9.794	5.491	3.732	2.036	0.404	0.420	88.72	61.74	7.30	6.94	2.99	2.78
IH17-1H	4	1	8.074	4.811	3.612	1.596	0.504	0.320	64.72	51.74	7.33	7.38	2.51	2.04
AH10-2H	5	1	7.818	5.463	3.596	2.858	0.484	0.500	72.72	61.64	6.90	6.76	2.72	2.09
IH4-3H	1	2	7.498	4.491	3.380	2.164	0.424	0.500	68.12	50.24	7.46	7.32	2.74	2.28
DH2-5H	5	5	9.924	2.687	3.292	0.820	0.304	0.300	82.72	53.24	7.72	7.62	2.58	2.29
IH16-1H	5	2	8.044	3.521	3.262	1.714	0.404	0.500	72.72	46.24	6.59	7.55	2.55	2.44
IH16-L0	3	1	7.858	3.463	3.230	1.698	0.384	0.500	68.72	55.14	7.08	6.89	2.60	2.09
AH10-3H	3	1	7.368	4.657	3.226	2.354	0.424	0.480	63.12	57.64	7.11	7.19	2.10	2.19
IH17-L0	4	5	7.284	3.183	3.206	1.418	0.484	0.400	76.32	44.14	6.59	6.76	2.32	1.83
IH4	1	2	6.888	3.531	3.180	1.804	0.424	0.500	67.12	50.24	7.44	7.48	2.67	2.23
DH2-1H	1	5	8.758	4.577	3.170	1.270	0.324	0.300	72.12	57.74	8.96	7.69	2.81	2.17
MH18-L0	1	5	6.668	3.887	3.050	1.700	0.424	0.400	78.12	61.74	6.57	7.01	2.11	2.19
DH12	4	3	9.104	4.601	3.036	0.986	0.284	0.220	76.32	54.74	8.15	7.80	2.45	2.57
DH2-4H	5	4	8.714	3.737	3.022	1.194	0.404	0.280	88.72	57.14	7.31	6.84	2.24	1.67
DH2-2H	1	3	7.378	5.961	2.920	1.386	0.424	0.220	83.12	57.74	7.93	8.62	2.52	2.76
IH16	3	2	6.938	3.911	2.896	1.824	0.384	0.500	71.72	54.74	6.27	6.77	2.08	1.91
MH18-1H	5	4	8.174	4.307	2.762	2.004	0.304	0.480	48.72	65.14	7.05	6.74	2.58	1.93
AH9-L0	4	5	7.614	2.477	2.726	0.810	0.384	0.300	85.32	50.24	8.29	7.08	2.80	2.08
IH4-L0	2	4	6.674	4.417	2.716	1.994	0.404	0.480	52.12	55.14	7.41	6.25	2.51	1.56
Imen	NA	NA	6.864	4.168	2.712	1.760	0.420	0.420	55.80	51.20	7.01	6.86	2.03	2.15
DH12-1H	1	1	7.358	3.413	2.670	0.928	0.324	0.300	77.12	58.14	6.92	6.75	2.52	2.13
IH16-3H	2	2	6.534	3.511	2.656	1.544	0.404	0.400	57.12	47.74	6.70	6.92	2.09	1.89
IH17-2H	5	3	7.818	3.401	2.650	1.794	0.324	0.500	64.12	49.24	7.27	7.33	2.30	2.40
IH4-2H	3	4	7.148	3.847	2.616	1.824	0.384	0.480	78.72	57.14	6.97	6.78	2.59	2.03
IH16-2H	5	1	6.794	3.073	2.552	1.468	0.404	0.500	72.72	55.64	6.23	6.39	2.19	1.58
AH10-1H	4	3	8.234	2.363	2.492	0.478	0.304	0.200	89.72	59.64	8.00	6.89	2.44	2.62

RH8-VS	2	1	5.634	1.593	2.456	0.508	0.404	0.300	71.12	55.64	6.64	5.99	1.39	1.99
AH10-L0	1	4	6.094	2.851	2.436	1.196	0.384	0.420	74.32	48.24	7.94	8.07	2.48	2.10
IH5-VS	3	4	5.728	4.057	2.426	1.674	0.384	0.380	77.72	53.64	7.58	8.98	2.70	3.32
ML0	2	3	7.984	2.851	2.366	0.956	0.304	0.320	76.12	54.74	7.96	8.20	2.33	2.39
DH12-2H	3	4	6.268	3.137	2.266	0.874	0.384	0.280	67.72	58.64	7.27	7.64	2.53	2.34
Rihane	NA	NA	5.966	3.724	2.212	1.588	0.380	0.440	71.40	58.50	6.12	6.38	2.36	2.02
DH12-3H	2	5	7.904	3.237	2.096	1.010	0.304	0.300	72.12	58.74	5.76	7.23	1.93	1.77
IH17-3H	1	2	5.294	3.557	2.056	1.690	0.384	0.500	66.32	54.24	7.54	6.99	2.68	2.33
Djebali	NA	NA	6.792	3.156	1.902	0.968	0.300	0.300	73.80	59.10	7.61	7.15	2.33	1.99
DH2	2	5	6.414	1.447	1.886	0.260	0.304	0.200	64.12	53.74	7.79	7.09	2.26	1.77
IL0	4	1	7.304	2.993	1.826	0.868	0.284	0.300	74.32	49.64	7.71	7.27	2.07	2.67
DH14-VS	4	2	5.274	1.271	1.726	0.344	0.284	0.200	79.32	41.74	8.46	7.41	1.89	1.74
MH18-3H	1	2	5.558	1.371	1.720	0.444	0.324	0.300	71.12	45.24	6.37	7.95	2.50	2.16
Ardhaoui	NA	NA	5.146	3.852	1.720	1.570	0.340	0.400	71.00	57.40	6.68	6.32	2.00	1.69
AH9	3	4	7.028	4.177	1.706	1.874	0.184	0.480	57.72	58.64	6.77	6.97	1.99	1.64
Manel	NA	NA	6.494	4.216	1.704	1.472	0.280	0.340	67.60	55.00	8.65	7.34	2.16	2.68
AH9-3H	2	3	6.024	5.561	1.606	2.306	0.304	0.420	69.12	53.24	7.33	8.06	1.92	2.30
AH9-2H	3	4	6.288	4.367	1.596	1.784	0.284	0.380	67.72	50.14	6.89	6.99	1.84	1.59
DL0	2	5	6.994	1.547	1.336	0.360	0.204	0.300	75.12	47.74	7.21	7.11	2.12	1.96
AL0	4	3	5.234	4.001	1.166	1.226	0.184	0.320	69.32	43.74	7.88	7.74	2.12	1.83
AH9-1H	4	2	4.004	4.411	0.996	2.324	0.284	0.500	79.32	56.24	7.33	8.28	2.02	1.87
DH2-L0	4	4	4.074	1.147	0.856	0.204	0.184	0.180	64.32	38.14	8.00	6.63	1.79	1.58
DH12-L0	3	3	3.598	2.761	0.746	0.506	0.184	0.220	72.72	43.74	7.05	7.11	1.83	2.06
S.E. difference														
Between checks			0.6542	0.5104	0.2531	0.2402	0.0276	0.0265	4.808	4.799	0.320	0.374	0.178	0.273
Between augmented entries (same block)			1.4629	1.1413	0.5660	0.5371	0.0616	0.0592	10.750	10.730	0.716	0.836	0.398	0.610
Between augmented entries (different block)			1.6025	1.2502	0.6200	0.5884	0.0675	0.0648	11.776	11.754	0.785	0.916	0.436	0.668
Between an augmented entry and a check			1.2063	0.9411	0.4668	0.4429	0.0508	0.0488	8.865	8.848	0.591	0.690	0.328	0.503

Blocks are indicated to facilitate comparisons for appropriate S.E.

For the number of fertile tillers per plant though, Ardhaoui is performing check indicating a different response checks having almost same number of fertile tillers with Manel and Djebali (Table 4). In this case, the best performing check was Imen, showing 2.25 fertile tillers per plant (Table 4). Thirteen lines, out of them the nine lines selected for high yield at the second cycle of selection and the four lines selected for low yield either at the first either at the second cycle, outperformed significantly the best check. Five among these lines has been derived from Ardhaoui (AL0, AH9-L0, AH10-H1, AH10-H2, and AH9-H3), three from Djebali (DH2-H2, DH2-H3, and DH2-H4), three from Imen (IH4-H1, IH16-H1, and IH17-L0) and two from Manel (ML0 and MH18-H2) (Table 4).

Table 4. Entries' BLUEs values for agronomic performance traits and photosynthesis related parameters measured only at Kef location

Entries	Block	HD	T/P	FT/P	SPAD_FL
IH4-4H	1	87.44	2.892	1.957	48.73
DH2-3H	5	87.84	5.298	3.029	41.44
MH18	2	85.24	3.316	2.609	49.35
MH18-2H	5	85.84	5.498	3.849	44.44
IH17	2	86.24	3.546	2.229	56.50
AH10	3	83.84	3.228	2.243	50.70
IH4-1H	5	88.84	4.788	3.119	42.22
IH17-1H	4	85.84	3.638	2.409	54.32
AH10-2H	5	85.84	5.848	2.803	52.23
IH4-3H	1	86.44	2.922	1.827	54.23
DH2-5H	5	90.84	3.978	2.199	45.59
IH16-1H	5	86.84	4.118	2.859	47.49
IH16-L0	3	88.84	2.678	2.213	51.75
AH10-3H	3	88.44	3.122	2.257	48.45
IH17-L0	4	85.64	3.606	2.653	53.12
IH4	1	86.44	3.302	2.327	50.53
DH2-1H	1	93.44	3.572	2.237	46.80
MH18-L0	1	86.44	2.872	2.067	44.93
DH12	4	92.64	3.106	2.193	45.95
DH2-4H	5	92.84	4.958	3.139	51.27
DH2-2H	1	91.44	3.922	2.667	47.53
IH16	3	85.84	2.558	1.743	50.13
MH18-1H	5	84.84	3.928	2.119	47.22
AH9-L0	4	86.64	5.246	3.053	54.75
IH4-L0	2	87.24	3.886	2.319	52.93
Imen	NA	89.00	3.412	2.250	49.70
DH12-1H	1	93.44	3.992	2.187	41.90
IH16-3H	2	87.24	2.986	2.049	49.55
IH17-2H	5	89.44	3.742	2.467	50.15
IH4-2H	3	86.84	2.008	1.313	50.53
IH16-2H	5	86.84	3.788	2.099	46.67
AH10-1H	4	94.84	4.058	3.019	54.02
RH8-VS	2	85.24	2.716	1.389	48.43
AH10-L0	1	86.64	3.976	2.593	51.75
IH5-VS	3	87.84	3.338	1.743	49.78
ML0	2	93.24	4.556	2.939	50.60

DH12-2H	3	94.84	1.508	1.123	49.10
Rihane	NA	87.40	3.384	2.060	49.44
DH12-3H	2	86.24	3.786	2.249	45.53
IH17-3H	1	86.64	4.056	1.993	48.15
Djebali	NA	95.20	2.968	1.816	46.86
DH2	2	93.24	2.696	1.999	45.05
IL0	4	90.64	4.756	2.443	42.50
DH14-VS	4	94.64	2.666	1.653	52.05
MH18-3H	1	87.44	2.332	1.157	56.40
Ardhaoui	NA	89.60	3.984	1.858	49.29
AH9	3	83.84	4.218	2.363	43.38
Manel	NA	95.00	3.032	1.930	46.85
AH9-3H	2	85.24	4.296	2.759	47.78
AH9-2H	3	84.84	3.618	2.643	43.28
DL0	2	96.24	3.206	2.299	49.60
AL0	4	84.64	4.556	2.823	49.70
AH9-1H	4	84.64	2.756	2.173	47.62
DH2-L0	4	96.64	4.226	1.613	48.25
DH12-L0	3	95.84	2.108	1.273	47.78
S.E. difference					
Between checks		1.406	0.2790	0.2166	2.341
Between augmented entries (same block)		3.143	0.6239	0.4843	5.235
Between augmented entries (different block)		3.443	0.6834	0.5306	5.735
Between an augmented entry and a check		2.592	0.5145	0.3994	4.317

Blocks are indicated to facilitate comparisons for appropriate S.E.

Physiological parameters of selected lines.

There were no significant differences for the PSII related parameters among lines (Table 5). Differences were only detected between the locations for F_0, F_v/F_m and F_v/F_0 values (Table 5). For the Kef station, among all the checks, Djebali showed the highest values for the ratios F_v/F_m and F_v/F_0, while Imen showed the lowest ones (Table 6). For Mornag trial, among all the checks, Rihane showed the highest F_v/F_m and F_v/F_0 ratios and Imen showed the lowest ones (Table 6). A number of lines showed also high values of F_v/F_m and F_v/F_0 at both locations. The IH4-H4, second cycle high yielding line derived from Imen, showed high F_v/F_m and F_v/F_0 ratios at Kef station and was ranked second for both of these ratios at Mornag (Table 6). Other lines, scoring high values at both locations for the above-mentioned ratios, were DH2-H5 and DH12-H1 originated from Djebali and AH10-H1 issued from Ardhaoui (Table 6).

Regarding F_0 value, among the checks, Rihane showed the highest value for Kef, while Ardhaoui the highest value for Mornag (Table 6). The latter though was the one among all checks by scoring the lower F_0 value at Kef station, while at Mornag station the lower F_0 value, among all checks, was recorded by Djebali (Table 6). For one more time also, line IH4-H4

showed high values for the PSII related parameters and particularly at Mornag, this line was ranked as first for the F_0 value, among all the entries of the trial (Table 6).

No significant differences were detected among entries for the SPAD values and leaf canopy temperature recorded at the middle of tillering stage, as well as at the heading time (Table 5). However, significant effects were observed among locations for the leaf canopy temperature and the SPAD at the middle of tillering stage (Table 5).

Ranking of lines for SPAD values measured at the two different growth stages (i.e. middle of tillering stage and heading time), did not reveal any significant correlation and the Spearman's rho was not significant ($r = 0.115$, $P > 0.05$). During the tillering stage, Manel showed among the checks the highest SPAD value. However, during heading time, Manel showed the lowest SPAD value (Table 6).

For Mornag station, Rihane indicated the higher and Djebali the lower SPAD values when assessed during tillering stage (Table 6). Two lines, one selected for high yield from Imen at the first cycle (IH17) and the other selected for low yield from Ardhaui at the second cycle (AH9-L0), recorded high SPAD values at Kef station for both stages (Table 6). These lines were also above the best check Rihane at Mornag trial in terms of SPAD value (Table 6).

Canopy temperature was on average 19.5ºC at Kef and 23.7ºC at Mornag (Table 6). Among the checks Rihane, showed the highest canopy temperature in both of the locations, while Djebali for Kef and Manel for Mornag recorded the lowest values for this physiological parameter (Table 6). The majority of lines evaluated at Kef (i.e. 30 out of a total of 50 lines), showed lower values for leaf canopy temperature than Djebali, while for Mornag a total of 18 selected lines showed lower leaf canopy temperature than Manel (Table 6).

Principal component analysis and partial correlations between traits.

Principal component analysis (PCA) provided six principal components (PCs) that explained more than 80% of the total variation among the entries tested (Table 7). The first three components accounted together for 59.56% of the total variation (Table 7). For the first PC, growth and yield parameters, such as plant height, biological yield and grain yield were those with high positive loadings along with the PSII related parameter of F_0, while SPAD and leaf canopy temperature, scored at the middle of tillering stage, were the ones with high negative loads (Table 8). The second PC gathered except from F_0, all the other PSII related parameters, all contributing with high positive loads (Table 8). The third PC is mainly associated with agronomic performance traits.

The harvest index followed by grain yield, number of fertile tillers per plant and number of total tillers per plant were the ones with high positive loads, while heading time and spike length the traits with high negative loads (Table 8). The rest of the parameters, such as SPAD measured at the heading time, powdery mildew resistance and spike grain weight, accounted for the other minor PCs and their contribution to the performance assessment and differentiation among the entries tested was considered negligible (Table 8).

Pairwise correlations between phenotypic traits revealed significant coefficients in 74 out of the 324 trait combinations. Correlation coefficient (r) ranged between 0.009 up to 0.977 (Table 9). High positive correlations were

revealed among biological yield, grain yield and plant height for both locations (r = 0.484-0.838, $P < 0.01$). A high correlation was also found between harvest index and grain yield (r = 0.484, $P < 0.01$) (Table 9). In addition, a negative correlation was revealed between grain yield and days to heading (r = -0.408, $P < 0.01$), implying that early flowering lines were more productive than the late flowering ones (Table 9).

High negative correlations were found for leaf canopy temperature and SPAD (measured at the middle of tillering stage) with the biological and grain yield (r = -0.417 up to r = -0.628, $P < 0.01$). The SPAD measured at heading time did not reveal any significant correlation with any of the yield related parameters (Table 9). Among the PSII related parameters, F_0 and F_m were highly correlated with grain yield (r = 0.516 and r = 0.389 respectively, $P < 0.01$) (Table 9).

Powdery mildew resistance.

Powdery mildew scores at both locations revealed on average a moderate susceptibility for the entries. None of the entries scored was characterized as resistant to the disease. Among the two locations, 24.67% of the entries were ranked as moderately resistant, 58.00% as moderately susceptible, and 17.35% as susceptible to powdery mildew. Disease symptoms noted at Mornag were more severe than those observed in Kef ($X^2 = 26.714$, $P < 0.01$). Grouping of the entries, based on cycle and direction of selections, showed that within the group of the first cycle high yielding selected lines, no line was scored as susceptible to the disease and all lines were characterized either as moderately resistant or as moderately susceptible (Fig. 3). However, for all the other group of lines as well as for the checks, there were cases of entries that at least in one location were scored as susceptible to the disease (Fig. 3).

Table 5. Combined analysis of variance for physiological parameters measured in both locations (Kef and Mornag) indicating values for degrees of freedom (DF) and mean squares (MS)

Source of variation	DF	MS						
		SPAD_M	LCT	F_0	F_v	F_m	F_v/F_m	F_v/F_0
Entries (adj.)	54	17.50	15.29	254.78	8154.46	12035.67	0.003	0.214
Location (unadj.)	1	2814.73**	573.75**	83986.31**	57783.85	283437.37	0.034**	4.189**
Entries x Location	54	19.43	14.31	291.77	6749.78	10575.96	0.002	0.144
Block (unadj.)	4	10.86	12.08	756.04	3381.00	6303.36	0.004	0.191
Residuals	36	8.78	10.44	783.71	5549.90	9862.88	0.001	0.102

*Significant differences for $\alpha = 0.05$; **Significant differences for $\alpha = 0.01$

Table 6. Entries' BLUEs values for photosynthesis related parameters at both locations (Kef and Mornag)

Entries	SPAD_M		LCT		F_0		F_v		F_m	
	El Kef	Mornag	El Kef	Mornag	El Kef	Mornag	El Kef	Mornag	El Kef	Mornag
IH4-4H	46.13	50.64	24.94	23.48	197.30	176.82	591.91	639.12	789.21	815.94
DH2-3H	42.48	50.21	16.95	22.63	223.45	154.12	441.96	307.27	665.41	461.39
MH18	41.82	55.21	18.39	23.01	183.85	163.82	353.91	431.37	537.76	595.19
MH18-2H	40.78	53.17	18.07	25.05	180.20	144.52	392.21	335.52	572.41	480.04
IH17	45.45	55.24	20.11	24.04	189.10	163.42	418.16	310.77	607.26	474.19
AH10	46.31	51.24	15.85	22.43	217.50	164.77	465.31	385.77	682.81	550.54
IH4-1H	41.28	54.16	18.52	23.18	207.95	150.12	418.96	398.52	626.91	548.64
IH17-1H	39.53	53.98	17.97	25.15	217.70	154.37	377.71	284.52	595.41	438.89
AH10-2H	43.84	52.92	15.17	23.83	216.50	154.02	453.56	313.77	670.06	467.79
IH4-3H	41.16	49.46	19.87	24.67	197.80	133.92	364.91	299.27	562.71	433.19
DH2-5H	38.08	47.81	18.05	24.06	214.95	163.82	471.71	465.37	686.66	629.19
IH16-1H	39.83	53.34	18.17	22.79	206.45	145.67	392.46	273.02	598.91	418.69
IH16-L0	45.36	50.19	19.12	24.70	208.75	139.27	497.81	325.02	706.56	464.29
AH10-3H	48.01	53.38	24.47	24.76	196.55	156.12	403.16	355.57	599.71	511.69
IH17-L0	40.84	47.99	22.82	22.13	197.15	152.77	305.41	333.27	502.56	486.04
IH4	37.66	53.69	16.89	22.89	175.55	123.67	451.66	256.77	627.21	380.44
DH2-1H	42.11	49.51	18.94	22.18	196.55	170.82	437.16	350.87	633.71	521.69
MH18-L0	39.81	52.46	15.57	23.43	187.80	140.82	473.16	318.37	660.96	459.19
DH12	45.24	48.08	17.85	24.80	196.40	131.12	359.66	291.52	556.06	422.64
DH2-4H	44.33	47.73	19.25	21.91	209.20	164.12	430.96	413.57	640.16	577.69
DH2-2H	44.23	54.58	14.59	23.25	187.05	160.62	318.66	409.77	505.71	570.39
IH16	47.19	54.49	15.97	23.02	213.50	141.67	432.06	376.27	645.56	517.94
MH18-1H	39.28	51.20	19.37	23.38	216.20	143.62	442.71	336.07	658.91	479.69
AH9-L0	47.01	53.69	18.27	25.73	220.90	152.32	446.41	402.62	667.31	554.94
IH4-L0	42.92	51.98	21.11	20.73	173.85	160.37	325.41	398.32	499.26	558.69
Imen	41.86	52.12	20.77	24.76	194.45	158.65	351.85	354.00	546.30	512.65
DH12-1H	41.13	46.69	21.49	23.05	186.80	161.02	491.66	408.27	678.46	569.29
IH16-3H	43.40	49.29	18.96	23.72	196.85	122.92	398.66	280.52	595.51	403.44
IH17-2H	40.98	52.66	21.54	22.54	190.80	142.67	392.16	290.77	582.96	433.44
IH4-2H	43.51	48.53	18.17	19.71	211.75	141.12	458.81	290.32	670.56	431.44
IH16-2H	42.11	50.54	18.82	23.78	219.95	131.77	411.46	310.77	631.41	442.54
AH10-1H	38.56	46.54	19.35	23.53	180.95	146.52	399.21	380.02	580.16	526.54
RH8-VS	44.92	52.14	23.89	25.08	182.85	141.77	351.41	357.77	534.26	499.54
AH10-L0	44.91	55.16	22.55	25.65	220.15	140.62	406.41	432.02	626.56	572.64

IH5-VS	39.71	54.38	16.70	24.66	210.00	152.37	451.56	363.82	661.56	516.19
ML0	39.87	55.06	22.16	25.50	192.35	147.87	295.16	360.77	487.51	508.64
DH12-2H	38.89	51.95	16.50	21.03	218.25	127.87	455.81	304.07	674.06	431.94
Rihane	44.59	53.62	23.01	24.87	212.45	155.10	393.85	389.45	606.30	544.55
DH12-3H	39.82	52.21	19.71	23.53	204.10	168.32	408.91	436.12	613.01	604.44
IH17-3H	40.71	52.09	19.75	22.76	214.90	176.57	326.66	637.37	541.56	813.94
Djebali	41.65	48.43	19.80	23.72	195.15	149.35	382.65	349.10	577.80	498.45
DH2	41.05	53.04	17.96	25.41	195.35	141.07	357.66	373.37	553.01	514.44
IL0	47.06	48.79	19.27	25.05	221.15	159.27	421.16	393.27	642.31	552.54
DH14-VS	40.26	47.01	23.07	23.67	214.15	154.67	394.41	336.77	608.56	491.44
MH18-3H	43.38	50.31	16.32	24.87	194.80	155.67	359.41	314.27	554.21	469.94
Ardhaoui	39.93	52.99	22.17	24.02	175.00	161.10	334.25	391.65	509.25	552.75
AH9	40.51	54.85	17.30	22.21	221.25	165.37	424.06	348.07	645.31	513.44
Manel	46.68	51.22	22.93	23.09	197.20	154.40	390.70	343.15	587.90	497.55
AH9-3H	38.65	58.33	20.51	23.30	199.35	160.12	361.16	293.52	560.51	453.64
AH9-2H	45.34	51.50	15.80	22.98	217.50	160.37	402.81	406.07	620.31	566.44
DL0	42.20	51.04	22.16	24.46	184.85	163.32	394.66	434.87	579.51	598.19
AL0	42.66	52.53	21.85	22.98	221.15	156.12	423.41	409.02	644.56	565.14
AH9-1H	39.06	50.59	24.00	24.82	242.65	174.67	409.66	431.02	652.31	605.69
DH2-L0	38.64	50.20	22.62	24.68	220.90	156.62	448.16	292.82	669.06	449.44
DH12-L0	44.21	50.61	18.15	24.43	188.00	160.62	354.56	340.27	542.56	500.89
S.E. difference										
Between checks	1.565	1.345	1.944	0.893	11.952	11.068	21.598	36.016	31.091	42.464
Between augmented entries (same block)	3.500	3.007	4.346	1.996	22.038	24.749	48.294	80.578	69.521	94.952
Between augmented entries (different block)	3.834	3.294	4.761	2.187	29.276	27.111	52.903	88.269	76.156	104.014
Between an augmented entry and a check	2.886	2.480	3.584	1.646	22.038	20.408	39.824	66.447	57.328	78.299

Blocks are indicated to facilitate comparisons for appropriate S.E.

Table 7. Principal components analysis (PCA) based on correlations between all traits.

Number	Eigenvalue	Percent	Cum. %
1	5.1471	28.595	28.595
2	3.2367	17.982	46.577
3	2.3363	12.980	59.556
4	1.7325	9.625	69.182
5	1.3045	7.247	76.429
6	0.8974	4.986	81.415

Table 8. Factor loads for the first 6 PCs accounting for more of the variation revealed among entries

Trait	PC1	PC2	PC3	PC4	PC5	PC6
HD	-0.11943	-0.10394	**-0.48519**	0.20566	0.19535	-0.25555
PH	**0.33671**	0.01153	-0.14204	0.05204	-0.04342	-0.01531
BY	**0.41117**	0.02136	0.02356	0.08919	0.00893	-0.04861
GY	**0.34518**	0.06582	**0.27569**	-0.02128	0.23136	-0.17406
HI	0.04550	0.05050	**0.49269**	-0.07202	0.35104	-0.33976
SPAD M	**-0.33373**	0.07574	0.17597	0.15543	0.08852	-0.06745
LCT	**-0.31044**	0.09885	0.03900	0.12972	-0.06420	-0.24854
SPAD FL	-0.00898	-0.09404	0.27642	-0.13216	0.41756	0.15835
T/P	0.10806	0.01960	**0.20128**	0.48545	-0.40438	-0.10216
FT/P	0.18386	-0.02187	**0.26625**	0.50864	-0.24178	0.05775
PM	-0.15973	0.19673	0.14597	-0.07998	-0.12534	0.71755
Fo	**0.36563**	0.11616	-0.14245	-0.23168	-0.12157	0.02617
Fv	0.13934	**0.51151**	-0.10353	-0.05820	0.02520	-0.04526
Fm	0.22484	**0.44324**	-0.13075	-0.11928	-0.01314	-0.03196
Fv/Fm	-0.17311	**0.46878**	-0.00495	0.15600	0.09249	-0.02538
Fv/Fo	-0.18129	**0.47319**	0.01351	0.14101	0.09769	-0.05604
SL	0.05437	-0.03849	**-0.36626**	0.37705	0.39176	0.13128
SGW	0.18390	-0.03276	0.03239	0.35286	0.41467	0.37622

Table 9. Pearson correlation coefficients analysis for agronomic and physiological related traits and powdery mildew symptoms across both locations (Kef and Mornag)

	HD	PH	BY	GY	HI	SPAD_M	LCT	SPAD_FL	T/P	FT/P	PM	Fo	Fv	Fm
HD	1.000													
PH	0.105	1.000												
BY	-0.156	0.676**	1.000											
GY	-0.408**	0.486**	0.838**	1.000										
HI	-0.442**	-0.037	0.113	0.582**	1.000									
SPAD_M	0.052	-0.594**	-0.628**	-0.417**	0.193*	1.000								
LCT	0.195	-0.551**	-0.609**	-0.462**	0.002	0.573**	1.000							
SPAD_FL	-0.194	-0.139	-0.066	0.161	0.295*	-0.022	-0.032	1.000						
T/P	-0.213	0.143	0.253*	0.169	0.012	-0.120	0.085	-0.026	1.000					
FT/P	-0.290*	0.305**	0.512**	0.374**	0.144	-0.056	-0.236*	-0.050	0.664**	1.000				
PM	-0.225*	-0.226**	-0.286**	-0.204*	-0.003	0.316**	0.221**	0.014	-0.100	-0.075	1.000			
Fo	-0.320**	0.600**	0.710**	0.516**	-0.105	-0.667**	-0.486**	-0.128	0.050	0.058	-0.247**	1.000		
Fv	-0.155	0.249**	0.306**	0.286**	0.013	-0.167*	-0.052	-0.152	0.034	-0.028	0.130	0.537**	1.000	
Fm	-0.208	0.386**	0.466**	0.389**	-0.023	-0.342**	-0.193*	-0.158	0.041	-0.009	0.026	0.740**	0.965**	1.000
Fv/Fm	0.057	-0.223**	-0.312**	-0.194*	0.030	0.412	0.361	-0.093	0.003	-0.076	0.350**	-0.293**	0.621**	0.403**
Fv/Fo	0.039	-0.261**	-0.327**	-0.188*	0.079	0.432**	0.386**	-0.097	-0.006	-0.088	0.358**	-0.308**	0.629**	0.405**
SL	0.535**	0.080	0.121	-0.054	-0.271**	-0.089	-0.094	-0.087	-0.004	0.060	-0.199*	0.095	0.053	0.072
SGW	-0.089	0.305**	0.389**	0.363**	0.090	-0.188*	-0.233**	0.087	0.149	0.300**	-0.129	0.153	0.029	0.071

*Significant differences for $\alpha = 0.05$; **Significant differences for $\alpha = 0.01$

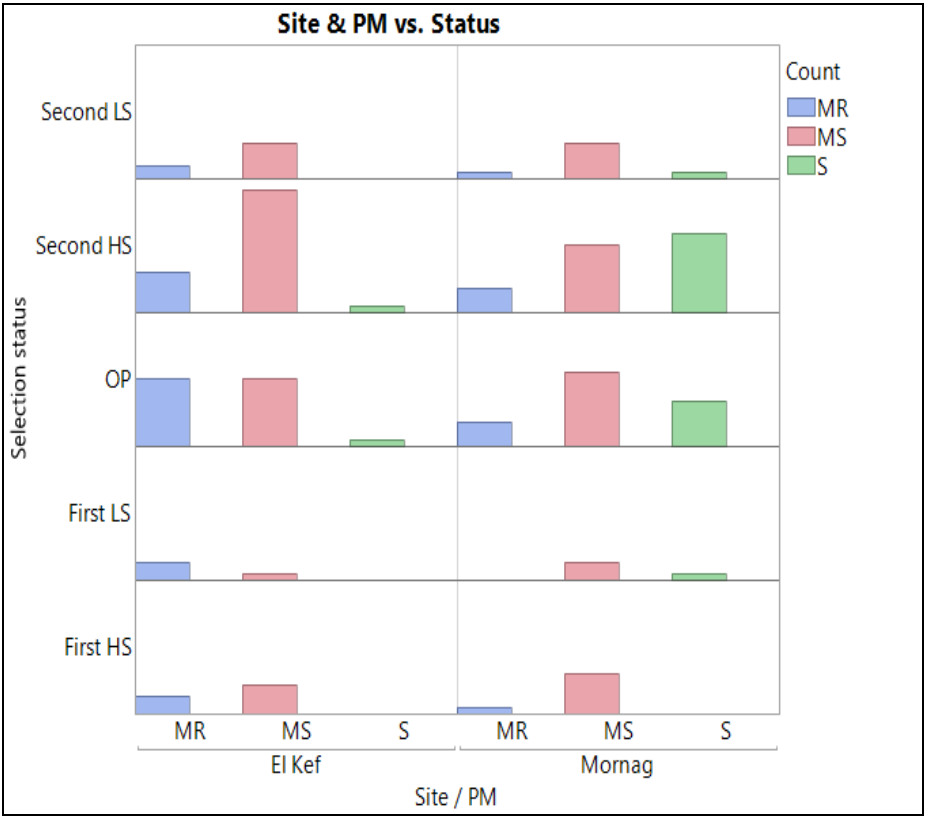

Fig. 3. Entries' response to powdery mildew (PM) at the two sites of experimentation; based on the selection status of the entries. First HS: First cycle of selection high yielding lines; First LS: First cycle of selection low yielding lines; OP: Original populations; Second HS: Second cycle of selection high yielding lines; Second LS: Second cycle of selection low yielding lines.

DISCUSSION

Intra-cultivar heterogeneity has long been recognized in crop species; this phenomenon is often ignored because most researchers assume that elite monogenotypic cultivars are composed of relatively homogeneous genetic pools (Haun et al. 2011). Attributed to genetic homogeneity a priori, only plants obviously of incorrect type are removed in breeder-seed treatment for cultivar maintenance (Parlevliet 2007). Nevertheless, evidence from selection experiments within fairly homogeneous genetic pools suggests that the genome is more flexible and plastic than previously assumed (Yates et al. 2012). Mechanisms that create new variations may accumulate undesirable mutations and gradually contribute to cultivar degradation (Fasoula 1990, 2012; Tokatlidis et al. 2006, 2011).

Regarding its reproductive system, barley is an inbred crop and as such, elite barley cultivars are considered to be genetically homogeneous. Nevertheless, even within fairly homogeneous gene pools, an intrinsic amount of latent genetic variation may still occur, whereas

mechanisms that generate de novo variation may also be present. Residual heterozygosity, due to segregation of parental loci during the breeding process is presumably one source of genetic variation (Haun et al. 2011; Tokatlidis 2015). On the other hand, additional heterogeneity might stem from de novo generated variation, resulting from spontaneous mutations (Ossowski et al., 2010; Shaw et al. 2000) or via genetic and epigenetic mechanisms, such as intragenic recombination, unequal crossing over, gene duplications or deletions, DNA methylation, excision or insertion of transposable elements, chromatin alterations, etc. (Cavrak et al. 2014; Kim and Zilberman 2014; Rasmusson and Phillips 1997; Sani et al. 2013). The original populations (checks) included in this study comprised three commercially released cultivars in Tunisia, i.e. Imen, Manel and Rihane and two Tunisian landraces, i.e. Ardhaoui and Djebali. The study targeted to investigate the stability of yield and plasticity of agronomic and phenological traits in two diversified environments (Kef and Mornag) in Tunisia. In fact, the mean rainfall recorded at Kef during the whole season (325 mm) was higher than that registered at Mornag (295 mm) especially for January and March.

The results showed that the effects due to environment, genotype and genotype×environment (G×E) interaction were significant, which indicates the existence of differences among genotypes for plasticity. Biological and grain yield ranged from 3.72 to 7.13 t/ha in Kef and from 1.46 to 2.66 t/ha in Mornag.

Based on PCA of the phenotypic traits, these barley lines were mainly grouped with respect to their plant height, biological yield and grain yield and the PSII related parameter of F_0. There was strong variability response among the genotypes. According to Fang and Xiong (2015), to overcome drought stress at the physiological level, plants can adjust their rates of photosynthesis by modifying photosystem II, stomatal closure, and low electron transport, carbohydrate and nitrogen metabolism, nucleic acid and protein activity, and growth as a whole.

High positive correlations were revealed between spike grain weight and grain yield, biological yield and grain yield, for both locations. In dry areas, moisture stress is prevalent at all stages, especially grain filling; thus, breeders tend to select material based on grain weight.

The potential of this novel approach to exploit latent or de novo variation within barley cultivars for the development of high-yielding lines under drought stressed conditions is also discussed. On average, high yielding selected lines outperformed in terms of grain yield performance their original populations (i.e. the checks), while the first cycle low yielding lines showed a performance that ranked them underneath the checks. Similar results were found by Tokatlidis et al. (2010) when a set of seven maize hybrids were grown at a range of four densities, in comparison with the normal density (8.33 plants/m), an eightfold greater seed yield per plant at nil competition (0.74 plants/m) was accompanied by a top-to-bottom genotype gap that was 15 times higher. In another one study, two high-yielding bread wheat families were significantly superior over source material in both generations under either low density and/or typical crop density, as well as averaged across the four densities in the split plot trials (Tokatlidis et al. 2005). Second cycle high yielding lines did not differ from the first cycle high yielding lines. On the contrary, the second cycle low yielding lines outperformed the ones of the first cycle, as well as the checks, since they have been subjected

during the first cycle to selection for high yield.

Rasmusson and Phillips (1997) reported that in barley, incremental gains for several traits were made in a very narrow gene pool, attributable to variation present in the original gene pool as well as to de novo variation. The continuous selection within a cultivar is necessary to exploit the potential existence of variation for either cultivar conservation or upgrading, and this target is feasible at the single plant level in the absence of competition (Fasoulas 1993). Christakis and Fasoulas (2002) found exploitable genetic variation for yield in tomato that was uncovered in advanced generations, after the point of achieving theoretical homozygosity (F7 generation). The selection study for modified oil and protein in maize, with selection being practiced effectively for more than 90 generations (Dudley and Lambert 1992), highlights the importance of continuous selection. Fasoula and Fasoula (2000) stated, ‘‘Continuous selection after the release of cultivars is imposed by the need to eliminate deleterious mutations and exploit any positive source of existing and newly derived variation, either genetic or epigenetic”.

Results from multi field evaluation indicated that the selection process applied within each commercial cultivar and landrace succeeded in isolating single-plant progeny lines of high performance. Selection within cultivars, especially for those released earlier, may prove to be a useful technique either to upgrade gradual degeneration of genetic background. Our data fully agrees with the conclusion coming from Tokatlidis (2015) who suggests that selection should be a perpetual process, so that any existing or newly developed variation is exploited and optimal quality of breeder’s seed is secured.

RESUME

Ben Ghanem H., El Felah M., Najar A., Kehel Z., Amri A., Rezgui S. et Tsivelikas A.L. 2018. Performances de lignées d'orge sélectionnées dans des conditions de stress hydrique et à très faible densité. Tunisian Journal of Plant Protection 13 (1): 1-25.

L'imprévisibilité des précipitations et de la température dans la région méditerranéenne est à l'origine d'une irrégularité des conditions environnementales influençant les cultures et engendrant un contexte d'incertitude pour les agriculteurs. Dans cette étude, des lignées d’orge sélectionnées pour la stabilité du rendement en grains en conditions semi-arides et à faible densité de semis "Honeycomb design", ont été évaluées pour leurs performances agronomiques dans des régions semi-arides (Kef et Mornag) suivant le dispositif expérimental "Augmented design" au cours de la campagne 2015/16. La comparaison a été réalisée par rapport aux parents témoins (Manel, Rihane, Imen, Ardhaoui et Djebali). L'analyse de la variance a montré que les rendements, biologique et grainier sont influencés par l’environnement, le génotype et l’interaction génotype × environnement (G × E). En effet, les variations respectives ont été de 3,72 à 7,13 t/ha et de 1,46 à 2,66 t/ha et les valeurs enregistrées au Kef étaient supérieures à celles notées à Mornag. Il est à signaler que cinq lignées-plantes sélectionnées pour leur rendement élevé (IH17 and IH4-H4 provenant de Imen, AH10-H2 et AH10-H3 de Ardhaoui et MH18 de Manel) dépassent les populations d'origine.

Mots clés: Evaluation, faible densité, orge, performances, sélection, semi-aride

ملخص

بن غانم ، هاجر ومودي فتاح وأسماء نجار وزكريا كحيل وأحمد عمري وصلاح رزقي وأثناسيوس ل. تسيفيليكاس، تقييم سلالات شعير منتقاة تحت ظروف الإجهاد المائي وكثافة ضئيلة.
Tunisian Journal of Plant Protection 13 (1): 1-25.

تتميز مناطق المتوسطية بعدم الانتظام في ظروفها المناخية خاصة فيما يتعلق بالأمطار والحرارة. هذا الاضطراب تؤثر سلبا على الزراعات وتؤدي الى الإحساس بالشك لدى المزارعين بما في ذلك المتخصصين في ميدان الحبوب. في هذا الصدد، اعتمدت الدراسة الحالية على طريقة تجريبية معروفة باسم "تصميم قرص العسل" (Honeycomb design) لاختيار السلالات الأفضل ضمن مجموعة معينة وقع بذرها بكثافة ضئيلة. اثر ذلك تمت عملية تقييم المردودية الزراعية للسلالات المنتخبة طبق مثال تجريبي يسمى "التصميم المعزز" (Augmented design) وذلك خلال الموسم 2016/2015 في جهتين مختلفتين من المناطق شبه الجافة (الكاف ومرناق) وقد مكن هذا التقييم بالمقارنة مع الخمس أصناف الأم (إيمان ومنال وريحان وعرضاوي وجبالي) من التأكد من تأثر عناصر الإنتاج بالمناخ والصنف ومدى التفاعل بين هذين العاملين. من ذلك فان المردود البيولوجي قد تراوح بين 3.72 و 7.13 طن/هك بينما تراوح مردود الحب بين 146 و 2.66 طن/هك مع أفضلية لمنطقة الكاف. من ضمن هذه النتائج سجلنا تفوق خمسة سلالات على الأصناف الأصلية منها اثنان وقع انتقاؤهما من ايمان (IH17 و IH4-H4) و اثنان من عرضاوي (AH10-H2 و AH10-H3) وواحدة من منال (MH18).

كلمات مفتاحية: انتقاء، تقييم، شبه الجاف، شعير، كثافة ضئيلة

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Novel Source of Resistance against Root Necrosis and Plant Wilting Disease Caused by *Phytophthora nicotianae* in *Capsicum annuum*

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ABSTRACT

Elbaz, M., Allagui, M.B., and Harbaoui, S. 2018. Novel source of resistance against root necrosis and plant wilting disease caused by *Phytophthora nicotianae* in *Capsicum annuum*. Tunisian Journal of Plant Protection 13 (1): 27-38.

Phytophthora nicotianae the causal agent of root necrosis in pepper was isolated and identified for the first time in Tunisia in 1995. It causes plant wilting and might induce considerable damage especially in infested soils grown with pepper varieties lacking genetic resistance. This disease was observed in green houses as well as in open fields. The present study aimed to identify sources of resistance against *P. nicotianae* in a collection of pepper accessions from the WorldVeg (The World Vegetable Center, previously named AVRDC for Asian Vegetable Research and Development Center) gene bank. These WorldVeg accessions include 28 chili and 12 sweet peppers together with 8 chili and one sweet local cultivars. Seedling reaction to the pathogen was determined one month after inoculation by the zoospores of a highly aggressive isolate deposited at the seedling crown. Results showed that 12 accessions (11 chili and 1 sweet peppers) displayed good resistance to this oomycete. However, fruit quality and productivity of these resistant accessions need to be verified. It is of importance to carry out an analysis on genetic control of such resistance by performing crosses between susceptible and resistant ones, besides studying the suitable use of the unveiled resistance according to the pathogenicity and virulence of the pathogen strains. Therefore, appropriate resistance sources can be used as genitors in a breeding program.

Keywords: Accessions, pepper, resistance, *Phytophthora nicotianae*

Phytophthora nicotianae has been described as a pathogen of a wide range of hosts worldwide covering herbaceous and woody plants. It has been reported on 90 plant families and 255 genera, under this name or as *P. parasitica* (Cline et al. 2008). In India and South Asia, it is considered as one of the most important among *Phytophthora* species. Its host range includes citrus, tobacco, betel, black pepper, eggplant, coconut, guava, orchids, periwinkle, pineapple, and vanilla (Guha Roy and Grünwald 2014). In Cuba, it has been reported on avocado (Machado et al. 2013), while in South

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Africa, it was associated to eucalyptus plants (Nagel et al. 2013).

Recent surveys conducted in Tunisia revealed that, together with various *Pythium* spp., *P. nicotianae* was associated with the apple tree dieback disease while *P. cactorum* the most known causal agent of this disease was no longer detected (Souli et al. 2014). These authors also reported that *P. nicotianae* was highly aggressive on several apple cultivars. In Tunisia, this pathogen was found to be responsible for root and crown necrosis leading to wilting and death of pepper (*Capsicum annuum*) plants (Allagui et al. 1995; Kendrick 1923; Nolla 1929; Tasugi and Ikeda 1939), even though *P. capsici,* the major pathogen in the main pepper-growing areas worldwide, was not detected in the country.

This oomycete is occurring in most pepper production areas in Tunisia (*i.e.* Cap-Bon and East-Center regions) in both protected and open field crops leading to considerable yield losses and fruit size alterations. Chemical control of this soilborne disease is slightly efficient. Resistant varieties are an essential component of the sustainable disease management approaches aiming to preserve environment and to reduce production cost. A few reports only could be cited as researches dedicated to identify pepper resistance sources against *P. nicotianae* (Allagui 1994; Andrés et al. 2003; Andrés et al. 2006; Saadoun and Allagui 2008). In Spain, a screening of local *C. annuum* germplasm was performed in order to search potential sources of resistance to *P. nicotianae* but no complete resistance was found (Andrés et al. 2006). In Tunisia, Saadoun and Allagui (2008) tested a collection of 15 *P. nicotianae* isolates having different pathogenic degrees against four pepper local varieties together with a susceptible (Baker) and a resistant landrace (CM334) controls. None of the tested local plant material (namely Baklouti, D'hirat, Beldi and Nabeul II varieties) showed a good level of resistance to *P. nicotianae*. In order to search for other sources of resistance to *P. nicotianae*, a collection of *C. annuum* germplasm was acquired from The WorldVeg and tested in the current study for its response to the target pathogen.

MATERIALS AND METHODS

Plant material.

The pepper plants used represented 40 genotypes (Tables 1 and 2) acquired from WorldVeg gene Bank (Taiwan), 28 of which were chili pepper accessions and 12 sweet pepper accessions. The remaining were local cultivars, including Baklouti, Chaabani, Beldi, Doux de Teboulba and Baker, with Baker and CM334 being chosen as susceptible (Saadoun and Allagui, 2008) and resistant (Bnejdi et al. 2009) control cultivars, respectively.

Pepper seeds were surface-sterilized with 5% sodium hypochlorite, rinsed twice with sterile distilled water and dried on filter paper. Then, they were sown in a sterilized substrate mixture of clay soil, sand and peat (1:1:1 in volume). The sowing trays were kept in growth chamber at 22-28ºC with a photoperiod of 16 h at 30,000 lux. Same conditions were applied during seedling growth period and post-inoculation period. At two cotyledon stage, seedlings were transplanted into plates with the same substrate used for sowing.

Table 1. Chili pepper accessions tested for their response to *Phytophthora nicotianae* infection

Code	Original code		Number of inoculated plants	Origin
INC-1	1.	VI041280 (PBC142)	10	WorldVeg (Taiwan)
INC-2	2.	AVPP0302	9	
INC-4	3.	AVPP9813	5	
INC-5	4.	AVPP0304	4	
INC-6	5.	AVPP0305	10	
INC-7	6.	AVPP9905	10	
INC-8	7.	AVPP0306	10	
INC-11	8.	VI041280 (PBC142-A)	10	
INC-12	9.	AVPP0903	6	
INC-13	10.	AVPP0904	7	
INC-14	11.	AVPP9813	6	
INC-15	12.	AVPP0905	10	
INC-16	13.	AVPP0906	10	
INC-19	14.	AVPP0908	7	
INC-20	15.	AVPP0909	10	
INC-21	16.	AVPP9703	10	
INC-22	17.	AVPP9704	10	
INC-23	18.	AVPP9801	10	
INC-24	19.	AVPP9806	10	
INC-25	20.	AVPP9811	10	
INC-26	21.	AVPP0606	8	
INC-27	22.	AVPP0706	10	
INC-28	23.	AVPP9805	9	
INC-29	24.	AVPP0507	10	
INC-30	25.	AVPP0710	6	
INC-31	26.	AVPP0711	10	
INC-32	27.	AVPP0516	10	
INC-33	28.	AVPP9911	10	
TN-1	29.	Baklouti	10	Tunisia
TN-2	30.	Arbi 1	10	
TN-3	31.	Arbi2	10	
TN-4	32.	Beldi	6	
TN-5	33.	RLO	10	
TN-10	34.	Arbi 3	10	
Baker	35.	Baker	10	
TN-8	36.	Chaabani	10	

Pathogen isolates.

Twenty seven *P. nicotianae* isolates recovered from diseased pepper plants were used in the current study. They were isolated from north eastern region in Tunisia (Cap-Bon): 25 of them (Pnt372-4, Pnt367-0, Pnt367-10, Pnt373-2, Pnt374-1, Pnt376-2, Pnt367-3, Pnt373-1, Pnt367-8, Pnt370, Pnt367-4, Pnt367-1, Pnt368-1, Pnt369-1, Pnt372-2, Pnt372-3, Pnt367-9, Pnt367-6, Pnt372-5, Pnt374-2, Pnt367-7, Pnt368', Pnt367-5, Pnt369-3 and Pnt368) were collected in 2009, one collected in 2005 (Pnt365) and the last one in 2011 (Pnt371).

Pathogen was isolated from the roots of infected plants on $P_{10}VP$ selective pythiaceous medium (Tsao and Ocana

1969), and pure isolates were conserved on Potato Dextrose Agar (PDA) medium until use. According to analysis based on morphological and molecular criteria, the above mentioned isolates were identified as *P. nicotianae* (Allagui et al. 1995; 2000). Pepper plants infected by this oomycete usually show general wilting associated with root necrosis. These 27 isolates showed similar symptoms as those observed during field prospection. In addition, their morphological criteria were typical of those of *P. nicotianae*.

Table 2. Sweet pepper accessions tested for their response to *Phytophthora nicotianae* infection

Code	Original code		Number of inoculated plants	Origin
INS-1	1.	AVPP9807	4	WorldVeg (Taiwan)
INS-2	2.	AVPP0110	4	
INS-8	3.	AVPP0114	10	
INS-14	4.	AVPP0910	4	
INS-16	5.	AVPP9904	10	
INS-17	6.	AVPP0006	10	
INS-18	7.	AVPP0108	10	
INS-19	8.	AVPP0118	10	
INS-22	9.	AVPP0601	10	
INS-23	10.	AVPP0602	10	
INS-24	11.	AVPP0503	10	
INS-25	12.	AVPP0504	10	
TN-7	13.	Doux de Teboulba	10	Tunisia

Inoculum preparation.

P. nicotianae isolates were grown on PDA at 25°C for 10 days. Each inoculum was prepared by growing isolate samples on pea agar medium in 90 mm Petri dishes (Allagui and Lepoivre 2000). Sporangia were formed by growing cultures under light at 25±3ºC for 7 days. Then, sterile distilled water was poured in the Petri dishes, under aseptic conditions. The Petri dishes were kept at 25ºC for 2 days, followed by a short incubation at 4°C for 30 min, and finally they were kept at room temperature during few minutes to release zoospores. Zoospore counting was performed under a light microscope (Gx100) using a hemocytometer (Malassez cell) where the final concentration of the inoculum was adjusted to 8 10^4 zoospores/ml. At the 4-true-leaf stage, the crown of each plant was inoculated with 4 ml of the zoospore suspension.

Pathogenicity tests.

All of the 27 *P. nicotianae* isolates were tested for their pathogenicity on pepper Baker susceptible cultivar (Saadoun and Allagui 2008). In pathogenicity and cultivar response screening tests, pepper plants were inoculated at the 4-true-leaf stage by dripping a suspension of 320,000 zoospores (in 4 ml) onto the crown of each plant.

At one month post-incubation, the root system of each seedling was delicately separated from the substrate and washed under running water. The

intensity of root necrosis was evaluated according to an arbitrary scale from 0 to 5 (Fig. 1) where 0 (healthy plant), 0.5 (necrosis limited to the extremity of radicles), 1 (necrosis on the lower half of primary roots), 2 (necrosis all over the primary roots), 3 (necrosis reaching the crown and the lateral roots), 4 (hypocotyl rotten), and 5 (whole plant dead) (Allagui and Lepoivre 2000; Kim and Hwang, 1992). For each tested pepper cultivar, the number of plants evaluated varied from 4 to 10 (Tables 1 and 2).

Pepper cultivars were classified at different resistance levels depending on their respective mean root necrosis scores (mrns) as highly resistant (HR): mrns < 1; resistant (R): 1 ≤ mrns < 1.5; merely resistant (MR): 1.5 ≤ mrns < 2; susceptible (S): 2 ≤ mrns < 2.5 and highly susceptible (HS): mrns ≥ 2.5.

Fig. 1. Disease rating scale used for the classification of the response of pepper plants to *Phytophthora nicotianae* infection.

Resistance assessment at late growth stage.

According to Allagui and Lepoivre (1996), the death of the plant following *P. nicotianae* infection may occur late at the stage of full production. Hence, pepper plants with disease intensity scores ≤ 1.5 were transplanted and grown for another period under greenhouse conditions, and periodically surveyed for any symptom development.

Statistical analysis.

Data were subjected to one-way analysis of variance (ANOVA) using IBM SPSS (Statistical Package for the Social Sciences) software for Windows version 20.0 (IBM Corp. 2011). Mean values of root rot necrosis intensity were calculated for each pepper cultivar. Means were separated using Student–Newman–Keuls (SNK) test to identify significant pairwise differences at $P = 0.05$.

RESULTS

Preliminary inoculation tests, including Baker susceptible cultivar, showed that among the 27 isolates only 18 were pathogenic (Fig. 2). The 18 *P. nicotianae* isolates induced root necrosis symptoms with different intensities: the mrns varied from 0.4 to 5. *P. nicotianae* Pnt367-3 isolate from Korba region induced the highest level of disease intensity as compared to the other isolates. Thus, this isolate was selected to be used for the screening of chili and sweet pepper germplasm collections for their response to *P. Nicotianae* infection.

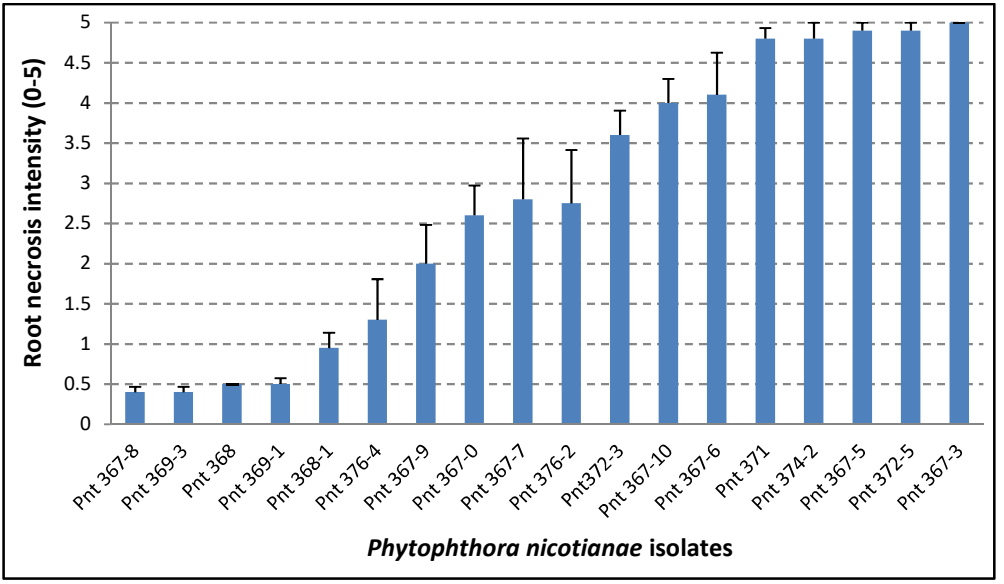

Fig. 2. Root necrosis intensity induced on pepper cv. Baker following infection with different *Phytophthora nicotianae* isolates as scored at one month after seedling inoculation.

Fig. 3 illustrates the results from the chili pepper collection challenge. Six pepper cultivars had mean root necrosis scores lower than 1 (namely INC-8, INC-19, INC-23, INC-26, INC-29 and INC-32) and were considered as highly resistant (HR) to *P. nicotianae*. Four cultivars exhibited mean root necrosis scores higher than 1 and lower than 1.5 (INC-6, INC-7, INC-11 and INC-27) and ranked as resistant (R). Two more cultivars had their mean root necrosis scores higher than 1.5 and lower than 2 (INC-2 and INC-28) and were qualified as merely resistant (MR). The three pepper cultivars (INC-1, INC-33 and TN-1) showing root necrosis level higher than 2 and less than 2.5 were classified as susceptible (S). All of these cultivars are pepper accessions acquired from the WorldVeg gene bank, except TN-1 (Baklouti) which is a local cultivar. All the remaining 21 cultivars showed root necrosis intensity higher than 2.5 and were ranked as highly susceptible (HS). Therefore, all local pepper cultivars (namely TN-1, TN-2, TN-3, TN-4, TN-5 and TN-8) are among the HS and S classes of cultivars.

Statistical mean comparison using SNK test (at P=0.05) showed that pepper cultivars labeled as HR and R to *P. nicotianae* Pnt367-3 isolate were clearly separated from the S and the HS ones (Fig. 3). However, differences between HR, R and MR chili pepper cultivars were not significant. The MR cultivars do not separate significantly from the S cultivars, neither from some of the HS cultivars.

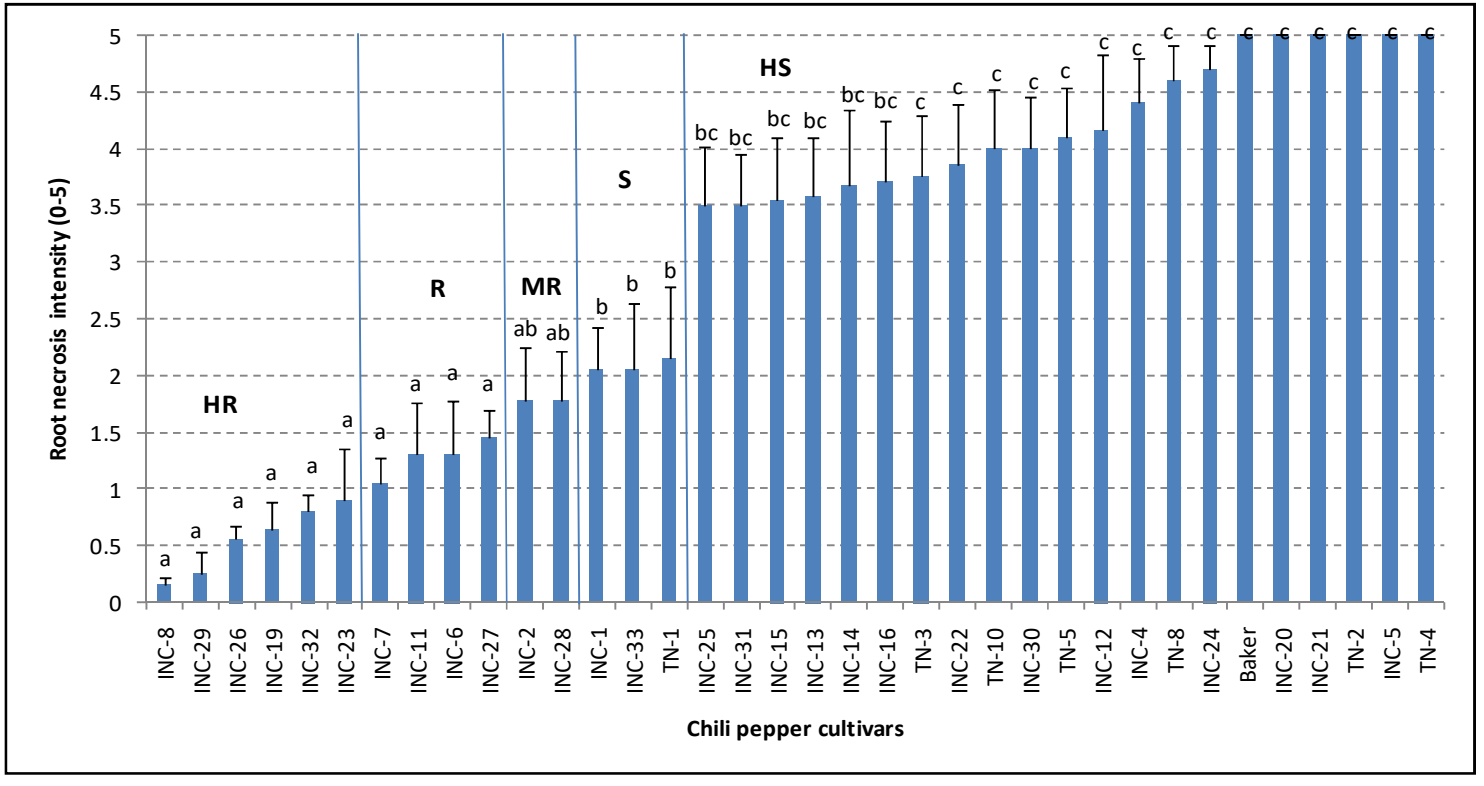

Fig. 3. Root necrosis intensity caused by *Phytophthora nicotianae* isolate Pnt367-3 on chili pepper collection (23 cvs) as scored at one month after seedling inoculation. HR: Highly Resistant; R: Resistant, S: Susceptible; HS: Highly Susceptible. Means sharing the same letter are not significantly different based on SNK test at $P < 0.05$.

Among the sweet pepper germplasm collection tested, all cultivars, except two (INS-14 and INS-19) were classified as HS to *P. nicotianae* Pnt-367-3 isolate. INS-19 was ranked as HR cultivar whereas INS-14 was found to be MR to infection with this isolate (Fig. 4). None of these two is a local cultivar. Both HR and MR cultivars were significantly separated from the cultivars classified as HS including the local TN-7 (Doux de Teboulba) cultivar. In addition, differences in the intensity of root necrosis induced by INS-19 and INS-14 were statistically significant.

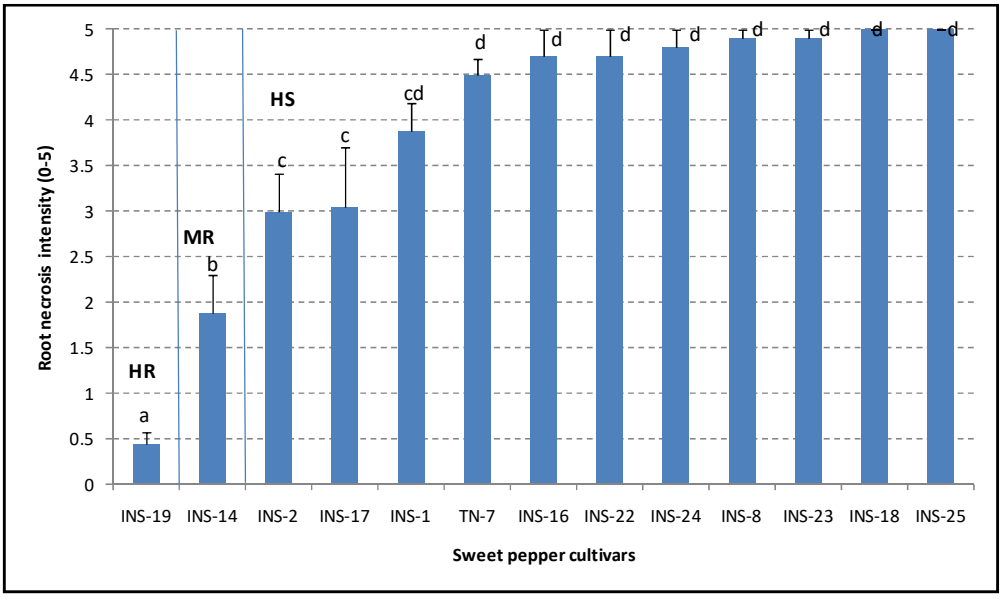

Fig. 4. Root necrosis intensity caused by *Phytophthora nicotianae* isolate Pnt367-3 on sweet pepper collection noted at one month after seedling inoculation. HR: Highly Resistant; MR: Merely Resistant; HS: Highly Susceptible cultivars. Means sharing the same letter are not significantly different based on SNK test at $P< 0.05$.

Illustration of pepper cultivars expressing good resistance levels to *P. nicotianae* is given in Fig. 5 where obvious differences in the root necrosis were noted when compared to resistant and susceptible cultivars.

Moreover, based on results from inoculation tests, all pepper cultivars with disease severity scores ≤ 1.5 were under observation after transplantation in greenhouse. None of them had shown any wilting symptoms during the growing period until full fruit maturity.

To our knowledge, this is the first research work allowing identification of pepper genotypes with complete resistance to *P. nicotianae*.

Fig. 5. Average scoring of pepper root necrosis caused by *Phytophthora nicotianae* isolate Pnt367-3 noted at one month after seedling inoculation. (A) INC-23, chili pepper with root necrosis intensity scoring 0.9; (B) TN2, chili pepper with root necrosis intensity scoring 5 and (C) INS-19, sweet pepper with root necrosis intensity scoring 0.45.

DISCUSSION

In Tunisia, *P. nicotianae* is considered as the main collar and root rot pathogen of pepper. It is a serious threat to pepper crop in both greenhouses and open fields. The disease management is generally ineffective especially when it is only based on chemical control. During the last decade, investigation efforts have been deployed to suggest complementary alternatives for disease control. Soil solarization was shown to be effective in reducing the percentage of wilted pepper plants and in improving plant vigor and yield (Boughalleb and El Mahjoub 2005). Grafting was also investigated and evidence was given on resistance enhancement, but not complete resistance, with two different rootstocks: namely Brutus (M'Hamdi et al. 2009) and CM334 (Saadoun and Allagui 2013). A more recent work reported on the efficacy of *Brassica* green manures combined with solarization against the viability and the infectivity of *P. nicotianae* chlamydospores in the soil (Lacasa et al. 2015). Nonetheless, all these alternatives are laborious techniques and require supplementary production cost and technical knowledge as well. Accordingly, the use of resistant varieties remains an inexpensive and easy mean for disease control. From previous investigations intending to screen Tunisian local pepper cultivars for resistance to *P. nicotianae*, none of them showed a complete resistance (Saadoun and Allagui 2008). Indeed, Baklouti cultivar was the local pepper performing the best, compared to Nabeul II, D'hirat and Beldi, varieties, showing a mean intensity of root necrosis estimated at 2.4 following its inoculation with the most aggressive *P. nicotianae* used isolate (Saadoun and Allagui 2008). Our data showed similar results as Baklouti cultivar (TN-1) exhibited a mean root necrosis intensity equal to 2.15. Ultimately, the present work came out with 7 highly resistant pepper genotypes (6 chili peppers and 1 sweet pepper), in addition to 4 chili resistant genotypes: total of 10 genotypes of chili pepper and 1 genotype of sweet pepper are so

selected for further research. Obviously, resistance level as evaluated during this work is promising since pepper genotypes were challenged by the most aggressive *P. nicotianae* isolate from our pathogen collection. Under natural growing conditions, it is expected that inoculum infectivity is lower and consequently pepper selected genotypes may display better resistance performance to *P. nicotianae*. Our results provide valuable information useful in breeding programs. Moreover, some of the selected pepper genotypes might be submitted to registration in the national official catalog as new pepper varieties, based on their agronomic traits and yield performance.

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RESUME

Elbaz M., Allagui M.B. et Harbaoui S. 2018. Nouvelle source de résistance contre la maladie de la nécrose racinaire et du flétrissement des plantes causée par *Phytophthora nicotianae* chez *Capsicum annuum*. Tunisian Journal of Plant Protection 13 (1): 27-38.

Phytophthora nicotianae, l'agent causal de la nécrose racinaire et du flétrissement du piment, a été isolé et identifié en Tunisie pour la première fois en 1995. Cet agent phytopathogène provoque le flétrissement des plantes et peut causer des dégâts considérables surtout dans le cas des sols infestés en présence de variétés cultivées dépourvues de résistance vis-à-vis de ce pathogène. Cette maladie a été observée dans les serres ainsi que dans les cultures de plein champ. La présente étude vise à identifier des sources de résistance contre *P. nicotianae* parmi une collection d'accessions de piment provenant de la banque de gènes du WorldVeg. Ces accessions comprennent 28 piments forts et 12 piments doux ainsi que 9 cultivars de piments locaux dont 8 forts et 1 doux. La réaction des plants de piment à l'agent pathogène a été déterminée un mois après l'inoculation au collet des plantules, par des zoospores d'un isolat hautement agressif. Les résultats ont montré que 11 accessions (10 piments forts et 1 piment doux) présentent une bonne résistance à cet oomycète. Cependant, la qualité des fruits et la productivité de ces accessions résistantes doivent être vérifiées. Il est important de mener une analyse portant sur la détermination du contrôle génétique de cette résistance par des croisements entre individus sensibles et résistants, en plus d'une étude de l'utilisation adéquate de la résistance identifiée tenant compte de la pathogénie et de la virulence des souches du pathogène. Ainsi, les sources de résistance appropriées peuvent être utilisées en tant que géniteurs dans un programme de sélection.

Mots clés : Accessions, piment, résistance, *Phytophthora nicotianae*

ملخص

□باز، منيرة ومحمد □بشير علاقي وسرور □رباوي. 2018. مصدر جديد□لمقاومة ضد مرض تعفن□جذور وذبول □نبتة□ناجم عن *Phytophthora nicotianae* في□فلفل *Capsicum annuum*.

Tunisian Journal of Plant Protection 13 (1): 27-38.

تم عزل شبه الفطر *Phytophthora nicotianae* العامل السببي لتعفن الجذور وذبول النبتة على الفلفل وشُخص لأول مرة في تونس في عام 1995. وهو يسبب ذبول النباتات ويمكن أ ينتج أضرارا كبيرة خاصة في حالة تربة مصابة مع استعمال أصناف تفتقر إلى مقاومة جيدة عند الزراعة. وقد لوحظ هذا المرض الفطري في البيوت المحمية وكذلك في الحقول. تهدف هذه الدراسة إلى تشخيص مصادر المقاومة ضد *P. nicotianae* ضمن مجموعة من أصناف الفلفل وقع

جلبها من بنك الجينات WorldVeg. وتشتمل هذه الأصناف من الفلفل على 28 صنفا من الفلفل الحار و12 صنفا من الفلفل الحلو إلى جانب 9 أصناف محلية من بينها 8 أصناف من الفلفل الحار وصنف واحد من الفلفل الحلو. وقد تم تحديد تفاعل الشتلات مع المرض بعد شهر من الإلقاح بمعلق أبواغ هدبية لسلالة عالية الضراوة على المستوى القاعدي لتاج النبتة. بينت النتائج أن 12 صنفا من الفلفل (11 من الفلفل الحار وصنف واحد من الفلفل الحلو) أظهرت مقاومة جيدة لهذا المرض. ومع ذلك فإنه يجب التحقق من جودة الثمار وإنتاجية هذه الأصناف المقاومة. ومن المهم أيضا وضع دراسة يتم من خلالها تشخيص العوامل الوراثية المحددة لهذه المقاومة عن طريق التهجين بين أصناف ذات مقاومة جيدة وأخرى حساسة، إضافة إلى دراسة الاستخدام المناسب للأصناف المقاومة المكتشفة وفقا لقدرة سلالات المرض على التسبب في ظهور الأعراض ودرجاتها. يمكن استخدام تلك الأصناف المقاومة ضمن برنامج للتحسين الوراثي.

كلمات مفتاحية: أصناف، فلفل، مقاومة، *Phytophthora nicotianae*

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Antifungal Activity of Essential Oils of *Origanum majorana* and *Lavender angustifolia* against *Fusarium* Wilt and Root Rot Disease of Melon Plants

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ABSTRACT

Dhaouadi, S., Rouissi, W., Mougou-Hamdane, A., Hannachi, I., and Nasraoui, B. 2018. Antifungal activity of essential oils of *Origanum majorana* and *Lavender angustifolia* against Fusarium wilt and root rot disease of melon plants. Tunisian Journal of Plant Protection 13 (1): 39-55.

The objective of this study was to evaluate the antifungal activity of essential oils of marjoram (*Origanum majorana*) and lavender (*Lavender angustifolia*) against eleven isolates of *Fusarium oxysporum* f. sp. *melonis* and ten isolates of *Fusarium solani*, the causal agents of Fusarium wilt and root rot disease of melon. The effect of essential oils on disease development under in vivo conditions was also tested. GC-MS analysis of marjoram essential oils showed that terpinen-4-ol (34.94%) is the major component, followed by *γ*-terpinene (24.66%), *α*-terpinene (13.22%), *β*-terpinene (5.84%), *α*-terpineol (3.98%), and *β*-phellandrene (3.16%). Chemical analysis of lavender essential oils showed that *α*-terpinene (48.76%) is the major component, followed by linalool (16.79%), *γ*-terpinene (7.00%), *β*-trans-ocimane (6.47%), *β*-caryophyllene (5.83%), and lavandulol (3.23%). All essential oils tested in vitro using the disk diffusion method revealed a significant antifungal effect against mycelium growth of all *F. oxysporum* f. sp. *melonis* and *F. solani* isolates. The volatile compounds of essential oils have completely inhibited spore germination of both pathogens. In vivo, the essential oils applied as biofumigant significantly reduced disease severity on melon plants 20 days post-incubation. Lavender essential oils significantly reduced disease severity by almost 60% as compared to control melon plants while Marjoram essential oils reduced disease severity by almost 23% under controlled conditions. These results showed that lavender essential oils may contribute to the development of new antifungal compounds to protect melon crops from Fusarium wilt and root rot disease.

Keywords: Essential oils, disease severity, *Fusarium oxysporum*, *Fusarium solani*, lavender, marjoram, melon

Melon (*Cucumis melo*) is a vegetable crop of the Cucurbitaceae family with wide botanical varieties and native to Africa (Janick et al. 2007; Pitrat et al. 2000). Worldwide, almost 28,3 million of tons are produced each year and nearly half produced in China (FAOSTAT, 2014). Like all warmer regions of the Mediterranean Sea, melon is widely grown in Tunisia. It is considered as the most economically important cucurbit crop after watermelon

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and known by its valuable nutritional components (GIL 2015). It is mainly cultivated in open fields (8060 ha) in the regions of Beja, Jendouba, Sfax, Gafsa, Tozeur's oases, Gabes, Kairouan, Sidi Bouzid with limited acreage in the Sahel (Trimech et al. 2013).

Melon production in Tunisia is mostly affected by vascular wilt disease caused by *Fusarium oxysporum* f. sp. *melonis* and root and crown rot caused by *Fusarium solani* f. sp. *cucurbitae* (El Mahjoub and Ben Khedher 1987). Fusarium wilt disease of melon remains a constant problem and there is no effective strategy to eradicate it as causal agents survive in the soil for years and even on roots of symptomless plants (Martyn 1996). The management strategies of Fusarium-incited diseases are generally limited to harmful and inefficient chemical products, as for all soil-borne diseases. Therefore, the search of non-chemical strategies, reducing pesticide use and the development of resistant genes to fungicides by certain pathogens, limited the application of synthetic chemicals. Resistant rootstocks and cultivars (Nelson 1981), crop rotation and pre-planting soil disinfestations (Baker 1981) are the most effective control methods for the control of wilt diseases. Additional non-chemical strategies including antagonistic strains, bio-organic fertilizers, natural plant extracts and essential oils have been proved to be effective to control Fusarium disease (De Cal*et al*. 1995; Larkin and Fravel 1998). Antifungal and disease control effects of essential oils and their major active compounds responsible of their antifungal properties have been reported by many studies in the world (Silva et al. 2011; Tserennadmid et al. 2011). Several reports indicate that essential oils can effectively control pathogenic microorganisms without promoting the acquisition of resistance (Ohno et al. 2003). Therefore, using essential oils as an alternative to control Fusarium disease of many Cucurbitaceae and Solanaceae crops has been expanded during the last decades. In fact, Nosrati et al. (2011) found that antifungal activity of spearmint essential oils controlled *F. oxysporum* f. sp. *radicis-cucumerinum*, the causal agent of stem and crown rot of greenhouse cucumber. To control Fusarium crown and root rot and Fusarium wilt of tomato caused respectively by *F. oxysporum* f. sp. *radicis-lycopersici* and *F. oxysporum* f. sp. *lycopersici*, Arici et al. (2013) demonstrated that cumin, thymus, lavender, eucalyptus, rosemary, nigella and dill essential oils, showed interesting antifungal activity against these pathogens. Volatile compounds responsible of this antifungal activity are mostly molecules of terpenes, terpenoids and phenol, derived aromatic and aliphatic compounds, which have not only fungicidal activities but also bactericidal and viricidal properties as well (Rao et al. 2010). Indeed, many authors (Gergis et al. 1990; Panizzi et al. 1993; Pellecuer et al. 1980; Sivropoulou et al. 1996) reported that essential oils extracted from Lamiaceae plants contain phenolic compounds, which are well known for their antimicrobial activities. Among these plants, marjoram, belonging to the genus *Origanum*, is known for the production of essential oils rich in phenolic compounds, like thymol and its isomer carvacrol which have strong antifungal and antimicrobial properties. Lavender also belonging to this family, and its main components, carvacrol, linalool and linalyl acetate, displayed strong fungistatic and fungicidal activity against *Candida albicans* clinical strains (D'Auria et al. 2005). However, the information of the antifungal activity of lavender and

marjoram essential oils against Fusarium wilt disease of melon is limited. Therefore, this study was conducted to: (1) identify the major chemical components of lavender and marjoram essential oils; (2) evaluate their in vitro antifungal activity toward *Fusarium oxysporum* f. *melonis* and *F. solani* isolates; and (3) assess their ability to reduce disease severity under greenhouse conditions.

MATERIALS AND METHODS

Extraction and yield of essential oils.

Plant materials selected in the present study consisted of aerial parts (leaves and stems) of aromatic plants of marjoram (*Origanum majorana*) and lavender (*Lavender angustifolia*). The Botany laboratory in the Regional Research Centre on Horticulture and Organic Agriculture of Chott-Mariem (CRRHAB), University of Sousse, Tunisia, identified and deposited the plants in the department of herbarium. Fresh aerial part of marjoram and lavender (1000 g of each sample) were subjected to the Clevenger-type apparatus hydro-distillation for approximately 4 h in accordance with the method recommended by Kumar and Tripathi (2011). Three replicates of each fresh biomass were subjected to hydro-distillation for estimation of essential oil composition and yield. The average yield of essential oils extracted (EO_y (%) by Clevenger apparatus was determined as follows:

$$EOy(\%) = \frac{\text{Weight of the oil}}{\text{Weight of the material taken}} \times 100$$

Extracted oils were stored at 4°C and protected from light prior to chemical analysis and use.

GC analysis.

Elmer Autosystem XL gas chromatograph (Perkin Elmer, Shelton, CT, USA) equipped with two flame ionization detectors (FIDs) was used in this study. A data handling system and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused silica column (30 m × 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific Inc., Rancho Cordova, CA, USA) and a DB-17HT fused silica column (30 m × 0.25 mm i.d., film thickness 0.15 µm; J & W Scientific Inc.). The oven temperature was programmed at 45-175°C, at 3°C/min, then subsequently at 15°C /min up to 300°C, and held isothermal for 10 min; injector and detector temperatures were 280 and 300°C, respectively; the carrier gas, hydrogen, was adjusted to a linear velocity of 30 cm/s. The samples were injected using the split sampling technique, ratio 1:50. The volume of injection was 0.1 µl of a pentane-oil solution. The percentage composition of the oils was computed by the normalization method from the GC peak areas, calculated as mean values of two injections from each oil, without using correction factors.

GC/MS analysis.

The GC-MS unit consisted of a Carlo Erba 6000 Vega gas chromatograph, equipped with a DB-1 fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 µm; J&W Scientific Inc.), interfaced with a Finnegan MAT 800 Ion Trap Detector (ITD; software version 4.1). The oven temperature was programmed to 45-175°C, at 3°C /min, subsequently at 15°C /min up to 300°C, and then held isothermal for 10 min. The temperature conditions of the transfer line and ion trap were 280 and 220°C, respectively. The linear velocity of the carrier gas, helium, adjusted to 30 cm/s and the split ratio to 1:40. Furthermore, the ionization energy was 70 eV, the ionization current, 60 µA, the scan range,

40-300 u and the scan time, 1 s. The identity of the components was assigned by comparison of their retention indices, relative to C_9-C_{16} n-alkanes, with GC-MS corresponding data of reference oil components, laboratory synthesized components and commercially available standards from a home-made library, constructed based on the analyses of reference oils, laboratory-synthesized components and commercial available standards.

Isolates of plant pathogenic fungi.

Eleven isolates of *F. oxysporum* f. sp. *melonis* and ten isolates of *F. solani* used throughout the trials are represented in Table 1 and belonged to Laboratory of Phytopathology of the *Institut National Agronomique de Tunisie* (INAT), Tunisia. Fungal isolates were originally isolated and identified from melon plants grown in different open fields in Tunisia and exhibiting typical symptoms of Fusarium wilt and root rot disease as described by Champaco et al. (1993). Monosporal cultures were transplanted in Petri dishes on Potato Dextrose Agar medium (PDA, Tunmedia, SARL, Tunisia) and incubated for 7 days at 25°C until sporulation.

Table 1. *Fusarium* spp. isolates used in this study

Isolate code	Species	Organ	Sampling site
S1	*F. oxysporum* f. sp. *melonis*	Stem	Beja
S2	*F. oxysporum* f. sp. *melonis*	Collar	Beja
S3	*F. oxysporum* f. sp. *melonis*	Root	Beja
S4	*F. oxysporum* f. sp. *melonis*	Leaf	Beja
S5	*F. oxysporum* f. sp. *melonis*	Stem	Beja
S6	*F. oxysporum* f. sp. *melonis*	Leaf	Beja
S7	*F. oxysporum* f. sp. *melonis*	Stem	Slimane
S8	*F. oxysporum* f. sp. *melonis*	Stem	Slimane
S9	*F. oxysporum* f. sp. *melonis*	Collar	Slimane
S10	*F. oxysporum* f. sp. *melonis*	Root	Mahdia
S11	*F. oxysporum* f. sp. *melonis*	Root	Mahdia
S12	*F. solani*	Stem	Beja
S13	*F. solani*	Root	Beja
S14	*F. solani*	Stem	Beja
S15	*F. solani*	Stem	Beja
S16	*F. solani*	Stem	Beja
S17	*F. solani*	Leaf	Slimane
S18	*F. solani*	Collar	Slimane
S19	*F. solani*	Root	Slimane
S20	*F. solani*	Root	Grombalia
S21	*F. solani*	Root	Grombalia

In vitro antifungal activity bioassay on mycelial growth.

Antifungal effect of marjoram and lavender essential oils on mycelium growth of *Fusarium* spp. isolates was performed using agar disk diffusion method (Lahlou 2004). For this aim, a plug of 6 mm diameter was removed from the periphery of a 7-day-old culture of each isolate. The plugs were placed upside down on the edge of PDA Petri dishes. On the opposite side, a well of 6

mm diameter was made in the agar medium and filled with 5 µl of undiluted essential oils. The control sets were prepared using equal amounts of sterile distilled water instead of oils. The plates were sealed with polyethylene film and incubated at 25°C until the growth in the control plates reached the edge of the plates. The plates were used in triplicate for each treatment. Percentage inhibition of the radial growth by different oils compared to control was calculated using the following formula (Albuquerque et al. 2006):

Percentage mycelial inhibition = [(DC – DS) / DC] × 100; where DS is the mean colony diameter in control plates and DS is the mean colony diameter in treated plates. Fungal colony diameter values were analyzed separately for each *Fusarium* isolate due to their different growth rates.

In vitro antifungal activity bioassay on spore germination.

To evaluate the antifungal activity of volatile compounds of essential oils on pathogen spore germination, bioassay was performed using a modified method of evaporation of the essential oils from the filter paper on the top of agar plate. Conidial suspension obtained by washing fungal culture with sterile distilled water. Spore concentration was prepared under microscope using haemacytometer following appropriate dilutions and adjusted to 10^7 spores/ml of each fungal isolate. PDA plates were inoculated with 100 µl of conidial suspension. The Petri plates were placed with the lid upside down. A volume of 2.5 µl of each undiluted essential oils was added to a sterile filter paper placed on the lid of plates without agar neither direct contact with the inoculated agar plate. Control plates without essential oils contained sterile distilled water and pathogen spore suspension. The plates were sealed with polyethylene film and incubated at 25°C for 24 h. Tests were undertaken in triplicate. To determine the percentage of spore germination inhibition, the number of spores germinated was scored using haemocytometer and compared to control plates.

Effect of essential oils on Fusarium wilt and root rot disease in planta.

Melon seedlings 'Afamia' grown under greenhouse conditions in Select Plant (El Haouaria, Nabeul, Tunisia) were used in this experiment. These seedlings were previously treated by foliar sprays with standard insecticides (Tracer 240 SC, INNOVA AGRI) and fungicides (Fungastop, AGRIPROTEC) under national regulations. Five isolates of *F. oxysporum.* f. sp. *melonis* and five isolates of *F. solani* were selected based on their sensitivity to tested essential oils previously demonstrated in vitro (around 50% of inhibition). Fungal culture was grown on PDA medium at 25°C for 7 days, washed from the plates, and suspended in sterile distilled water. Fungal conidial suspension was adjusted to approximately 10^6 spores/ml. The essential oil treatment was performed using the biofumigation method with slight modifications. Thirty melon seedlings of five-week-old per replicate, grown in PEAT MOSS FINE mix (Général horti services, Tunisia) were first inoculated by injecting 1 ml of spore suspension of each isolate around roots. Two hours after pathogen inoculation, a volume of 1 ml of each essential oil was dissolved in 100 ml of 0.1% Tween 20. Diluted essential oils were placed in sterile bowls and kept with the inoculated seedlings in a closed room at 27 ± 2°C and under high relative humidity (90-100%). One hundred milliliters of distilled water were placed in bowls

instead of essential oils for the control treatment (ten seedlings used for each control). A randomized complete block design was employed with 30 seedlings per treatment: three replications of ten seedlings were used for each individual treatment.

Disease severity assessment.

Disease severity was assessed visually and weekly, starting at 20 days post-inoculation and calculated when control plants had completely wilted. For *F. oxysporum* f. sp. *melonis*, symptom severity on plants was assessed based on the number of yellowed, wilted or dead seedlings using a 0-4 rating scale where (0) no symptoms, (1) beginning of yellowing or wilting on leaves, (2) leaves heavily affected, (3) stem standing and leaves completely wilted, and (4) death of plant (Chikh-Rouhou et al. 2007, 2010). Another scale was used the assessment of disease severity induced by *F. solani*. Estimation was recorded via the progress of crown necrosis or roots of each plant; 0 = asymptomatic crown; 0.5 = some necrotic traces; 1 = necrosis of 1/3 of the crown; 2 = necrosis of 2/3 of the crown; 3 = necrosis of more than 2/3 of the crown (Ayed et al. 2007).

On the basis of these notations, we calculated the disease severity (DS %) for both *Fusarium* species using the following formula (Doumbouya et al. 2012):

$$\text{DS (\%)} = \frac{\sum \text{Scores} \times \text{Nip}}{\text{Hss} \times \text{Tnp}} \times 100$$

with ∑Scores: total of scale scores, Nip: Number of infected plants, Hss: Highest scale score; Tnp: Total number of plants.

The reduction of the disease severity (RDS %) was calculated as following:

$$\text{RSD (\%)} = \frac{\text{severity of control} - \text{severity of treatted plants}}{\text{severity of control}} \times 100.$$

Statistical analyses.

Statistical analyses were performed with SAS software version 7 (SAS Institute, Cary, NC). Data were expressed as means ± standard deviation. Means were compared by Least significant difference (Fisher's LSD).

RESULTS

Yield and chemical composition of essential oils.

Marjoram essential oils. Marjoram essential oils showed a transparent color. The average extraction yield of marjoram essential oils was determined as 0.69 ± 0.20%, achieved after about 4 h of extraction. The rates and retention indices of the identified components of tested marjoram essential oils are listed in Table 2. Among several compounds recorded by gas chromatography and GC-MS analysis, marjoram essential oils were found to be rich in terpinen-4-ol (34.94%) which is the major component, followed by *γ*-terpinene (24.66%), *α*-terpinene (13.22%), *β*-terpinene (5.84%), *α*-terpineol (3.98%) and *β*-phellandrene (3.16%).

Lavender essential oils. Lavender essential oils showed yellow color. The average extraction yield was estimated at 0.43 ± 0.15%, achieved after 4 h of hydro-distillation. Chemical composition of our lavender essential oils was determined (Table 3) where *α*-Pinene (48.76%) was the major component, followed by linalool (16.79%), *γ*-terpinene (7.00%), *β*-tran-ocimane (6.47%), *β*-caryophyllene (5.83%) and lavandulol (3.23%).

Table 2. Chemical composition of *Origanum majorana* essential oils

No	RT[a]	Compound	Rate (%)
1	3.178	Octane	0.04
2	5.398	*α*-Thujen	0.46
3	5.576	*α*-Pinene	0.60
4	6.543	*β*-Terpinene	5.84
5	6.634	*β*-Pinene	0.53
6	6.943	*β*-Myrcene	1.28
7	7.367	*α*-Fellandrene	0.33
8	7.710	*α*-Terpinene	**13.22**
9	7.962	o-Cymene	2.03
10	8.071	*β*-Phellandrene	3.69
11	9.844	*α*-Terpinolen	3.16
12	10.331	*γ*-Terpinene	24.66
13	12.934	Terpinen-4-ol	34.94
14	13.392	*α*-Terpineol	3.98
15	15.257	3-Caren	1.94
16	17.867	P-Cymene	0.08
17	20.676	*β*-Caryophyllene	2.47
18	21.763	*α*- Caryophyllene	0.10
19	23.068	Bicyclogermacrene	1.45
20	25.694	4,11,11-Trimethyl-8-methyl-enebicyclo[7.2.0]undec-3-ene	0.14
Total			99.49

[a] RT = Retention Time

Table 3. Chemical composition of *Lavender angustifolia* essential oils

No	RT[a]	Compound	Rate (%)
1	6.635	*β*-Pinene	0.25
2	6.829	3-Octanone	0.72
3	6.938	*β*-Myrcene	0.27
4	7.510	Bicyclo[4.1.0]hept-3-ene	0.42
5	8.111	1, 8-Cineol	0.46
6	8.271	*β*-Trans-Ocimene	6.47
7	8.586	cis-*β*-Ocimene	1.84
8	9.398	cis-Linalool oxide	0.43
9	10.337	Linalool	16.79
10	10.508	1-Octen-3-ol acetate	2.70
11	12.551	Borneol	1.28
12	12.889	*γ*-Terpinene	7.00
13	13.386	4-Isopropenyl-1-methyl-1-cyclohexe	0.93
14	15.326	*α*-Pinene	48.76
15	16.408	Lavandulol	3.23
16	19.429	3-Caren	0.50
17	20.676	*β*-Caryophyllene	5.83
18	21.729	(Z)-*β*- Farnesene	1.46
19	25.706	Alloaromadendrene	0.67
Total			100

[a] RT = Retention time

Antifungal effect of essential oils in vitro on mycelial growth.

The antifungal effect of tested essential oils was determined based on the inhibition rate of mycelial growth of *F. oxysporum.* f. sp. *meloni* and *F. solani* isolates as compared to control (Tables 4). The essential oils of marjoram and lavender inhibited significantly ($P < 0.05$) the mycelial growth of all tested fungal isolates compared to control plates. Analysis of variance (ANOVA) revealed a significant interaction between essential oils and tested isolates ($P < 0.05$). Marjoram essential oils had a significantly ($P < 0.05$) higher inhibitory effect than lavender essential oils. The obtained results showed that the antifungal activity depends on essential oil extract and the type of isolate. The highest rates of mycelial growth inhibition by both plant essential oils were observed in *F. oxysporum*. Isolates S4 (Fig. 1A) and S11 of the latter were completely inhibited by essential oils of marjoram. The rates of mycelial growth inhibition by marjoram essential oils for the rest of *F. oxysporum* isolates still high and ranged between 49.95 and 85.71% (Table 4).

All *F. oxysporum* and *F. solani* isolates were found to be sensitive to lavender essential oils and especially *F. oxysporum* isolates which growth was considerably reduced by 50-90% compared to controls (Table 4; Fig. 1B). As for *F. solani* isolates, growth inhibition induced by lavender essential oils varied between 35 and 85% (Table 4).

Table 4. Inhibition rates of mycelial growth of *Fusarium oxysporum* f. sp. *melonis* and *F. solani* isolates by lavender and marjoram essential oils (EOs) noted after 7 days of incubation at 25°C

***Fusarium* species**	**Isolate**	***Marjoram* EOs**	***Lavender* EOs**
F. oxysporum.* f. sp. *melonis	S1	70.05 ± 2.21	81.16 ± 1.15
	S2	49.95 ± 1.00	64.32 ± 0.06
	S3	66.19 ± 1.65	78.05 ± 1.25
	S4	**100 ± 0.49**	**89.90 ± 0.85**
	S5	81.21 ± 1.05	63.64 ± 0.55
	S6	84.13 ± 0.00	87.83 ± 0.68
	S7	61.11 ± 1.60	77.78 ± 1.62
	S8	65.93 ± 1.72	55.56 ± 0.62
	S9	44.84 ± 1.08	52.72 ± 0.95
	S10	85.71 ± 0.93	63.16 ± 0.21
	S11	**100 ± 0.00**	61.29 ± 0.52
F. solani	S12	44.74 ± 1.82	55.26 ± 1.51
	S13	46.84 ± 1.71	38.40 ± 0.46
	S14	79.66 ± 0.95	72.88 ± 0.44
	S15	74.15 ± 1.53	83.51 ± 0.06
	S16	59.62 ± 2.14	59.36 ± 0.64
	S17	35.90 ± 0.96	35.90 ± 0.87
	S18	74.92 ± 1.00	51.46 ± 0.20
	S19	33.00 ± 0.75	66.00 ± 1.44
	S20	65.00 ± 1.21	46.67 ± 0.21
	S21	39.95 ± 1.76	40.82 ± 0.76

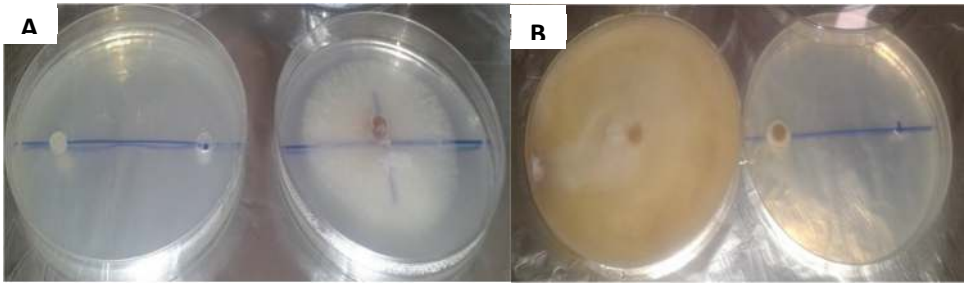

Fig .1. Complete inhibition of *Fusarium oxyporum* mycelial growth by Marjoram and Lavender essential oils. **A**: Isolate S_4 completely inhibited by Marjoram essential oils. **B**: Isolate S_1completely inhibited by Lavender essential oils.

Effect of tested essential oils on spore germination.

The volatile compounds of marjoram (Fig. 2A) and lavender (Fig. 2B) essential oils completely inhibited spore germination of both *F. oxysporum*. f. sp. *melonis* and *F. solani* compared to controls after 24 h of incubation (Fig. 2C).

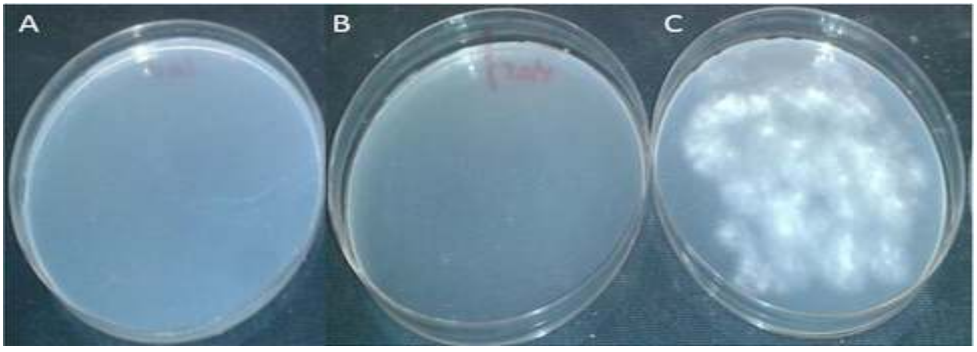

Fig. 2. Volatile effect of plant essential oils on spore germination of *Fusarium oxysporum* f. sp. *melonis* and *Fusarium solani*. A: Complete inhibition by Marjoram essential oils. B: Complete inhibition by Lavender essential oils. C: Untreated control.

Effects of tested essential oils on disease severity.

The effect of essential oils on Fusarium wilt severity varied significantly depending on essential oils only. No significant effect of Fusarium isolates was observed. Essential oils, applied as biofumigant, under growth chamber conditions significantly ($P < 0.05$) reduced disease severity on melon plants 20 days post-inoculation (Fig. 3). The highest efficacy in reducing over 57% of disease severity was achieved by lavender essential oils compared to

control plants (Fig. 4C) whereas slightly lower inhibitory activity (23%) was detected using marjoram essential oils (Fig. 3, D).

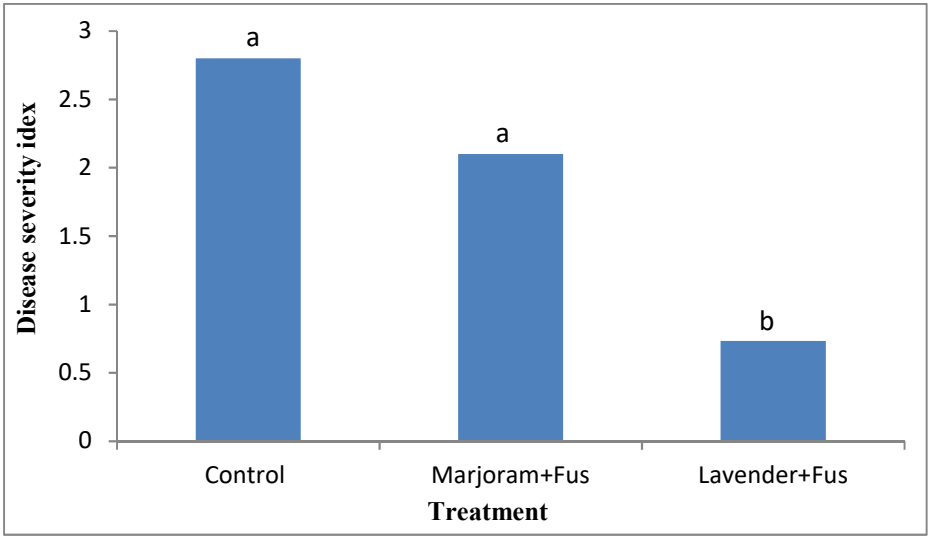

Fig. 3. Effect of marjoram and lavender essential oils on Fusarium wilt and root rot disease under control conditions. Treatments are mean disease indices of disease severity. Values followed by common letters do not differ according to LSD tests ($P < 0.05$).

DISCUSSION

The yield and the chemical composition of marjoram and lavender essential oils in this study were determined. Compared to our results, Busatta et al. (2008) recorded 1.33 ± 0.18% of oils extracted after 60 min from dried marjoram leaves. These variations may be due to several factors including environmental effects (type of climate, soil) and the time of harvest and method and time of extraction (Besombes 2008). The chemical composition of our marjoram essential oils is quite similar to those of Vera and Chane-Ming (1999) where marjoram essential oils are rich in terpinen-4-ol (38.4%), *cis*-sabinene hydrate (15.0%), *p*-cymene (7.0%) and γ-terpinene (6.9%). Busatta et al. (2008) detected carvacrol (11.67%) and thymol (9.45%) components after terpinen-4-ol (21.43%) and g-terpinene (12.32%) in marjoram essential oils. Both results are in accordance with several reports (Baser et al. 1993; Komaitis et al. 1992; Lawrence 1989; Nykanen 1986a; Ravid and Putievsky 1986) investigating the composition of oils from various *Origanum* species. They found that marjoram essential contain terpinen-4-ol and sabinene hydrate as major components and the other with thymol and/or carvacrol as predominant compounds.

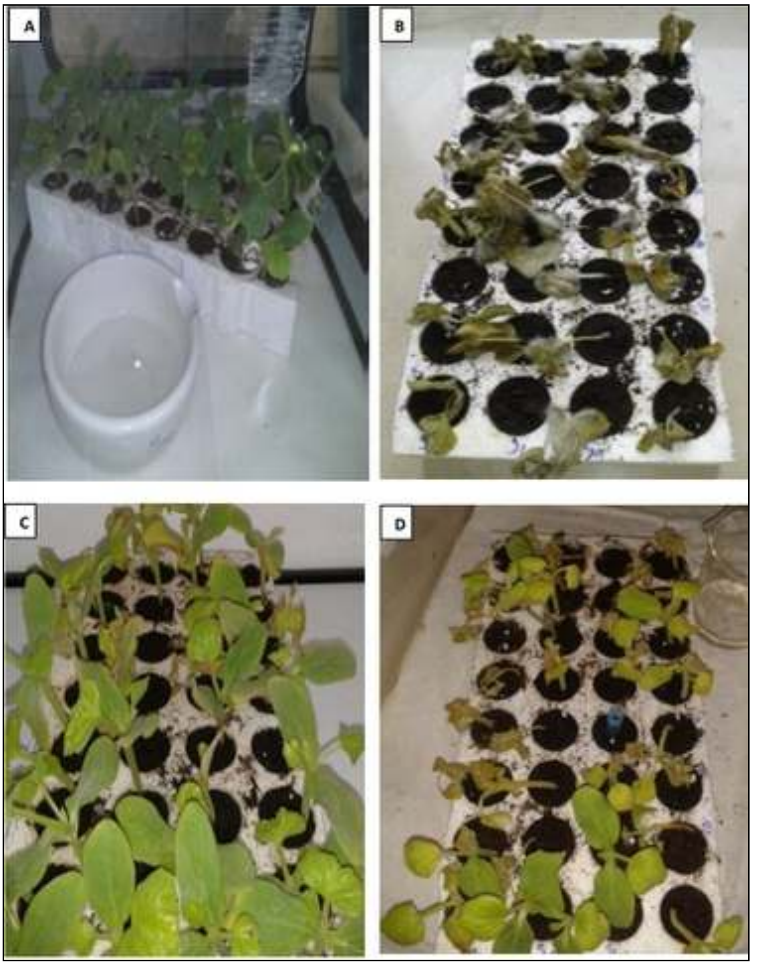

Fig. 4. Effect of volatile compounds of lavender and marjoram essential oils on severity of Fusarium wilt and root rot disease of melon. A: Application of essential oils by bio-fumigation; B: Melon plants inoculated with *F. oxysporum* f. sp. *melonis* and *F. solani* isolates (positive control). C: Effect of lavender essential oils. D: Effect of marjoram essential oils.

In our study, the lavender essential oils content was lower as compared to the yield found by Denys et al. (2002) recording 2.3 to 5% of lavender essential oils. Extraction method and time may be considered as the principal reasons of essential oils yield comparison. Périno-Issatier et al. (2013) studied eight extractions of lavender essential oils techniques ranging from conventional methods (hydrodistillation, steam distillation...) through innovative techniques. They found that the best extraction method (microwave hydrodiffusion and gravity) did not affect the essential oils yield but just decreased the extraction time (30 min). In fact, essential oils yield is influenced by the season, the aerial part of the plant collected and by the drying process. Dried

leaves contained higher oil content in summer than the fresh leaves, flowers and fruits collected in winter in both seasons (Mendes Silva et al. 2011). Hui et al. (2010) identified 1,5-dimethyl-1-vinyl-4-hexenylbutyrate, 1,3,7-Octatriene, 3,7-dimethyl, eucalyptol and camphor as the main constituents of lavender essential oils. The observed differences in the constituents of lavender essential oils across our results may be due to different environmental and genetic factors, different chemotypes and the nutritional status of the plants. In addition, chemical composition of the essential oils, both quantitative and qualitative, differs widely according to the extraction technique. For example, the steam distillation method yields oils rich in terpene hydrocarbons, while the supercritical extracted oils contain higher percentages of oxygenated compounds, owing to the polarity of supercritical CO_2 (Danh et al. 2013).

In in vitro experiments, our results indicated that the application of marjoram and lavender essential oils reduced mycelial growth and spore germination significantly for all tested *Fusarium* isolates. The antifungal effect of marjoram essential oils noted in vitro was found to be very strong. This effectiveness may be due to its richness of terpinen-4-ol compound which is in accordance with Vági et al. (2005) work. Similar studies (Carmo et al., 2008; Gallucci et al., 2014, Griffin et al. 1999; Kalemba and Kunicka 2003) confirmed that terpene components are the agents with the highest antifungal potential of essential oils. Different results (Gergis et al. 1990; Panizzi et al. 1993; Pellecuer et al. 1980; Satrani et al. 2007; Sivropoulou et al. 1996; Trombetta et al. 2002) reported that the antimicrobial activity of marjoram essential oils against many strains of Gram-positive and Gram-negative bacteria was due to its high level of thymol and carvacrol compounds. Also, it was found that the inhibition of phytopathogenic fungi is due to carvacrol and thymol compounds (Dikbas et al. 2008).

Additionally, the antifungal action of essential oils of lavender might be due to its major active compound α-Pinene. This compound, not commonly found as a major component in lavender essential oils, was found to be a major constituent of Pine essential oils and showed a high antifungal effect against *F. oxysporum* (Tullio et al. 2007). Linalool, major component of lavender essential oils and responsible of the antifungal activity against *F. oxysporum* in the latter author's investigation, was detected as second major component in our lavender essential oils and may be also responsible of the recorded inhibition of *Fusarium* spp. mycelial growth. Dorman and Deans (2000) also reported that linalool and carvacrol components of lavender essential oils are known for their fungicidal effects. However, more experiments are necessary to identify the main components of essential oils that are responsible of the antifungal effect and further study their effects and toxicity in vivo to better understand their safety usage under environment conditions.

Ten microliters of pure essential oils of marjoram and lavender tested in vitro showed 100% inhibition of spore germination. Different essential oil concentrations and dilutions are further to be tested on spore germination inhibition. Various plant essential oils exhibited antifungal activity in vitro without direct contact on spore germination as is the case of *Colletotrichum gloeosporioides* (Hong et al. 2015). Many studies have shown that essential oil vapors are also able to control various plant pathogens (Kumar et al. 2008). Tullio et al (2007)

studied the antifungal activity of essential oils against filamentous fungi using broth microdilution and vapor contact methods and demonstrated that the inhibitory effects of essential oils in vapor phase were generally higher than those in liquid state. The complete inhibition of our lavender and marjoram essential oils on spore germination of *F. oxysporum* and *F. solani* isolates by vapor contact method can be explained by the fact that thymol and carvacrol components have strong volatility rather than solubility into agar layers (Inouye et al. 2001a).

Further studies in planta demonstrated the efficacy of lavender essential oils on reducing over 57% of disease severity caused by *F. oxysporum* f. sp. *melonis* and *F. solani*. This finding is original as no previous reports were found on the efficacy of lavender essential oils in vivo reducing Fusarium wilt disease nor other phytopathogen fungal diseases.

Marjoram essential oils were very toxic to melon seedlings and additional phytotoxicity studies using different doses of these essential oils are required. Because Mediterranean essential oils and their volatile compounds, including marjoram essential oils were used for weed management eradicating the radical emergence of many seeds due to their phytotoxic properties (de Almeida et al. 2010), the lowest inhibitory activity of our marjoram essential oils in vivo could be only associated to its phytotoxicity to young plants not to its inefficacy. Also, in our study, we tried to apply marjoram essential oils to melon seedlings by injecting undiluted essential oils around roots, but within 24 h all the seedlings died unable to stand for such harsh treatment. Although, the vapors produced by marjoram essential oils in the biofumigation method still harsh to herbaceous young plants, they should be further tested for their toxicity at different doses in in vivo bioassays. Few studies were performed on the efficacy of marjoram essential oils in vivo. Doumbouya et al. (2012) demonstrated the efficacy of *Ocimum gratissimum* essential oils in reducing severity of Fusarium wilt of tomato caused by *F. oxysporum* f. sp. *lycopersici* by almost 40%. To conclude, marjoram and lavender essential oils may be suggested as a new potential source of a natural antifungal product able to control Fusarium wilt disease in melon crops.

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RESUME

Dhaouadi, S., Rouissi, W., Mougou Hamdane, A., Hannachi, I., and Nasraoui, B. 2018. Activités antifongiques des huiles essentielles d'*Origanum majorana* et de *Lavender angustifolia* contre le flétrissement vasculaire et la pourriture racinaire et du collet du melon. Tunisian Journal of Plant Protection 13 (1): 39-55.

L'étude de l'activité antifongique des huiles essentielles de la marjolaine (*Origanum majorana*) et de la lavande (*Lavender angustifolia*) contre onze isolats de *F. oxysporum* f. sp. *melonis* et dix isolats de *F. solani*, causant le flétrissement vasculaire et la pourriture racinaire et du collet chez le melon a été évaluée *in vitro* et *in vivo* dans le but de rechercher de nouveaux produits non chimiques. La détermination des rendements des huiles essentielles a montré que le rendement le plus élevé a été obtenu chez la marjolaine avec 0,69%. Les compositions chimiques des huiles essentielles isolées par hydrodistillation des parties aériennes de la marjolaine et de la lavande ont été analysées par CG et CG-

SM. Ces analyses ont montré que le composant majeur des huiles essentielles de la marjolaine est terpinène-4-ol (34,94%), suivi par γ-terpinène (24,66%), α-terpinène (13.22%), β-terpinène (5.84%), α-terpinéol (3.98%), and β-phellandrène (3.16%). L'analyse chimique des huiles essentielles de la lavande a montré le composant prédominant est α-terpinène (48.76%), suivi par linalool (16.79%), γ-terpinène (7.00%), β-trans-ocimane (6.47%), β-caryophyllène (5.83%), and lavandulol (3.23%). Le α-terpinène (48,76%) et le linalool (16,79%) sont les composés prédominant des huiles de la lavande. Les huiles essentielles de la marjolaine et la lavande testées par confrontation directe sur milieu de culture contre les 21 isolats de *F. oxysporum* et *F. solani* ont significativement réduit la croissance mycélienne de tous les isolats par rapport au témoin. De plus, les composés volatiles des huiles essentielles ont complètement inhibé la germination des spores de tous les isolats. *In vivo*, la bio-fumigation par les huiles essentielles contre dix isolats de *F. oxysporum* et dix isolats de *F. solani* a montré que les huiles essentielles de la lavande sont les plus efficaces en réduisant de 60% la sévérité de la maladie par rapport aux plants non inoculés alors que les huiles essentielles de la marjolaine n'ont réduit que 23% la sévérité de la maladie. Ces résultats ont montré que les huiles essentielles de la lavande peuvent contribuer au développement de nouveaux composés antifongiques pour protéger le melon contre le flétrissement vasculaire et la pourriture racinaire et du collet.

Mots clés: Huiles essentielles, sévérité de la maladie, *Fusarium oxysporum*, *Fusarium solani*, lavande, marjolaine, melon

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ملخّص

صابرين الذّوادي، وفاء الرويسي، أميرة موقو-حمدان، ابتسام الحنّاشي وبوزيد نصراوي. نشاط الزيوت الأساسية المستخلصة من نباتات المردقوش (*Origanum majorana*) والخزامة (*Lavender angustifolia*) ضد الذبول الفوزاري وتعفن الجذور والعنق عند البطيخ/الشمام.

Tunisian Journal of Plant Protection 13 (1): 39-55.

تهدف دراستنا إلى استعمال الزيوت الأساسية المستخلصة من نباتات المردقوش (*Origanum majorana*) والخزامة (*Lavender angustifolia*) لمقاومة العامل المسبب لأعراض الذبول وتعفن الجذور والعنق على البطيخ تحت ظروف مخبريه وبيوت زراعية محكمة وذلك من أجل استعمالها كمبيدات بيولوجية. تم تحديد مردود الزيوت الأساسية المستخرجة من هذه النباتات فتحصلنا على أعلى مردود لدى نبتة المردقوش وذلك بنسبة 0,69%. تم كذلك تحليل التركيبات الكيميائية للزيوت المستخلصة من خلال عمليّة تقطير مائي من الأجزاء الهوائية للمردقوش والخزامة بواسطة تقنية GC و CG-MS. تبين أنّ المكون الرئيسي لزيوت المردقوش هو terpinène-4-ol (34.94%) ويتبعه γ-terpinène (24,66%) و α-terpinene (13.22%) و β-terpinene (5.84%) و α-terpineol (3.98%) و β-phellandrene (3.16%). أمّا بالنسبة للمركب السائد لزيوت الخزامة فهو α-terpinene (48.76%) متبوع بـ linalool (16.79%) و γ-terpinene (7.00%) و β-trans-ocimane (6.47%) و β-caryophyllene (5.83%) و lavandulol (3.23%). أثبتت التجارب المخبرية التي تمت فيها مجابهة واحد وعشرون عزلة من فطريْ *F. oxysporum* و *F. solani* مع الزيوت المستخلصة من المردقوش والخزامة على الوسط الغذائي أنها تكبح بشكل كبير النموّ الغزلي لجميع العزلات مقارنة مع الشاهد. بالإضافة إلى ذلك، منعت المكونات المتطايرة لهذه الزيوت تمنع بشكل كامل إنبات أبواغ كل العزلات. كما استعملت مستخلصات هذه النباتات في اختبار الفعالية داخل بيت بلاستيكي تحت ظروف محكمة. وأثبتت النتائج المتحصل عليها أن هناك تأخر في ظهور المرض بشكل ملحوظ مع تقليصه بنسبة 60% بإستعمال زيت الخزامة. كما أظهرت النباتات المعاملة مقاومة جيدة للمرض مقارنة مع الشاهد وأنّ انتاجية نباتات البطيخ تحسّنت. وأثبتت النتائج المتحصل عليها أنّه يمكن استخدام مستخلص زيت الخزامة كبديل لمكافحة الذبول الفيوزاري للبطيخ.

كلمات مفتاحية: بطيخ/شمام، حدّة المرض، خزامة، زيوت أساسية، مردقوش، *Fusarium oxysporum*، *Fusarium solani*

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Barley Net Blotch in Tunisia: Areal Distribution, Forms and Molecular Identification

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ABSTRACT

Mougou-Hamdane, A., Touati, R., Faddaoui, S., Garbouj, R., BenAraar, A., Nasraoui, B. 2018. Barley Net Blotch in Tunisia: Areal distribution, forms and molecular identification. Tunisian Journal of Plant Protection 13 (1): 57-68.

Net blotch is one of the most important diseases of barley in Tunisia and it frequently causes heavy yield losses. The causal fungal agent is *Pyrenophora teres* (anamorph: *Drechslera teres*) with two forms, *P. teres* f. *tere*s and *P. teres* f. *maculata* showing the net type and the spot type symptoms, respectively. In the current study, we characterized the distribution and the severity of the disease in Northern Tunisian areas. It was easy to differentiate the symptoms caused by the two fungal forms (net and spot), but no morphological difference between conidia of these forms was observed. The confirmation of the identity of the fungus was achieved based on molecular techniques, using two specific primers, PTT for the net form and PTM for the spot form.

Keywords: Disease severity, molecular identification, net form, *Pyrenophora teres*, spot form, symptoms, Tunisia

Barley (*Hordeum vulgare*) is an important cereal crop in Tunisia and plays a key role in the economy of the country (El Felah and Gharbi 2014). Surveys carried out in Northern Tunisia showed that net blotch is one of the major diseases of barley which causes heavy yield losses. Its fungal causal agent is *Pyrenophora teres* (anamorph: *Drechslera teres*) which develops under several climatic and agronomic factors (Chamekh 2007). *P. teres* occurs in two forms, *P. teres* f. *tere*s (Ptt) and *P. teres* f. *maculata* (Ptm) which differ in their induced symptoms: net type and spot type, respectively (Manninen et al. 2006). These two forms are morphologically similar but genetically distinct (Akhavan et al. 2015). Net blotch is also responsible for yield loss (up to 40%) and a total loss when susceptible barley varieties are grown under favorable environmental conditions (Marthre 1997; Murray and Brennan, 2010). This disease can also lead to a reduction in malting quality in most barley growing areas of the world

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(Weiland et al. 1999) and to a decrease in kernel size, plumpness and bulk density (Grewal et al. 2018; Marthre 1997).

The aim of this study was firstly to investigate the areal distribution of the net blotch disease on barley in Tunisia. This was performed through three year surveys of disease occurrence in different Northern regions of Tunisia and the observation of both symptom types on the collected samples (Ptt and Ptm). Then, the presence of the two forms was confirmed using the PCR technique.

MATERIALS AND METHODS

Sampling.

Barley samples were collected between March and May of three years (2014 to 2016) in six Tunisian governorates (Jendouba, Beja, Kef, Bizerte, Siliana, and Zaghouan) from symptomatic plants. All the samples consisted of leaves showing symptom lesions of both forms (net and spot) of the disease. Symptom types and GPS coordinates were noted for each collected sample.

Disease severity.

The severity of a disease is the amount of infected plant tissue on a single plant. Net blotch disease was evaluated in the visited plots based on a rating scale from 0 to 5 where 0 : no infestation), 1: very low infestation, 2: low infestation, 3: medium infestation, 4: large infestation), and 5: very large infestation (Fig. 1).

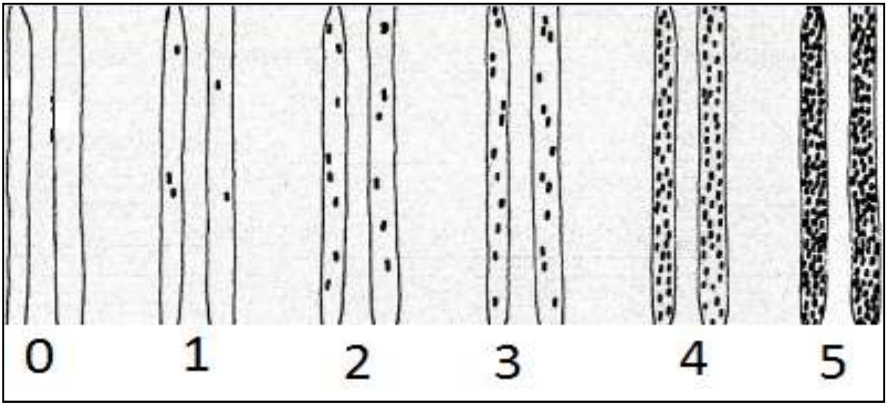

Fig. 1. Disease severity scale used for the estimation barley net blotch severity.

Fungal isolation.

Leaves with visible lesions of net blotch were cut, sterilized in hypochlorite solution during 3 min and in alcohol 70° for 3 s, then rinsed in sterile water for 3 min. Next, these fragments were dried and placed in Petri dishes on a V8-PDA (20%) medium (Andrie et al. 2006) and incubated for 10 days in an illuminated incubator at 22°C with a 12 h photoperiod. Single spore cultures were performed as described by Hansen and Smith (1932) and maintained under the same conditions.

Mycelial radial growth evaluation

The mycelial growth of 8 *P. teres* isolates (4 isolates for each form) on 3 different growth media (namely PDA,

barley-leaves (BL) and V8) was evaluated. For that, 3 disks of 5 mm in diameter were taken from a young culture and placed on each medium. Incubation was carried out at 23°C under continuous light. The mycelial growth was evaluated every 3 days for 15 days by measuring the mean of the 2 perpendicular growth diameters. The mycelial radial growth (MRG) on these different culture media was determined according to this formula: MRG (cm) = $[D_1/ T_1] + [(D_2-D_1)/T_2] + [(D_3-D_2)/T_3] +\ldots + [(D_n-D_{n-1})/T_n]$, with MRG: Mycelial radial growth on the day D_i and the incubation period T_i.

Molecular identification for net and spot forms

One fragment of ~0.5 cm^2 was cut out with a scalpel from a lesion in each leaf. These fragments were then transferred to 1.5 ml microtubes, labeled according to their origins and stored at -20°C before DNA extraction. The DNA of all isolates was extracted using a protocol previously described by Zolan and Pukkila (1986). The mechanical lysis of cells was achieved by a gentle grinding of mycelium and conidia in 800 µl of CTAB buffer, followed by incubation for at least 1 h in a 65°C water bath. Then, 200 µl of chloroform was added; Eppendorf tubes were shortly vortexed and centrifuged for 10 min at 14000 rpm. After transferring the supernatant to a new Eppendorf tube, 800 µl of isopropanol previously maintained at -20°C was added and mixed gently. The DNA was precipitated at -20°C for at least 1 h. The pellet, obtained by centrifugation for 10 min at 14000 rpm, was washed twice with 1 ml of 70% ethanol, then centrifuged for 5 min at 14000 rpm. The DNA pellet was finally dried at room temperature, suspended in 50 µl of ultra-pure water and stored at -20°C. PCR analyses orientated to the diagnostics of pathogen-specific DNA sequences were done using PCR primers cited in Table 1 (Williams et al. 2001).

Table 1. List of primers used for the identification of the net and spot form for the barley net blotch disease

Primer		Sequence (5'-3')	Length of the amplified fragment	*P. teres* form
PTT	**PTT-F**	CTCTGGCGAACCGTTC	378 pb	*P. teres* f. *teres*
	PTT-R	ATGATGGAAAAGTAATTTGTA		
PTM	**PTM-F**	TGCTGAAGCGTAAGTTTC	411 pb	*P. teres* f. *maculata*
	PTM-R	ATGATGGAAAAGTAATTTGTG		

The PCR for the identification of the net and spot forms of *P. teres* was performed using the following reaction mixture: 1×buffer, 1.5 mM Mg^{2+}, 1 µM primer PTT-F, 1 µM primer PTT-R, 1 µM primer PTM-F, 1 µM primer PTM-R, 0.2 mM dNTP and 0.8 U of Taq DNA polymerase. Amplifications were performed in a total volume of 15 µl. The PCR program for the amplification of PTM was 94°C for 1 min followed by 35 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s. The final step was 72°C for 7 min. The same cycle was used for the amplification of PTT however the annealing temperature was 58°C.

RESULTS

Disease distribution and severity.

The disease was observed in almost all surveyed plots (Fig. 2). Heavy

infestations were observed in Oued Zarga (Beja), Fernana and BouSalem (Jendouba), Seminja (Zaghouan) and Medien (Siliana). Limited infections were observed in the governorates of Kef (Touiref, Dahmeni, and Sers) and Siliana (Gaafour, Bourouis, and Makthar).

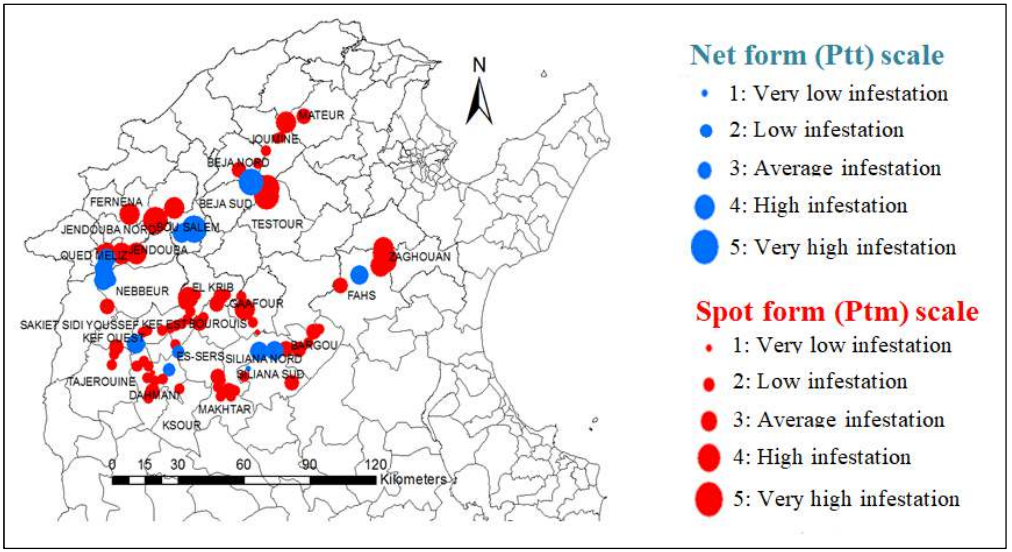

Fig. 2. Distribution and severity of the net and spot forms of the barley net blotch in the Northern Tunisia (Campaign 2015/16).

Symptom analysis.

We noticed the dominance of the net form (Ptt) in BouSalem (Jendouba) and Touiref (Kef), however the spot form (Ptm) was dominant in Kef area (except Touiref and Sers). In some visited fields located in Zaghouan and Siliana, net blotch was present with both forms in the same plot. The sampled barley leaves showed a dark brown color blotches on both sides. Leaves showing these symptoms usually undergo rapid necrosis.

The two different symptom types were observed (Fig. 3):

- Net type: Wide necrotic lesions along veins are of a light brown color becoming increasingly dark as the disease is severe. Through light, these lesions show a network of lines,
- Spot type: Dark brown lesions accompanied by an important chlorosis. These lesions can have linear, rectangular, oval or punctiform shape.

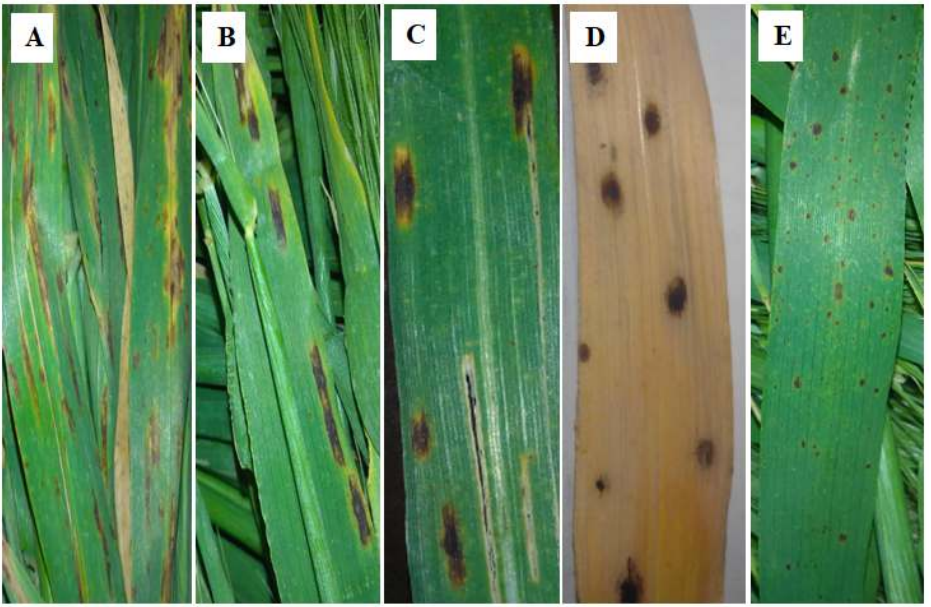

Fig. 3. Symptoms of *Pyrenophora teres* on barley leaves. Net type of Ptt: A (network) and spot type of Ptm: B (linear), C (rectangular), D (oval), and E (punctiform).

Morphological traits of *P. teres* cultures.

After few days of incubation, fungal isolates showed macroscopic differences when cultured on the three growth media. Different colors, textures and coremium productions were observed. Colors of fungal cultures were green, yellowish green, dark green, whitish, light gray or greenish gray. Mycelium generally appeared very thin and made of septate hyphae; it takes two aspects either cottony or flat. A more or less abundant coremium production was observed depending on the isolate (Fig. 4).

On BL medium, all isolates yielded green colonies (from light green to dark green). This green color was also noticed on V8 medium for all isolates except in one case where a greenish gray color was observed. On PDA, we noticed that the color of colonies ranged from whitish, whitish gray, greenish gray, light green, green to dark green (Table 2).

The fungus had a flat appearance on V8 medium except for one isolate that showed cottony appearance. On BL and PDA media, cottony was more frequent than flat type which was observed in colonies of two isolates only (Table 3).

Isolates produced coremia on all considered growth media but with a different level (Table 4). Abundant production of coremia was observed on V8 medium, whereas they were less present on BL. On PDA, coremia were quasi-absent except for few isolates.

Conidia of *P. teres* are straight cylindrical with rounded edges and a smooth cell wall. They are multicellular and cells are separated by 1 to 9 pseudosepta (often 4 to 6) usually carrying constrictions. The dimensions are 50-140 × 15-25 µm (Fig. 5).

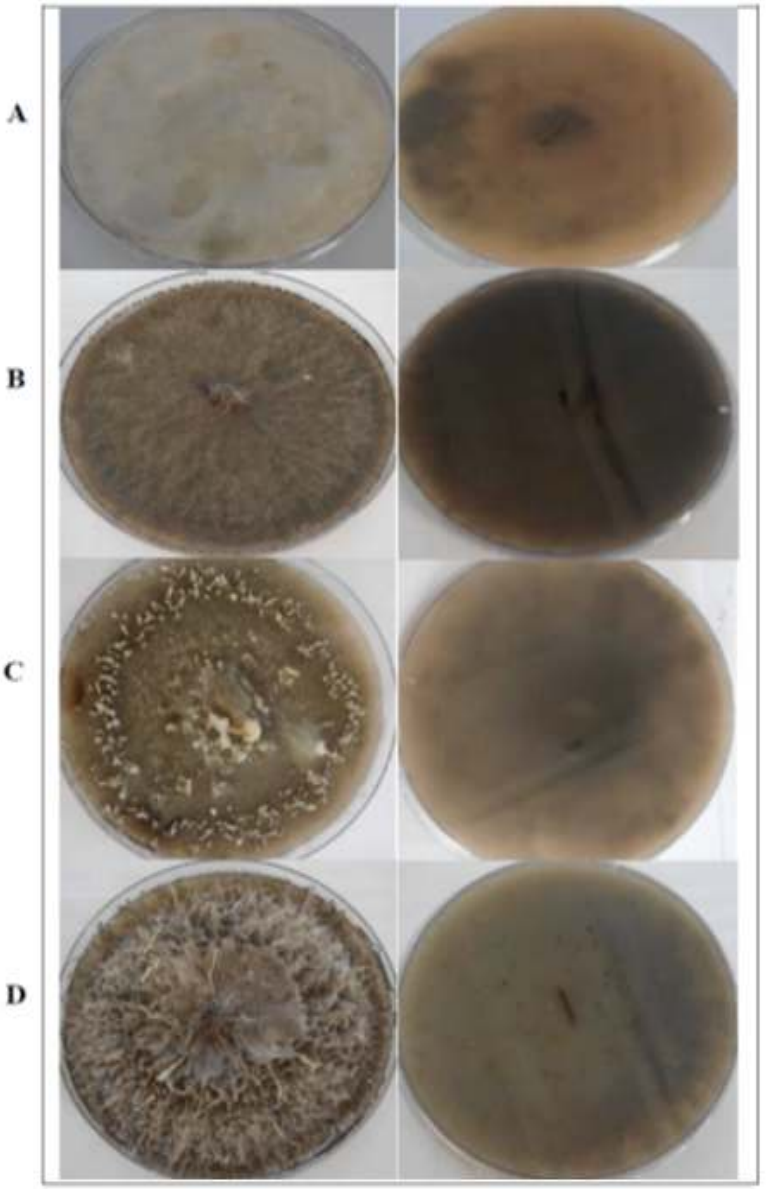

Fig. 4. Macroscopic traits of *Pyrenophora teres* colonies growing on different growth media. A: PDA medium / Whitish colony / cottony mycelium / absence of coremia, B: BL medium / Dark green colony / cottony mycelium / absence of coremia, C: BL medium / Light green colony / cottony mycelium / average production of coremia, D: V8 medium / Green colony / flat mycelium (Upper side on the left, lower side on the right)

Table 2. Color of *Pyrenophora teres* colonies growing on three different growth media

Form	Isolate	Growth medium		
		PDA	BL	V8
Net	1	Whitish	Dark green	Greenish gray
	2	Green	Green	Green
	3	Green	Dark green	Light green
	4	Dark green	Dark green	Dark green
Spot	5	Greenish gray	Dark green	Dark green
	6	Green	Green	Dark green
	7	Whitish gray	Dark green	Light green
	8	Whitish	Dark green	Green

Table 3: Mycelium aspect of *Pyrenophora teres* cultures grown on three different growth media

Form	Isolate	Growth medium		
		PDA	BL	V8
Net	1	Cottony	Cottony	Flat
	2	Cottony	Cottony	cottony
	3	Flat	Cottony	Flat
	4	Flat	Cottony	Flat
Spot	5	Cottony	Cottony	Flat
	6	Cottony	Cottony	Flat
	7	Cottony	Flat	Flat
	8	Cottony	Flat	Flat

Table 4 . Coremia production of *Pyrenophora teres* on three growth media

Form	Isolate	Growth media		
		PDA	BL	V8
Net	1	-	-	+++
	2	++	++	+++
	3	++	+	+++
	4	++	+	+++
Spot	5	-	+	+++
	6	+	+	+
	7	-	+	++
	8	-	++	+++

- : Absence of coremia; + : Less than 10 coremia/Petri dish; ++ : 10-40 coremia/Petri dish; +++ : More than 50 coremia/Petri dish.

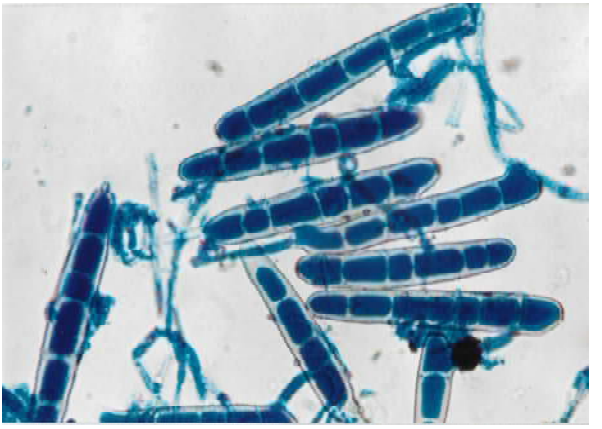

Fig. 5. Morpological traits of *Pyrenophora teres* conidia.

Mycelial radial growth.

P. teres mycelial radial growth on the three media (PDA, BL and V8) was found to be medium dependent (Fig. 6). Thus, the composition of the medium had a significant effect on the mycelial radial growth of the isolates at the threshold of 5%. BL medium was optimal for the radial growth of *P. teres* after 11 days of incubation with an average colony diameter of 6.53 cm, followed by V8 medium (6.39 cm) and finally PDA medium (5.46 cm). In addition, mycelial radial growth was not dependent on the pathogen form at the 5% threshold.

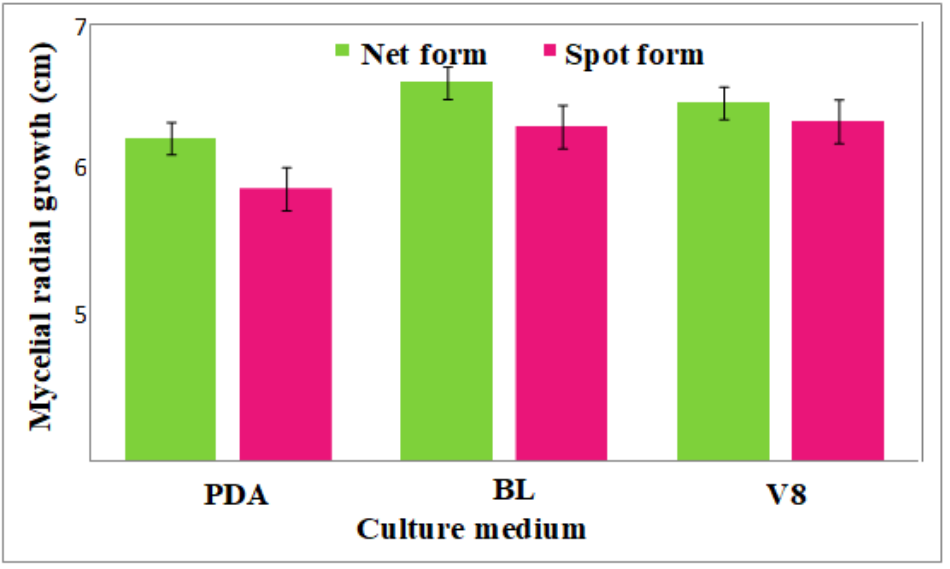

Fig. 6. Mycelial radial growth of *Pyrenophora teres* forms (net and spot) on PDA, BL and V8 media noted after 11 days of incubation (Segment = standard deviation).

Molecular identification of *P. teres* forms.

Molecular characterization based on the use of PTM and PTT primers allowed us to confirm the presence of both forms of the fungus; *P. teres* f. *teres* (Ptt) and *P. teres* f. *maculate* (Ptm). Indeed, primer set PTT specifically amplified a band of 378 bp from Ptt (net form) isolates whilst primer set PTM specifically amplified a band of 411 bp from Ptm (spot form) (Fig. 7).

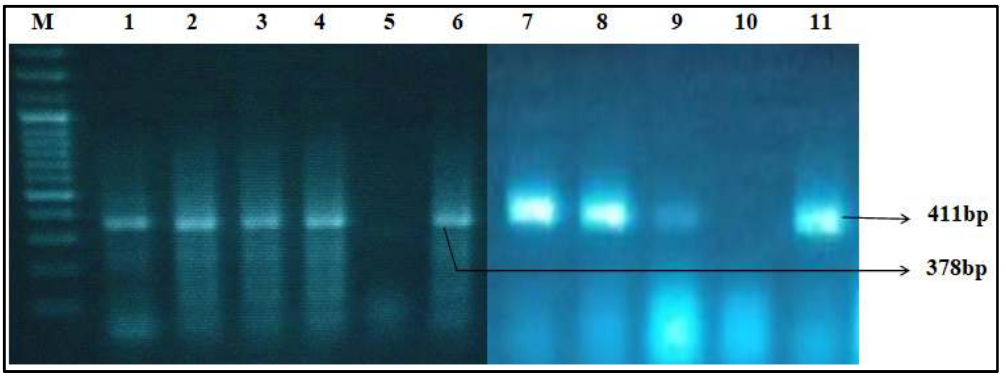

Fig. 7. Agarose gel showing PCR products amplified from the DNA of *Pyrenophora teres* isolates with the PTT and the PTM primers (M: Molecular marker 100 bp; 1, 2, 3, 4,: *P. teres* f. *teres* isolates; 6: Positive control for *P. teres* f. *teres*; 7, 8, 9: *P. teres* f. *maculate* isolates; 11: Positive control for *P. teres* f. *maculata*; 5 and 10: Negative controls).

DISCUSSION

The large prevalence of the disease in the surveyed plots is probably due to favorable Tunisian climate conditions. In fact, climatic factors of almost every year promoted the development and the spread of *P. teres* as described by Chamekh (2007). The heavy infections observed in some plots at Beja, Jendouba, Zaghouan and Siliana areas can be attributed to the high planting density, the absence of seed treatment, the lack of disease management and the presence of stubble and weeds when installing the culture. This can be also explained by the extended use of the variety "Rihane" instead of "Manel" known for its better resistance to the net blotch disease. The low infections in plots situated in Kef and Siliana governorates can be explained by more drought periods of these areas, not in favor of fungus development.

Symptoms observed on barley leaves are similar to those reported by Morvan (2006) or Manninen et al. (2006). The differentiation between the two forms (Ptt and Ptm) was easily done based on symptoms as described by Liu and Friesen (2010). As reported by Liu et al. (2011) and Boungab (2013), no significant difference in morphology between the two *P. teres* forms has been identified. However, the study of the morphological characteristics in vitro of *P. teres* made it possible to distinguish variability in the pigmentation, the colony texture and the mycelial radial growth between isolates of the two forms.

The analysis of the colony color confirmed the results of Frazzon et al. (2002) who obtained whitish colonies of *P. teres* on PDA medium. However, a predominance of green and gray colors was reported by Chamekh (2007). According to Chamekh (2007), mycelium of all *P. teres* isolates had a flat appearance on V8 medium but on PDA, only one isolate had a flat appearance while all the others are cottony. These descriptions were very close to those reported in our study.

The evaluation of the mycelial radial growth showed that BL medium allows the maximum of growth. However, Kim et al. (2005) reported that PDA promotes good growth whereas V8 medium allows average growth. We observed a quasi-absence of coremia on

PDA whereas Chamekh (2007) noticed an abundant presence of coremia on this medium. Frazzon et al. (2002) reported also that the frequency of coremia is different according to the isolate.

The obtained bands on agarose gel were at the expected size for both forms of *P. teres* and confirmed the identification based on the symptoms they caused, as noticed by Williams et al. (2001). Gubis et al. (2004) used a multiplex approach and the result of molecular analysis was also consistent with the net blotch symptoms. To the best of our knowledge, this is the first molecular identification of both forms of *P. teres* (Ptt and Ptm) in Tunisia.

RESUME

Mougou-Hamdane A., Touati R., Faddaoui S., Garbouj R., Ben Araar A. et Nasraoui B. 2018. La rayure réticulée de l'orge en Tunisie: Distribution régionale, formes et identification moléculaire. Tunisian Journal of Plant Protection 13 (1): 57-68.

La rayure réticulée est l'une des principales maladies de l'orge en Tunisie entraînant des pertes de rendement importantes. L'agent causal est *Pyrenophora teres* (anamorph: *Drechslera teres*) comportant deux formes, *P. teres* f. *teres* et *P. teres* f. *maculata* causant respectivement des symptômes de types réseau et spot. Dans notre étude, nous avons déterminé la répartition et la sévérité de la maladie dans le nord de la Tunisie. Une distinction entre les deux formes réticulée et maculée a été notée au niveau des symptômes sans révéler une différence au niveau de la forme des conidies. La présence de ces deux formes en Tunisie a été confirmée par des analyses moléculaires en se basant sur la technique PCR en utilisant deux amorces spécifiques, PTT pour la forme réticulée et PTM pour la forme maculée.

Mots clés: Forme maculée, forme réticulée, identification moléculaire, *Pyrenophora teres*, sévérité, symptômes, Tunisie

ملخص

موقو-حمدان، أميرة ريم تواتي سهام فدا ي ريم قربوج علاء دين بن عرعار بوزيد نصرا ي. 2018. تبقع شبكي لشعير في تونس: توزيع مساحي مميزات شكلية تشخيص جزيئي.

Tunisian Journal of Plant Protection 13 (1): 57-68.

التبقع الشبكي هو من أهم أمراض الشعير في تونس التي تتسبب في خسائر مرتفعة في الإنتاج. أما الفطر المسبب لهذا المرض فهو *Pyrenophora teres* (الطور اللاجنسي: *Drechslera teres*) بشكلين هما *P. teres* f. *teres* و*P.teres* f. *maculata* اللذان يسببان على التوالي أعراضا من النوع الشبكي والنوع التبقعي. في هذه الدراسة، تمكنا من توصيف انتشار وشدة المرض في الشمال التونسي. تبين تفاضل بين الشكلين الشبكي و التبقعي من ناحية الأعراض ولكن لم تكن هناك اختلافات مظهرية للأبواغ الكونيدية لهذين الشكلين للفطر. تم تأكيد تواجد شكلي الفطر عن طريق التقنيات الجزيئية باستعمال بادئتين خاصتين هما PTT للشكل الشبكي وPTM للشكل التبقعي.

كلمات مفتاحية: أعراض، شدة المرض، شكل تبقعي، شكل شبكي، *Pyrenophora teres*

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First Report of the Cyst Nematode *Heterodera mediterranea* on Olive Trees in Tunisia

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ABSTRACT

Guesmi-Mzoughi, I., Troccoli, A., Fanelli, E., Radicci, V., Regaieg, H., Hadj-Naser, F., Horrigue-Raouani, N., and De Luca, F. 2018. First report of the cyst Nematode *Heterodera mediterranea* on olive trees in Tunisia. Tunisian Journal of Plant Protection 13 (1): 69-77.

A survey was conducted in Tunisia to detect the presence of plant parasitic nematodes associated to olive trees. A high infection of olive roots and soil by the cyst nematode *Heterodera mediterranea* was detected in olive orchards located in the region of Moknine (Monastir, Sahel of Tunisia). Integrative taxonomic approaches (morphological, morphometrical and molecular analyses) were carried out in order to characterize the Tunisian population of *H. mediterranea*. Phylogenetic analyses of the ITS region, the D2-D3 expansion segments of the 28S rRNA gene and 18S rRNA gene highly supported that *H. mediterranea* from Tunisia belongs to the Schachtii group. So far, this is the first report of this nematode in Tunisia.

Keywords: 18S rRNA gene, D2-D3, *Heterodera mediterranea*, ITS, olive orchard, phylogenetic tree, taxonomy

Cyst forming nematodes belonging to the genus *Heterodera* are economically important as they are able to attack and to induce heavy damages to both annual and perennial plants all over the world. In 1981, Vovlas et al. found *Heterodera* cysts on the root of the bushy plant lentisc (*Pistacia lentiscus*), typical of the Mediterranean flora common in southern Italy. The nematode population was found to belong to an undescribed species and was named by these authors as *Heterodera mediterranea* (Vovlas et al. 1981). Later on, a greenhouse study revealed that besides lentisc, the nematodes was also able to attack olive (*Olea europaea*) and pistachio (*Pistacia*

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vera) (Vovlas and Inserra 1983), both typical Mediterranean plants. More recently, *H. mediterranea* was also reported from olive under field conditions in Spain (Castillo et al. 1999).

As olive is an important crop also in Tunisia, a survey was undertaken in 2013-2014 to investigate the nematode fauna associated with olive trees and able to cause damage on this crop. Among other nematodes, second-stage juveniles and cysts belonging to *Heterodera* genus were found in the rhizosphere of olive trees located in the region of Moknine (Sahel of Tunisia). Therefore, morphological, morphometrical, and molecular studies were undertaken to identify and characterize this nematode population.

MATERIALS AND METHODS

Sampling and nematode extraction.

Soil and root samples were collected from an olive orchard, cultivar Chemlali, with a shovel. They were removed from the upper 50 cm of soil from arbitrarily chosen olive trees in the rhizosphere of feeder olive roots. Samples were thoroughly mixed and nematodes were extracted from 500 g of soil and 1 g of root subsamples by centrifugal flotation method (Coolen 1979). Cyst nematode population showed a density of 60.4 juveniles and females/500 g of soil and 22.5 juveniles and females/1 g of roots.

Morphological and morphometrical characterization.

Extracted nematodes were observed under light microscopy, second-stage juveniles were killed by gentle heat, fixed in a solution of 4% formaldehyde and 1% propionic acid and processed to pure glycerine using Seinhorst's method (Seinhorst 1966). Second-stage juveniles were examined using Leitz Diaplan GMBH light microscope equipped with a digital camera (DFC 425) and connected to software of measurements called "LAS". Available non damaged cysts and females with good structure were picked out to take some morphometrical measurements.

Molecular characterization.

Individual cysts were crushed with a sterile micro-spatula under a stereo-microscope and the second-stage juveniles were recorded. Genomic DNA was extracted from fifteen individual nematodes as described by De Luca et al. (2004). The crude DNA isolated from each individual nematode was directly amplified. The ITS1-5.8S-ITS2 regions were amplified using the forward primer TW81 (5'-GTTTCCGTAGGTGAACCT GC-3') and the reverse primer AB28 (5'-ATATGCTTAAGTTCAGCGGGT -3') (Joyce et al. 1994); the 18S rDNA was amplified using the 18SnF (5'-TGGATAACTGTGGTAATTCTAGAG C-3') and 18SnR (5'-TTACGACTTTT GCCCGGTTC-3') primers (Kanzaki and Futai 2002); the D2A-D3B expansion segments of 28S rRNA gene was amplified using D2A (5'-ACAAGTA CCGTGGGGAAAGTTG-3') and D3B (5'-TCGGAAGGAACCAGCTACTA-3') primers (Nunn 1992). PCR cycling conditions used for amplification were: an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 50 s, annealing at 55°C for 50 s and extension at 72°C for 1 min and a final step at 72°C for 7 min. The size of the amplification products was determined by comparison with the molecular weight marker ladder 100 (Fermentas, St. Leon-Rot, Germany) following electrophoresis of 10 µl on a 1% agarose gel.

PCR products of the ITS containing region, the 18S rRNA gene

and the D2-D3 expansion segments from three individual nematodes were purified using the protocol given by the manufacturer (High Pure PCR elution kit, Roche, Germany). Purified DNA fragments were cloned and sent for sequencing, in both directions, at MWG-Eurofin in Germany.

Phylogenetic analysis.

A BLAST (Basic Local Alignment Search Tool) search at NCBI (National Center for Biotechnology Information) was performed in order to confirm nematode origin and most related species (Altschul et al. 1997). The newly obtained sequences for ITS containing region and the D2-D3 expansion domains of 28S gene were aligned using MAFFTv. 7 software (Katoh and Standley 2013) with default parameters with the corresponding published gene sequences of *Heterodera* species. Sequence alignments were manually edited using BioEdit in order to improve the multi-alignment. Outgroup taxa for each dataset were chosen according to the results of previously published data. Phylogenetic trees, obtained for ITS dataset and the D2-D3 expansion domains, were performed with Maximum Likelihood (ML) and Maximum Parsimony (MP) method using MEGA version 6 software (Tamura et al. 2013). ML analysis under a general time reversible and a gamma-shaped distribution (GTR + G) model was performed for ITS and 28S datasets. The phylograms were bootstrapped 1,000 times to assess the degree of support for the phylogenetic branching indicated by the optimal tree for each method. The newly obtained sequences were submitted to GenBank with the following accession numbers: LT990598 for the 18S rRNA gene; LT990599-LT990600 for the D2-D3 expansion domains of the 28S rRNA gene; LT990247-LT990248 for the ITS region.

RESULTS

Morphological and morphometrical characterization.

Observation under light microscope of second-stage juveniles of the recovered nematode (Fig. 1) showed that the species studied is morphologically close to the original population of *H. mediterranea* (Vovlas et al. 1981).

Second-stage juveniles of the Tunisian population are characterized by a hemispherical, slightly set-off lip region, 4 µm high and 7.5 µm wide, with three (rarely four) distinct post-labial annuli (Fig. 1D - E). The stylet is robust, with knobs measuring 2 µm in height and 5 µm across, with concave anterior surfaces (Fig. 1D - E.). Pharyngeal glands well developed, extending 134 to 152 µm from head end and overlapping intestine ventrally, ca. 35 µm in length. Lateral field bearing four lines. Tail stout and short, irregularly annulated, tapering abruptly to a finely rounded terminus (Fig. 1F).

Morphometrics of five second-stage juveniles of the Tunisian population of *H. mediterranea* are in agreement with the original species description (Vovlas et al. 1981) (Table 1) except for minor intraspecific differences with longer stylet (26.2-28.3 µm *vs* 25.0-27.0 µm), shorter tail (32.4-42.2 µm *vs* 38.5-45.0 µm) and shorter hyaline portion (15.0-26.5 µm *vs*19.0-26.0 µm).

The examination of three cysts of *Heterodera* has allowed measuring the length (excluding neck), 526 (418 - 583) µm and the width, 340 (257 - 381) µm. Ratio L/W is 1.6 (1.5 - 1.6) and agree with the original population (L/W:1.2 - 1.6). Cysts are lemon shaped (Fig 1B), with prominent vulval cone, provided with bullae, and with vulval fenestra

(ambifenestrate), 46 (38-51) µm long, which is in the range of measurements of original population (42 - 48 µm). The distances between vulval slit and anus, and vulval slit and underbridge was calculated on two cysts, measuring 52 (43 -61) and 49 (47 - 51.5) µm, respectively.

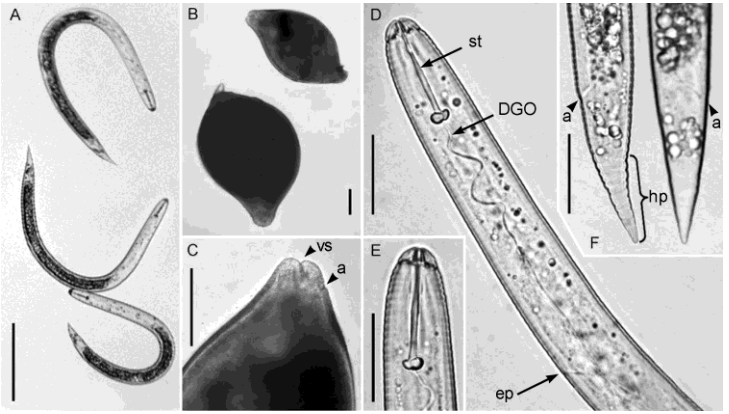

Fig. 1. Light micrographs of the Tunisian population of *Heterodera mediterranea*. A: Second-stage juveniles entire body; B: Mature cysts; C: Vulval cone in lateral view, showing vulval slit (vs) and anus (a); D: Second-stage juveniles anterior region, showing stylet (st), DGO and position of excretory pore (ep); E: Second-stage juveniles anterior end; F: Tail region showing anus (a) and hyaline portion (hp). (Scale bars: A-C = 100 µm; D-F = 20 µm).

An adult female of the Tunisian population of *H. mediterranea* was 418 µm long, which is in the range of the original population (380 - 670 µm), and 258 µm wide, shorter than the original population (280 - 540 µm) (Vovlas et al. 1981). The vulval slit measured 45 µm and the distance between anus and vulval slit is 53 µm, both slightly higher than the original population of *H. mediterranea* (36 - 42 µm) and (34 - 48 µm), respectively (Vovlas et al. 1981).

The sequenced ITS, D2-D3 expansion domains of the 28S rRNA gene and the 18S rRNA gene are 1028, 784, and 1626 bp long, respectively. BLAST search at NCBI revealed that the ITS, D2-D3 expansion domains of the 28S rRNA and the partial 18S rRNA sequences of *H. mediterranea* from Tunisia, newly obtained in this study, matched well with the corresponding sequences of *H. mediterranea* present in the database. The ITS sequences of *H. mediterranea* from Tunisia showed 99-98% similarity (9 to 16 bp different, 4-7 gaps, respectively) with the two populations of *H. mediterranea* from South Italy and one from Spain. The D2-D3 sequences of *H. mediterranea* from Tunisia, determined for the first time in this study, showed 98-99% similarity (4-11 bp different, 0 gap) with all *Heterodera* species belonging to the Schachtii group as previously reported in De Luca et al. (2013). The partial 18S rRNA gene (1626 bp) showed 99% similarity (2-3 bp different) with all *Heterodera* species of the Schachtii group.

For phylogenetic analyses, the ITS and D2-D3 sequences of *H. mediterranea* recovered from Tunisia were aligned with

the closest sequences of *Heterodera* species present in GenBank. Outgroup taxa for each dataset, *Globodera rostochiensis* and *G. pallida*, were chosen according to previous phylogenetic analyses for *Heterodera* genus. The phylogenetic trees of ITS and D2-D3 of *H. mediterranea* from Tunisia are shown in Figs. 2 and 3. Both phylogenetic trees confirmed that *Heterodera* species can be grouped in different groups and *H. mediterranea* grouped with the Schachtii group with high support (99%). Unfortunately, the 18S rRNA phylogenetic tree (result not shown) does not discriminate *Heterodera* species.

Table 1. Comparison between morphometrics of second-stage juveniles of the Tunisian population and the original population of *Heterodera mediterranea**

Population	Tunisian population	Original population (Vovlas et al. 1981)
Host-plant	*Olea europaea* subsp. *europaea* var. *europaea*	*Pistacia lentiscus*
n	5	20
L[a]	393.6 ± 22.7 (354.0 - 411.0)	405.0 (360.0 - 430.0)
a	19.7 ± 1.3 (17.5 - 20.5)	20.0 (18.0 - 22.0)
b	2.7 ± 0.1 (2.6 - 2.7)	3.0 (2.4 - 4.1)
c	10.2 ± 0.5 (9.5 - 10.9)	10.0 (9.0 - 11.0)
c´	2.8 ± 0.3 (2.4 - 3.1)	-
Stylet length	27.1 ± 0.9 (26.2 - 28.3)	26.0 (25.0 - 27.0)
DGO	4.7 ± 0.2 (4.4 - 5.0)	-
Max. body width	20.1 ± 0.5 (19.5 - 20.5)	20.0 (18.0 - 22.0)
Pharynx length	147.0 ± 7.3 (134.0 - 152.0)	-
Ant. end to Excretory pore	97.2 ± 3.6 (92.0 - 102.0)	98.0 (94.0 - 105.0)
Anal body width	14.0 ± 0.3 (13.6 - 14.5)	13.5 (13.0 - 14.0)
Tail length	39.0 ± 3.9 (32.4 - 42.2)	40.0 (38.5 - 45.0)
Hyaline tail length	20.5 ± 4.2 (15.0 - 26.5)	22.0 (19.0 - 26.0)

* Measurements are in µm and in the form: mean (range).

[a] L: Body length; a: Body length/maximum body width; b: Body length/pharyngeal length; c: Body length/tail length; c′: Tail length/body width at anus.

DISCUSSION

Our study reports for the first time the presence of *H. mediterranea* in olive trees in Tunisia. The morphology (Fig. 1) and the morphometrics (Table 1) of Tunisian *H. mediterranea* are in agreement with the original species description (Vovlas et al. 1981) except for minor intraspecific differences. This species has been detected in several countries: on *Pistacia* spp. in Italy (Vovlas et al. 1981; Vovlas and Inserra 1983), on olive trees in Spain (Castillo et al. 1999), and on cabbage in Turkey (Mennan and Handoo 2006). The occurrence of *H. mediterranea* in Tunisia extends the geographical distribution of this species to North Africa. As *Heterodera* spp. are known as very severe pathogens, a more extensive survey should be undertaken in the major Tunisian olive-growing areas to

understand the role that this nematode is playing in olive orchards. Castillo et al. (2010) demonstrated that *H. mediterranea* reduces the growth of the Spanish olive cv. Arbequina. Therefore, pathogenicity tests should be also undertaken with Tunisian olive cultivars to estimate the extent of damage that may be caused by this nematode. Finally, control measures should be implemented to avoid the further spread of this nematode in Tunisia. Among these, it is strongly suggested that olive nurseries release olive stocks free of plant parasitic nematodes, including *H. mediterranea*, and other pathogens.

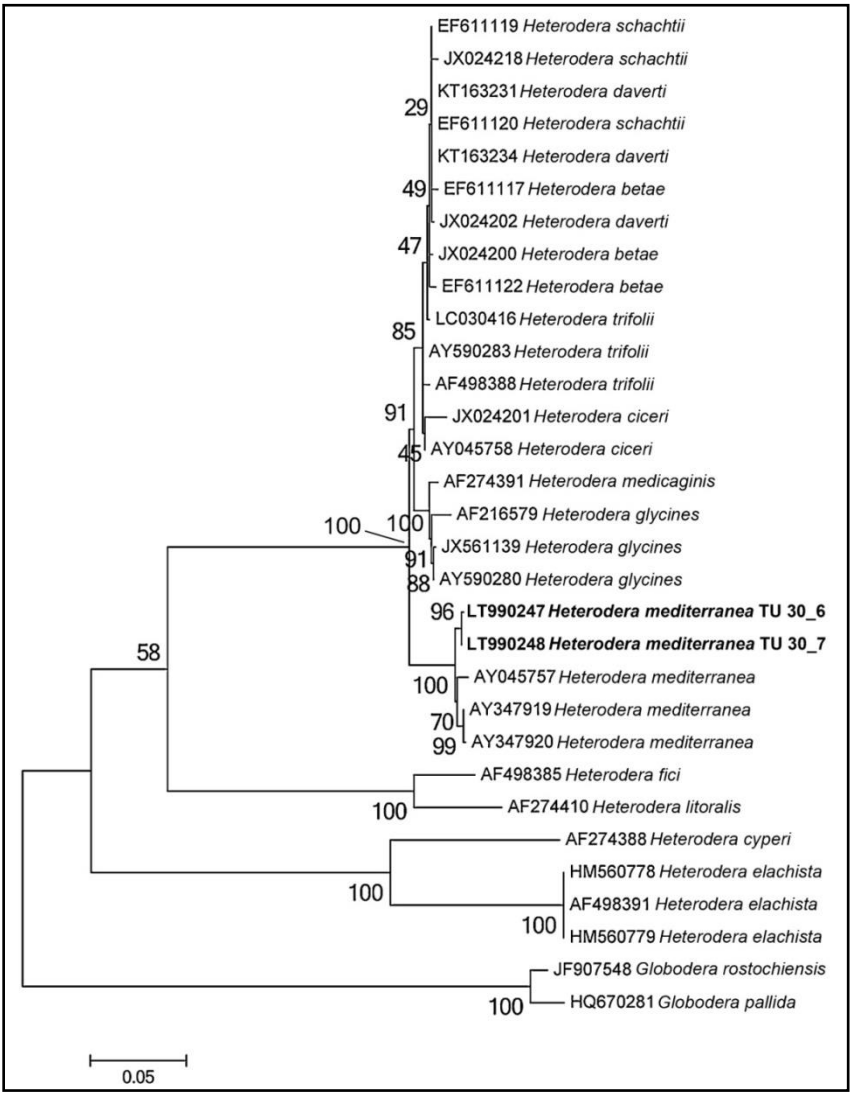

Fig. 2. Phylogenetic trees of ITS containing region of *Heterodera mediterranea* and the closest species. Sequences were analyzed using Maximum Likelihood method. Numbers at nodes indicate bootstrap values.

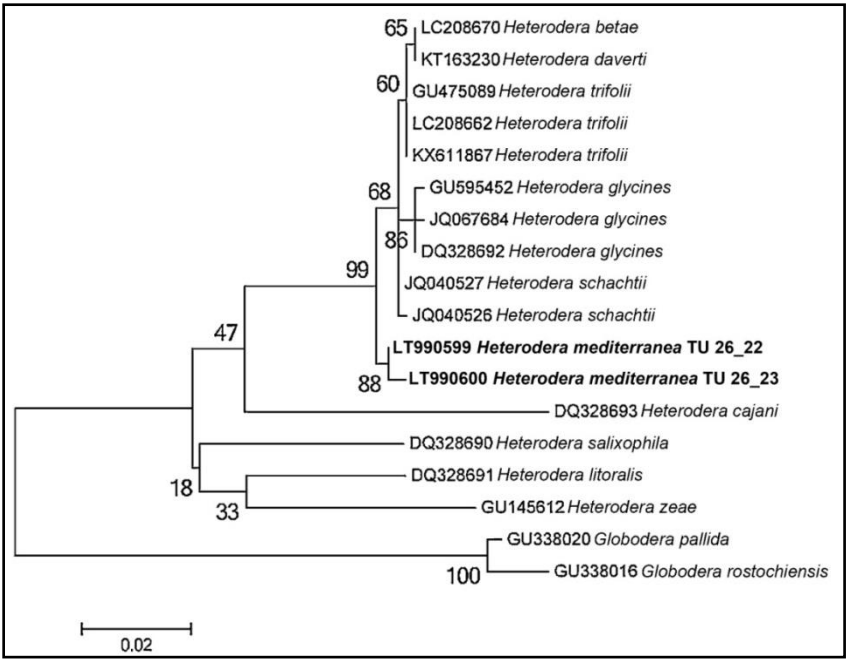

Fig. 3. Phylogenetic trees of the D2-D3 expansion domains of the 28S rRNA gene of *Heterodera mediterranea* and the closest species. Sequences were analyzed using Maximum Likelihood method. Numbers at nodes indicate bootstrap values.

ACKNOWLEDGMENTS

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RESUME

Guesmi-Mzoughi I., Troccoli A., Fanelli E., Radicci V., Regaieg H., Hadj-Naser F., Horrigue-Raouani N. et De Luca F. 2018. Premier signalement du nématode à kystes, *Heterodera mediterranea*, sur olivier en Tunisie. Tunisian Journal of Plant Protection 13 (1): 69-77.

Durant les enquêtes effectuées pour déterminer les nématodes phytoparasites associés à l'olivier en Tunisie, une forte infestation de l'olivier par les nématodes à kystes *Heterodera mediterranea* a été détectée dans une oliveraie située à Moknine (Monastir, Sahel Tunisien). Une approche taxonomique qui intègre les études morphobiométriques et les analyses moléculaires a été réalisée pour caractériser la population tunisienne de *H. mediterranea*. Les analyses phylogénétiques basées sur les régions ITS et les segments d'expansion D2-D3 de la grande sous-unité ribosomal 28S de l'ADN ribosomal ont montré que la population tunisienne de *H. mediterranea* appartient au groupe Schachtii. Ceci est le premier signalement de ce nématode en Tunisie.

Mots clés: 18S rRNA, D2-D3, analyse phylogénétique, *Heterodera mediterranea*, ITS, olivier, taxonomie

ملخص

قاسمي-مزوغي، إلهام وألبارتو تروكولي وايلينا فانيلي وفينسانزو راديتشي وهاجر رقيق وفتحية حاج-نصر ونجاة حريق-رواني وفرانكا دي لوكا.2018. أول تقرير حول النيماتودا الحوصلية *Heterodera mediterranea* على أشجار الزيتون في تونس. Tunisian Journal of Plant Protection 13 (1): 69-77.

خلال الدراسات التي أجريت لتشخيص أنواع النيماتودات الطفيلي على أشجار الزيتون في تونس، تم العثور على إصابة قوية لأشجار الزيتون بالنيماتودا الحوصلية *Heterodera mediterranea* في حقل زيتون بجهة المكنين (المنستير، الساحل التونسي). بالاعتماد على منهج تشخيص شامل يتضمن دراسات مورفوبيومترية وتحاليل جزيئية لتحديد المجتمعات التونسية للنيماتودا *H. mediterranea*، أثبتت التحاليل الفيلوجينية على أساس منطقة ITS ومقاطع التمدد D3-D2 من الوحدة الفرعية الريباسية 28S من الجينات الريباسية النووية أن المجتمع التونسي للنيماتودا *H. mediterranea* ينتمي إلى مجوعة Schachtii. هذا العمل هو أول تقرير حول هذا النوع من النيماتودا في تونس.

كلمات مفتاحية: أشجار الزيتون، تحاليل فيلوجينية، تصنيف، ITS، *Heterodera mediterranea*، D3-D2، 18S rRNA

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Aphids on Cultivated Cereals in Tunisia with a New Reported Species *Forda formicaria*

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ABSTRACT

Boukhris-Bouhachem, S., Ben Fekih, I., and Souissi, R. 2018. Aphids on cultivated cereals in Tunisia with a new reported species *Forda formicaria*. Tunisian Journal of Plant Protection 13 (1): 79-91.

A survey of the aphids associated with cultivated cereal in Tunisia was carried out during 2010 to 2012. Fourteen aphid species were recorded from eight regions (Cap Bon, Bizerte, Beja, Bousalem, Manouba, Zaghouan, Kef and Kairouan). For the first time a new species *Forda formicaria* was identified. Ten other winged species were also recorded in the suction trap of the Cap Bon region increasing the aphid richness to 24 species. From these aphid species listed, six are numerous and known for their economic importance: namely *Diuraphis noxia* as phloem feeder and *Metopolophium dirhodum, Rhopalosiphum padi, R. maidis, Schizaphis graminum*, and *Sitobion avenae* as virus vectors. Temporal activity of these species was established for 14 years by suction trap and their seasonal activity was discussed.

Keywords: Aphids, cereals, distribution, diversity, *Forda formicaria*, temporal activity

Cereals are the most important crop worldwide and the first source of food in Tunisia. Cultivated cereals (such as wheat and barley) are known to be infested by several aphid species. Aphids have the status of damaging insects as phloem-feeders, diverting for their own profit the nutrients necessary to plant growth and reproduction. They are considered as major pests particularly on winter wheat in Europe (Poehling et al. 2007). Mean annual losses induced by aphids were estimated at 700,000 t of wheat (Wellings et al. 1989). In Britain, losses in wheat attributed to aphids were about 10-13% (Tatchell 1989). Aphids also inject saliva that could be phytotoxic like that of *Diuraphis noxia* native from the southern former USSR. It subsequently spreads rapidly throughout wheat producing regions of the western USA where it caused hundreds of million dollars losses in wheat and barley crops, through reduced yields and increase pesticide treatment costs. Annual direct yield losses reached $274 million in 1988 and dropped to less than $10 million by 1993 (Michaud and Sloderbeck 2005). Aphid infestations not only reduce yield but also spoil the baking quality of the grains by inducing changes in their nutritional composition (Oakley et al. 1993). In addition, they can transmit numerous viruses. In fact, nearly 50% of insect-borne viruses (275 out of 600) are transmitted by aphids (Dedryver 2010). Barley Yellow Dwarf Virus (BYDV) is

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strictly and specifically transmitted by aphid vectors in a persistent and circulative manner. BYDV belongs to the Luteoviridae family which is widely spread throughout the world and responsible for considerable yield losses in major cereal crops particularly on barley (Rabbinge and Mantel 1981; Svanella-Dumas et al. 2013). BYDV infections induce fewer ears, less grain per tiller and lower thousand grain weights (Poehling et al. 2007). Even low populations of aphids can spread the virus. Four cereal aphids: *Rhopalosiphum padi, R. maidis*, *Sitobion avenae* and *Schizaphis graminum* are known as virus vectors of BYDV (Comas 1996).

In Tunisia, seven aphid species are reported by Dhouibi and Methnani (1990) among them the four cited vectors. Furthermore, a field survey showed that BYDV is most common in three regions (Zaghouan, Cap Bon and Bizerte) with an incidence estimated at 14-35% (Najar et al. 2017). Vector colonization and movement patterns influence the timing and pattern of virus epidemics linked to yield losses. Infections depend largely on aphid numbers, spread and weather conditions. The purpose of this study was to identify the species compositions of cereal aphids, list the potential vectors in Tunisia and describe the temporal flight activity of winged aphid by a suction trap.

MATERIALS AND METHODS

On cereal plants, heavy numbers of aphids have been recorded in 2010 at Kef region and the plants become sick and died; they were sampled to identify the species implicated in this big loss.

In March 2011, a damage of a mixed colony of aphids on roots was first observed at Manouba (North of Tunisia) and then in 2012 at Kef (North West). Aphids were collected from barley and wheat. Specimens were conserved on ethanol alcohol and then prepared as microscopic objects in Canadian balsam to be identified.

Furthermore, the inventory of aphid species was achieved through field surveys carried out in seven Tunisian regions. In total, 20 different fields were prospected during 2012: Cap Bon (2sites) (36°48′59″N10°34′07″E), Bizerte (3) (37°16′27″N9°52′26″E), Beja (3) (36°44’05’’N, 9°13’35’’E), Bousalem (2) (36°33’42”N 8°56’40”E), Manouba (1) (36°43’09”N 9°29’10”E), Zaghouan (2) (36°24′10″N 10°08′34″E), Kef (3) (36°11′10″N 8°42′00″E), and Kairouan (4) (35°39’50”N 9°59’10”E). In each site, 20 cereal plants infested with aphids were sampled between March and May. The frequency of aphid was calculated as the number of aphids divided by the total of collected aphids.

In addition, a 12.2 m suction trap is operated in Soliman (Cap Bon) from July 2002 until now. Aphids were collected every day from the suction trap. These aphid daily records allow us to know the aerial density of flying cereal aphids. We present here captures from 2003 to 2016 (there was a suction trap break during a long period of the year 2014 and little period of 2015, results are less in number compared to the other years). All aphid specimens were preserved in 96% ethanol until identification. Species were identified based on several keys (Blackman and Eastop 1994, 2000, 2006, 2012; Favret 2014; Jacky and Bouchery 1984; Nafría 2013; Remaudière and Seco Fernandez, 1990).

RESULTS

Aphid identification and distribution.

From more than forty cereal species worldwide, 14 aphid species (Table 1) were identified on cereals (wheat and barley) including *Diuraphis noxia*, *Metopolophium dirhodum*, *M. festucae,*

Rhopalosiphum padi, *R. maidis*, *R. rufulum*, *Sitobion avenae*, *Sitobion fragaria*, *Schizaphis graminum, Sipha maidis*, and *S. elegans*. Only at Manouba, *Geoica utricularia* was identified on roots. From Manouba and Kef samples, aphid colonies contain two more other *Forda* species on plant roots*:* namely *F. marginata* and *F. formicaria* recorded (Fig. 1). The latter is a new record added to the known Tunisian fauna.

The numbers of aphids recorded in 2012 show a real infestation of cereals sampled from Kairouan and Kef by aphids as compared to the other prospected regions. Generally, *R. maidis* was the most abundant species with 30% of the total aphids collected; *D. noxia* (26%) was the second abundant species, followed by *S. avenae* (22%) and *R. padi* (15%). However, *D. noxia* was the most frequent at Kef, *R. maidis* and *R. padi* the most prevalent at Kairouan, Beja, and Bousalem, *S. avenae* at Bizerte, Bousalem, Béja, Manouba and Cap Bon. *M. dirhodum* was only observed on Cap Bon, apterae and alatae on wheat. The Cap Bon region was the richest in terms of diversity of aphid species; it revealed 8 species out of 14 recorded.

Table1. Aphid identification and their distribution in the different prospected regions in 2012

Aphid species	Cap Bon	Zaghouan	Kairouan	Bizerte	Bousalem	Beja	Manouba	Kef	Total
Diuraphis noxia		14						1185	1199
Forda formicaria		18					62		80
Forda marginata							26		26
Geoica utricularia							47		47
Metopolophium dirhodum	69								69
Metopolophium festucae	12								12
Rhopalosiphum maidis			760	97	252	261		11	1381
Rhopalosiphum padi	31		245	65	165	177		9	692
Rhopalosiphum rufulum			5		26	14			45
Schizaphis graminum	4		4				7		15
Sipha elegans	2								2
Sipha maidis	44								44
Sitobion avenae	101	56	75	244	201	193	123	5	998
Sitobion fragaria	23								23
Total	286	88	1089	406	644	645	265	1210	4633
Aphids/plant	14	4	54	20	32	32	13	60	

The evolution of the numbers of the different species corresponds to the phenological development of wheat. In fact, *R. maidis* and *R. padi* occurred in early March while *S. avenae* colonies were observed late in the season (late April) especially in the ears at Kef region (Table 2).

Table2. Infested cereal organs by aphids depending on sampling month in Kef (2012)

Aphids/plant organ	Leaves		Ears	
	March	April	March	April
Diuraphisnoxia	326	859	0	0
Rhopalosiphumpadi	2	5	0	0
Rhopalosiphummaidis	1	8	0	0
Sitobionavenae	0	0	0	5

The specific information for the two species are reported below.

- *Diuraphis noxia* has occurred on 2010 at Kef region and all the plants in the field become sick, dwarf and diedleading to considerable yield losses to the farmers of this region (Fig. 1 a). In some years, Russian wheat aphid (RWA) can be the most serious insect pest on wheat and barley in the arid region of Kef resulting from damage to the flag leaf. High colonies of RWA induce yellow to purpling of the damaged leaves and plant remained weak, dwarf and younger stages killing of the plant. The aphid feeds on the youngest leaves within the whorl of the upper leaves, causing the leaf to remain tightly rolled. Its feeding has a rapidly toxic effect on the plant; the leaves become rolled (Fig.1 e) and desiccated and infested ears become bent. RWA is enclosed in the rolled leaf and inaccessible to natural enemies and insecticides. Severely damaged plants appear stunted and stressed, and tillers become prostrate.
- *F. formicaria,* is recorded for the first time in Tunisia. The apterae are medium-sized, oval with dorsal surface of body highly domed, and short appendages, varying in color from off-white to dull yellow, to various shades of dark green (Fig.1 f); they were observed on roots of cereals as their secondary host. Alata have a dull green abdomen with dark dorsal transverse bands. The plants attacked by this species are dwarf with dried leaves. Colonies of *Geoica utricularia* were also noted in a mixture with *F. formicaria* (Fig.1 f).

Aphid flight phenology.

A total of 28.133 cereal winged aphids were captured at Soliman site using suction trap between 2003 and 2016. Ten further species were identified and added to the species previously observed on cereal sampling foliage (*Triticum* and *Hordeum*): namely *Anoecia corni, Aploneura lentisci, Rhopalosiphum rufiabdominalis, Hysteroneura setariae, Schizaphis minuta, Tetraneura ulmi, Baizongia pistaceae, Geoica lucifuga, Paracletus cimiformis, Pemphigus* spp. The species richness reached now 24 cereal aphid species in Tunisia both on wheat/barley and aerial sampling. The mean aphid's numbers counted between 2003 and 2016 varied from 4 to 5896 individuals. Thus, they were separated into

three groups: group 1 with more than 1200, group 2 between 102-606 and group 3 with less than 90 individuals (Fig.2-A,B,C).

Fig. 2 also shows the aphid density patterns for 14 years, characterized by peak densities where *A. lentisci, R. padi, R. maidis* and *S. graminum* were the most abundant in the suction trap. The three last ones were frequent both on foliage and in the trap. They present a peak of populations on 2006, 2007, and 2008. A population decline was observed from 2010 to 2013 and then another peak recorded in 2016. The group 2 composed of *S. avenae*, *M. dirhodum* and *S. fragaria*, known as vectors of BYDV, were of less importance in numbers as compared to group1. Peaks were noted in 2004, 2007, 2010 and 2016 for these populations.

No vectors were known from the group 3 which contained a limited number of populations and seemed not damaging to cereals. Only in 2008, they showed a relatively important peak.

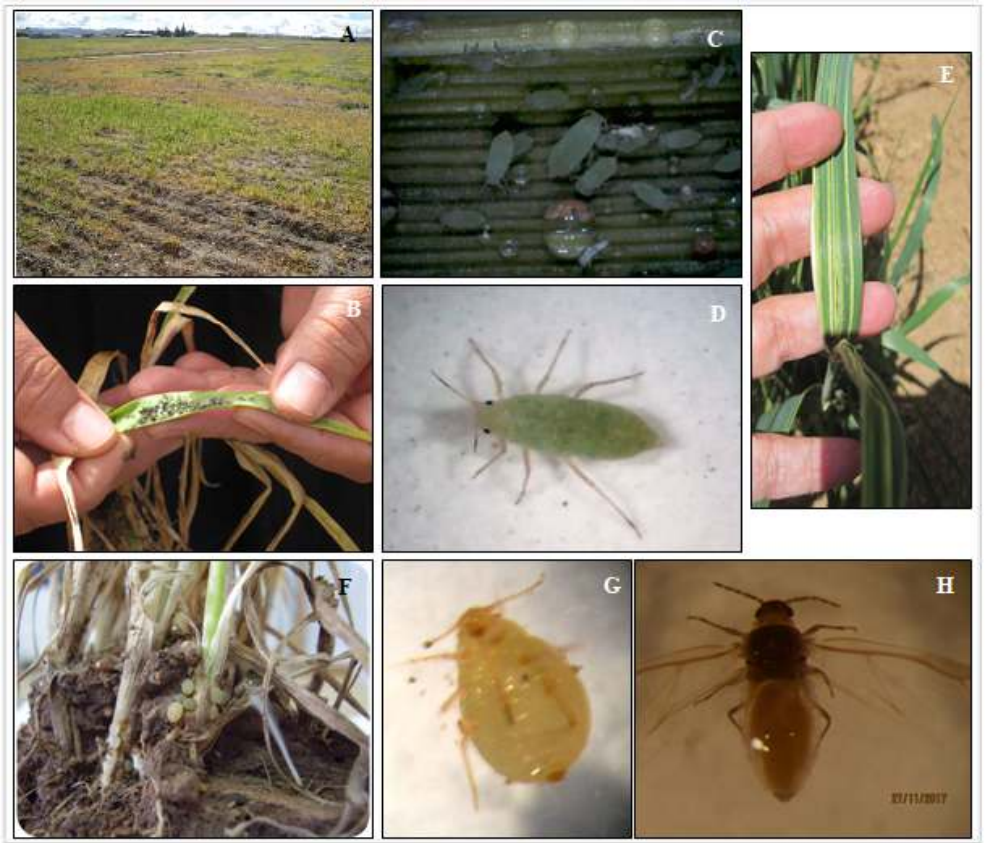

Fig. 1. Cereal aphids. *Diuraphis noxia*, damage at Kef region in 2010 (A), colonies (B, C), wingless adult (D), yellow chlorotic streaks on leaves (E); *Forda formicaria*, colony on roots (F), wingless adult (G), winged adult (H).

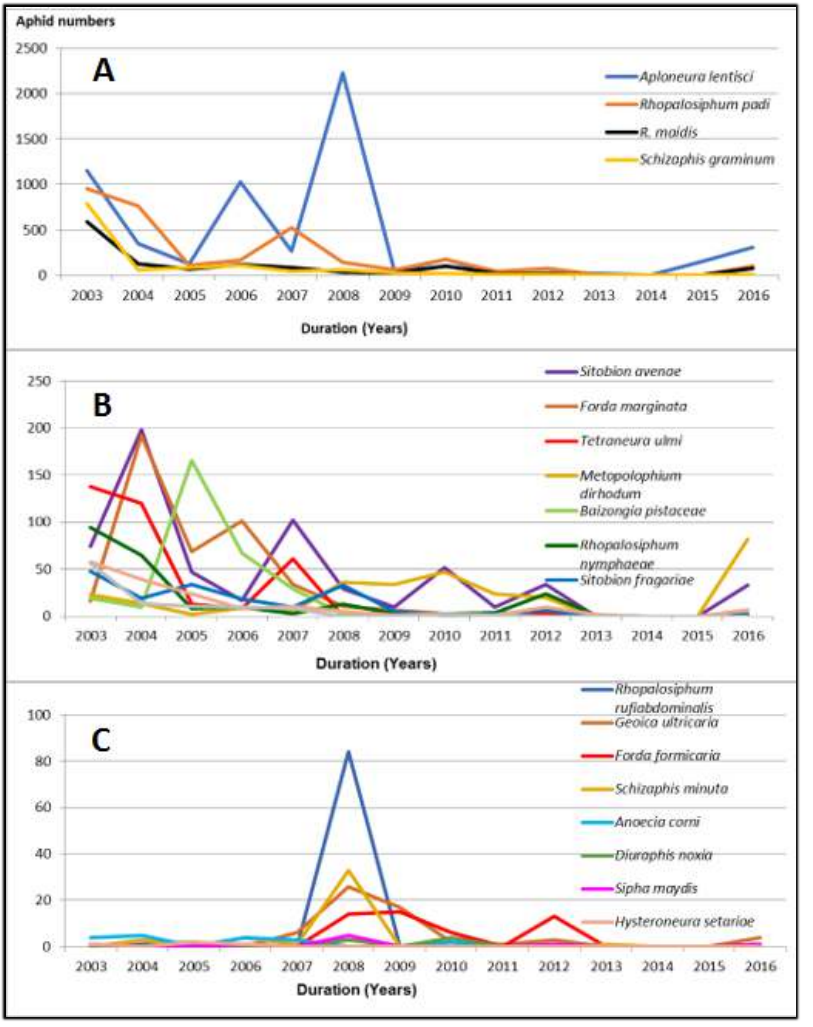

Fig. 2. Cereal aphid's flight in the suction trap of Soliman (Cap Bon) during 2003-2016 period. Group1 (A), group 2 (B), and group 3 (C).

Furthermore, flight activity of all aphid species generally occurred during two distinct seasons, both March and May during cereal growing season (spring) and a second one in the fall from September to November (Fig. 3). This is probably in relation with annual holocycle life cycle of heteroecious species which alternate between secondary and primary host. However, it appears that different species fly at different periods of the year. For example, *R. padi* flight was observed much earlier (in January) than most of other aphids. *R. maidis* was the second most frequent aphid after *R. padi* and was mainly observed in February and March. Both *M. dirhodum* and *S. graminum* have a peak population in March while *S. avenae* and *S. fragaria* in April.

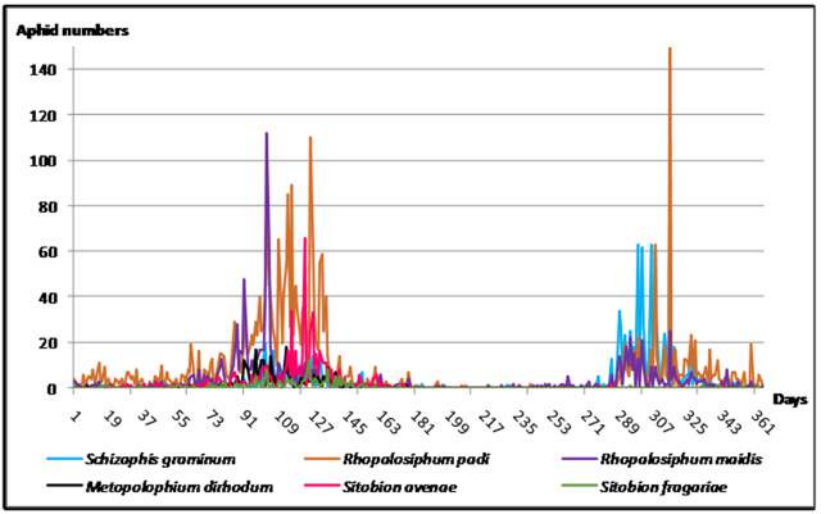

Fig.3. Temporal variation of different cereal aphid species in the suction trap of Soliman (Cap Bon), expressed as an average number by species, calculated during the 2003- 2016 period.

DISCUSSION

The present work brings to a total of 24 the number of aphid species associated with wheat and barley host cereal species. Five recovered species are of economic importance (namely *M. dirhodum, R. padi, R. maidis, S. graminum,* and *S. avenae*) and are known to transmit plant viruses in Tunisia. Their occurrence was high during the growing season suggesting their ability to propagate BYDV once present in the field. *R. padi* was captured in high numbers in April and in October but its flying decreased rapidly in the second half of October as also reported by Hullé et al. (1994).

Cereal aphidofauna does not show special characteristics and it consists of species already known in other European countries including those from the Mediterranean basin. According to Blackman and Eastop (1984, 2000), 32 aphid species are reported in the world while Holman (2009) mentioned 26 species on *Hordeum* and 41 on *Triticum*. In Tunisia, previous survey of aphid species showed the presence of seven species (Dhouibi and Methnani 1990), five of them were also listed by Harbaoui et al. (2008). A brief description of the identified species is presented below.

- *Diuraphis noxia* (Kurdjumov 1913), is an important pest of wheat and barley in several countries of North Africa and West Asia, e.g., Morocco, Algeria, Tunisia, Ethiopia, Yemen, Turkey, and Iran (El Bouhssini et al. 2011). *D. noxia* is originated in Europe and Central Asia. Feeding damage caused by *D. noxia* to plant leaves results in characteristic longitudinal white, yellow or red chlorotic streaks with a convoluted rolling of the leaf. Rolling of leaves reduces photosynthetic area and protects aphids from contact with insecticides and natural enemies (Khan et al. 2010). The symptoms observed on RWA infested plants are suggested to be partly due to the aphid's ability to

inflict severe damage on the phloem transport. This damage leads to noticeable reduction in the transport of assimilates within 72 h of RWA infestation and a significant reduction during prolonged feeding which, in most cases, results in total cessation of phloem transport (Saheed 2010).

- *F. formicaria* (von Heyden, 1837) is a very common Mediterranean species on *P. terebinthus* and was reported by many authors. *F. formicaria* is one of the gall-forming aphids on *Pistacia* trees (Anacardiaceae). The primary hosts of *F. formicaria* are *P. terebinthus* and *P. atlantica*. The aphid has a typical 2-year holocycle involving alternation between *P. terebinthus* and roots of the secondary hosts including Gramineae (*Agropyron, Agrostis, Bromus, Cynodon, Dactylis, Hordeum, Lolium, Poa, Triticum*…) in winter, various grasses (Zwolfer 1958). *F. formicaria* is also present in Europe, the Middle East, Central Asia, Siberia, and North America.
- *F. marginata* (Koch1957) apterae on grass roots are small to medium sized with reduced legs and antennae; body highly domed dorsally, brownish yellow or greenish yellow in color. Primary hosts are *Pistacia mutica, P. palestina*, and *P. terebinthus*. Secondary hosts are numerous species of Gramineae including *Agropyron, Agrostis, Avena, Bromus, Dactylis, Festuca, Hordeum, Poa, Secale*, and *Triticum* genera. The species is distributed in Europe, the Mediterranean region, the Middle East, Central Asia, Siberia, and North America. *F. marginata* is heteroecious holocyclic in the Mediterranean region and Middle East, forming galls on *Pistacia* by folding and swelling the edges of the leaflets (Blackman and Eastop 2006).
- *Geoica utricularia* (Passerini, 1856) is a species of temperate habitats that feeds mostly on root grasses. This aphid is globally distributed throughout most temperate regions of the world. It has a great range of hosts and is known to feed on roots of at least 40 species of grasses (Poaceae). It is particularly important on *Agrostis, Avena, Bromus, Festuca, Poa, Sorghum, Zea*, and other grasses. It also attacks 6 species of *Pistacia* (Anacardiaceae), some composite (Asteraceae), and *Plantago* (Plantaginaceae). It has not been involved in the transmission of any plant virus (Blackman and Eastop 2006).
- *Metopolophium dirhodum* (Walker 1849) is widely distributed in Europe, the Middle East, Africa, Central Asia, Japan, Australia, New Zealand, North America, and South America. Its primary hosts are cultivated and wild *Rosa* spp., and occasionally *Agrimonia* and *Fragaria* spp. Numerous species of grasses and cereals are secondary hosts of this species. *M. dirhodum* is a vector of Barley Yellow Dwarf Luteovirus (Blackman and Eastop 2000).
- *Metopolophium festucae* (Theobald 1917) is mainly found on cereals (*Avena, Hordeum, Secale, Triticum*) sometimes on other Gramineae especially *Lolium perenne* and *Phleum pratense*. This species is known from Europe and is monocious on Gramineae.
- *Rhopalosiphum maidis* (Fitch 1856) collected in Cap Bon, Beja, Kef, Kairouan, apterae on wheat and barley. It generally feeds in the whorl of the plant and extremely heavy infestation can induce honeydew. *R. maidis* is

Asiatic in origin but is now almost cosmopolitan in distribution. It is found on young leaves of grasses belonging to more than 30 genera. The species is heteroecious holocyclic with *Prunus* spp. as primary hosts in Asia, but apparently is entirely anholocyclic elsewhere although males occur sporadically (Blackman and Eastop 2006). *R. maidis* is probably the most important aphid pest of cereals in tropical and warm temperate climates (Blackman and Eastop 2000). It is an important vector of the BYDV.

- *Rhopalosiphum padi* (Linnaeus 1758) apterae and alatae occurs on leaves of wheat (*Triticum* sp.), on oats (*Avena sativa*) and colonies are in the underside of leaves from April to June. Alatae was captured in the suction trap. *R. padi* is a cosmopolitan species of Palaearctic origin. In Europe the species is heterocious holocylic between *Prunus* species, especially *P. padi* (the primary host) and Poaceae; it is anholocyclic on its secondary host plants in mild winter climates. *R. padi* is able to transmit a number of plant viruses (Blackman and Eastop 2000) and particularly the strain BYDV-PAV and Cereal yellow dwarf virus-RPV.
- *Rhopalosiphum rufulum* (Richards 1960) is a Nearctic species (Hidalgo et al. 2012) distributed in Canada and Europe (Denmark, England, Germany, Netherlands, former Czechoslovakia, Turkey) (Blackman and Eastop 2012; Holman 2009; Nafría 2013; Shenol et al. 2015). Primary host populations had not until recently been observed in the field in Europe, although gynoparae and alate males are produced in large numbers in autumn, and oviparae developed and laid numerous eggs which hatched to produce fundatrices on *Crataegus monogyna* under insectary conditions (Stroyan 1972). However, apterae of this species were recently collected in July on a *Crataegus* sp. in Turkey (Senol et al. 2015).
- *Schizaphis graminum* (Rondani 1852) (Aphidinae: Aphidini: Rhopalosiphina), apterae and alatae on *Triticum* sp. and is found in the suction trap. It is known from Southern Europe, the Middle East, Central Asia, parts of southern Asia, Japan, and North, Central and South America; it is often called greenbug. It has a monoecious holocycle in cold temperate climates but overwinters anholocyclically wherever winter conditions permit (Blackman and Eastop 2000). The aphids feed on the leaves of many species of grasses and cereal crops, often causing yellowing. Greenbug is a serious pest of cereal crops because it is the vector of several serious plant viruses including barley yellow dwarf Luteovirus (especially strain BYDV-SGV), miller red Luteovirus, sugarcane mosaic Potyvirus and Maize dwarf mosaic Potyvirus, among others that reduce yield (Blackman and Eastop 2000).
- *Sipha elegans* (Del Guercio 1905) usually forms colonies on the upper surfaces of the leaf blades of grasses and cereals, often causing them to roll upwards and develop yellow patches. Their plant hosts are *Agropyron* spp., *Festuca pratensis*, *Hordeum murinum* but sometimes on wheat and barley (Blackman and Eastop 2006).
- *Sipha maydis* (Passerini 1860) apterae were collected on *Hordeum* and *Triticum* in the Cap Bon region. Alatae were caught in the suction trap. *S. maydis* is found in Europe and the Mediterranean Region, the Middle East, Central Asia, India, Pakistan, North and South Africa. It occurs on numerous species in more than 30

genera of Poaceae. *S. maydis* is anholocyclic; it also has a monoecious holocycle with alatae males. It is a cereal crop pest in drier climates outside North-West Europe and is known to transmit Cucumber Mosaic Cucumovirus and Barley Yellow Dwarf Luteovirus (Blackman and Eastop 2000, 2006).

- *Sitobion avenae* (Fabricius 1775) was collected on *Triticum* sp. It occurs in Europe, the Mediterranean, the Middle East, Central Asia, India, Nepal, Pakistan, Africa, and North, Central and South America (Blackman and Eastop 2006). It feeds on both cultivated and wild species of Poaceae (Roberti 1993) and many other monocots. *S. avenae* has a monoecious holocycle, but in mild climates, it is anholocyclic, with asexual overwintering. This species is damaging to cultivated cereals and pasture grasses, and it is a vector of Barley Yellow Dwarf Luteovirus especially BYDV-PAV and BYDV-MAV and at least three other plant virus diseases (Blackman and Eastop 2000).
- *Sitobion fragariae* (Walker 1848) apterae and alatae on *Avena sativa*, on *Hordeum* sp., *S. fragariae* is found in Europe and Asia and has been accidentally introduced to South Africa, North and South America (Blackman and Eastop 2006). Its primary hosts are *Rubus* species, also *Fragaria*, *Rosa* and *Geum* spp., and it is polyphagous on secondary hosts in various unrelated plant families (Holman 2009). *S. fragariae* has a heteroecious holocycle (Blackman and Eastop 2006), an anholocycle and a possible paracycle (Roberti 1993). The species heavily infests blackberry and cereals and is a vector of Barley Yellow Dwarf Luteovirus (Blackman and Eastop 2000).

Few minorities (10%) of aphid species complete their life cycle on two completely different hosts and are known to be dioecious (Dixon1998). This requires that they often move between primary host (perennial) and secondary one (herbaceous) during their life cycle. Several species have this behavior such as *R. padi*. It migrates from *Prunus* and colonizes the cereals in February exploiting the complementary growth patterns of herbaceous and woody plants host-alternating and can overcome the constraints imposed by the seasonal changes in temperature.

The increase or the decline of the abundance of aphids between years (Fig. 2) is due mainly to variations in environmental conditions. It is well documented that a gradual change in climate, as well as local or regional climate characteristics, can affect population abundance (Martin 1998) and species distribution (Parmesan 1996). Aphids are tending to arrive earlier in the growing season (Fig.3) when crops are more susceptible to feeding damage and to viruses transmitted by aphids. For cereal, warmer winters increase the risk from BYDV because they lead to a greater prevalence of anholocycle and hence continued parthenogenesis on cereal (Harrington 2003).

Finally, it is important to comment the average of aphid numbers calculated by sampling field which is exceeding (except for Zaghouan) the nuisance threshold known around 7 to 10 aphids per tiller (Hansen 2000; Larsson 2005). The population density of aphids and hence the frequency and intensity of damages is expected to be amplified in the future by climate change. A large number of aphids were captured in early spring and in the fall. Suction trap catches indicate the aphid population abundance in the Cap Bon region. The cosmopolitan species *R. padi*,

R. maidis, S. graminum, S. avenae and *M. dirhodum* are among the most abundant species in the suction trap samples. They were found in wheat and barley and seem responsible for vectoring BYDV. English grain aphid, *S. avenae*, was the last species to colonize wheat each season, and the most abundant. *S. avenae*can be responsible for late-season virus transmission and causes direct yield loss by feeding on heads and flag leaves. Pest management efforts should focus on suppressing these aphid species to limit virus propagation and reduce yield losses.

Other aphids as *D. noxia* on leaves, *G. utricularia, F. formicaria* and *F. marginata* on roots are damaging to crop by their extraordinary capacity for population increase, mainly by parthenogenetic reproduction and their feeding on phloem of cereal plants. These species have also to be considered for reducing pest populations in wheat and barley in the future.

RESUME

Boukhris-Bouhachem S., Ben Fekih, I. et Souissi R. 2018. Pucerons sur les céréales cultivées en Tunisie avec le signalement d'une nouvelle espèce *Forda formicaria*. Tunisian Journal of Plant Protection 13 (1): 79-91.

Une étude sur les espèces de pucerons associées aux céréales cultivées en Tunisie a été réalisée entre 2010 et 2012. Quatorze espèces de pucerons ont été recensées dans 8 régions (Cap Bon, Bizerte, Béja, Bousalem, Manouba, Zaghouan, Kef et Kairouan). Pour la première fois une nouvelle espèce *Forda formicaria* a été identifiée. Dix autres espèces ailées ont également été enregistrées dans le piège à succion de la région du Cap Bon, ce qui élève la richesse des pucerons à 24 espèces. Parmi ces espèces, six sont abondantes et connues pour leur importance économique dont *Diuraphis noxia* comme suceur de sève et *Metopolophium dirhodum, Rhopalosiphum padi, R. maidis, Schizaphis graminum* et *Sitobion avenae* comme vecteurs de virus. L'activité temporelle de ces espèces a été établie pendant 14 ans par un piège à succion, la variation saisonnière des espèces de pucerons a été discutée.

Mots clés: Activité temporelle, céréales, distribution, diversité, *Forda fomicaria*, pucerons

ملخص

سنية بوريص-بوهاشم، ابتسام بن فقيه ورابحة السويسي. 2018. حشرات المن على الحبوب المزروعة في تونس مع تسجيل نوع جديد *Forda formicaria*. Tunisian Journal of Plant Protection 13 (1): 79-91.

قمنا بدراسة استقصائية عن حشرات المن التي تضر بمزارع الحبوب بثمانية مناطق من البلاد التونسية وهي الوطن القبلي وبنزرت وباجة وبوسالم ونوبة وزغوان والكاف والقيروان بين 2010 و 2012. سجلنا وجود 14 نوعا من هذه الحشرات. تم لأول مرة تسجيل نوع جديد من المن الذي يضر بجذور النبتة وهو *Forda formicaria*. إضافة إلى ذلك، تم الحصول على عشرة أنواع أخرى بواسطة مصيدة الالتصاص المركزة بجهة الوطن القبلي ما ساهم في إثراء عدد الأنواع إلى 24. من بين الأنواع المدرجة هناك ستة ذات أعداد وافرة ومعروفة من حيث دى خطورة تأثيرها على المحاصيل سواء كانت بواسطة تغذيتها على النسغ النباتي مثل *Diuraphis noxia* أو كناقلة للفيروسات مثل *Metopolophium dirhodum* و *Rhopalosiphum padi* و *R. Maidis* و *Schizaphis graminum* و *Sitobion avenae*. تم تأسيس النشاط الزماني لهذه الأنواع لمدة 14 عاما عن طريق مصيدة الالتصاص ومناقشة تغيراتها الموسمية.

كلمات مفتاحية: تنوع، توزيع، حبوب، حشرات المن، نشاط زماني، *Forda formicaria*

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First Report on the Occurrence of the Braconid parasitoid *Opius monilicornis* on the Chickpea Leaf Miner *Liriomyza cicerina* in Tunisia

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ABSTRACT

Soltani, A., Beyareslan, A., Haouel-Hamdi, S., Bousselmi, A., Amri, M., and Mediouni-Ben Jemâa, J. 2018. First report on the occurrence of the braconid parasitoid *Opius monilicornis* on the chickpea leaf miner *Liriomyza cicerina* in Tunisia. Tunisian Journal of Plant Protection 13 (1): 93-100.

Surveys were conducted during 2016 and 2017 in chickpea crops to document the parasitoid species of the chickpea leaf miner (*Liriomyza cicerina*) in Beja and Kef sites (north-west of Tunisia). One braconid wasp species namely *Opius monilicornis* was recorded for the first time as parasitoid on *L. cicerina* larvae. Larvae parasitism was observed from the end of March onwards and reached its peak during April coinciding with the second annual generation of the pest. Parasitism was noticed only on the second and third instars leaf miner larvae. The parasitoid abundance was higher in Beja site compared to Kef. In winter chickpea crops, parasitism rates during 2016 and 2017 ranged from 11.44 to 17.95% while in spring they fluctuated from 11.96 to 19.77%.

Keywords: Braconidae, chickpea, leaf miner, *Opius monilicornis*, parasitism

Leaf miner flies are a highly diverse group of exclusively phytophagous species and occur worldwide (Shahreki et al. 2012). The genus *Liriomyza* comprises numerous species that are economically important pests of many agricultural crops including chickpea (Naresh and Malik 1986). *Liriomyza cicerina* is the most important pest that causes significant damages on chickpea crops in West Asia and North Africa and the Mediterranean region (Cardona 1983; Çikman and Civelek 2006; El-Bouhssini et al. 2008; Soltani et al. 2016). Damages are caused by larvae consuming the mesophyll of leaves and the formation of holes and galleries with different shapes in the leaf tissue which reduce the photosynthetic capacity of infested leaves (Çikman 2006).

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In Tunisia, chickpea is ranked as the second major cultivated food legume crop after faba bean (Ouji et al. 2016). Fewer insecticidal treatments were undertaken in chickpea crops. Thus, as reported by Minkenberg and van Lenteren (1986), in such agricultural systems using infrequent pesticide applications, partial to complete control of agromyzid leaf miners is often the result of the abundant parasite fauna action. Consequently, in this work, we undertake a first survey to identify natural occurring parasitoids on *L. cicerina* larvae. In these regards, Heimpel and Meloche (2001) reported that a number of parasitoids of leaf miners have been recorded throughout the world. In addition, more than 140 species of parasitoids as natural enemies of *Liriomyza* have been reported (Liu et al. 2009). Braconid species (Hymenoptera: Braconidae) are among natural enemy's assemblages of agromyzid leaf miners (Gratton and Welter 2001). Among braconids, *Opius monilicornis* is a primary parasitoid on agromyzid leaf miners including *L. cicerina* (Çikma et al. 2008).

This paper reports first investigations on *L. cicerina* parasitoid species from the Braconidae family. Herein, we report the key morphological characters of *Opiu smonilicornis* and we present its parasitism rates in two regions of north-west Tunisia (Kef and Beja).

MATERIALS AND METHODS

Study sites.

The study was carried out over a 2-year period (2016 and 2017) in chickpea fields from two sites located in north-west of Tunisia: Beja (36°44'56.83"N 9°12'50.24"E) and Kef (36°07'13.96"N 8°43'21.39"E). The sites belonged respectively to sub-humid and semi-arid bioclimatic stages. For this study, the sampling was made from Beja 1 and Amdoun varieties (winter and spring varieties, respectively).

Sampling.

Sampling was carried out during the period from March to May each year. Thirty chickpea leaves (≈ 360-450 leaflets) harboring insect mines were weekly sampled from the two sites Beja and Kef. Samples were kept in a transparent plastic boxes closed with cotton ball and covered with muslin at 25± 2ºC, 70±5% RH and 14:10 h (L:D) photoperiod until the emergence of adults of both the pest and the parasitoids. After emergence, both pest and parasitoids specimens were conserved in 70% ethanol and stored at -4°C until identification.

Identification.

The emerged braconids were identified by Professor Ahmet Beyarslan (Department of Biology, Faculty of Arts and Sciences, Bitlis Eren University, Bitlis, Turkey). Specimens were morphologically identified based on taxonomic identification keys (Fischer 1972, 1977, 1986, 1987).

Assessment of parasitism rate.

For *O. monilicornis*, parasitism rate was determined according to Russell (1987) formula as following:

$$\text{Rate of parasitism (\%)} = \frac{Total\ parasitoids\ that\ emerged}{Total\ parasitoids\ that\ emerged + Total\ host\ emerged} \times 100$$

Statistical analysis.

All statistical analyses of individual number of parasitoids *O. monilicornis* were performed using the "SPSS statistical software version 20.0. Armonk, NY: IBM Crop". Differences in values of each year or season-sown crops and sites were tested by one-way ANOVA followed by Duncan test. All values given were the means of three

replications and were expressed as the mean ± standard deviation. Significant differences are reported as $P < 0.05$.

RESULTS

Parasitoid identification.

Opius monilicornis belongs to the order of Hymenoptera, super family Chalcidoidea, family Braconidae and subfamily Opiinae. This species has been reported as a parasitoid of *L. cicerina* in Syria (El-Bouhssini et al. 2008), Turkey (Çikman et al. 2006), Iran, Jordan, Morocco, Moldova, Spain, and Algeria (Ghahari et al. 2010; Khajeh et al.2014).

O. monilicornis had long antennae as body, 16- or 17-flagellomeres (Fig 1.1). The basal flagellomeres are 1.8 times as long as wide, middle flagellomeres and the rest not more than 1.5 times as long as wide; most flagellomeres clearly separated from each other, like a string of pearls, 2 or 3 sensillae visible in lateral view, the numerous hairs shorter than the flagellomeres wide (Fig 1.2).

The mesosoma is one-third times as long as high, upper side rather flat. The mesoscutumis about as wide as long, lateral lobes weakly rounded, declivity straight, dorsal fovea punctiform, notauli only anteriorly indicated; sides on posterior half margined. Only a few hairs occurred on the declivity and along the imaginary course of the notauli (Fig 1.3). The scutellum is longer than wide, axillae are absent. The rest of the mesosoma is smooth (Fig 1.4). Propodeumis fused with the metapleuron, no suture between them, lateral spiracles are present. All furrows of the side are smooth (Fig 1.5). Hind femur is 5 times as long as wide, also the other ones are short and thick (Fig 1.1).

For wings, pterostigma is cuneiform; radiusis arising from basal third. First section of radius is one-third as long as pterostigma wide, while second section of radius is about half as long as cubital vein; the third section of radius, is 2 times as long as second section of radius, curved inwards, radial cell ending before tip of wing, second radial cell narrowed distal, discoideus hardly long than nervusrecurrens, nervus almost interstitial, brachial cell closed, nervusparallelus arising from the middle of the distal side of brachial cell (Fig 1.7). For, the metasoma, first tergite 1.5 times as long as hind wide, parallel-sided behind, a little narrowed in front, smooth and shining, dorsal carinae developed on basal third (Fig 1.6). Ovipositor sheaths hidden or clearly visible, the rest of metasoma without sculpture, the projecting part a third as long as the metasoma, the hypopygium retracted (Fig 1.8).

Hosts and distribution.

This species has been reported as a parasitoid of *L. cicerina*in Turkey (Çikman et al. 2008). It was also reported from *L. congesta*, *L. pusilla*, *Ophiomyza* sp., *Phytomyza atricornis*, *Asphondylia verbasci* (Fischer and Koponen 1999). This parasitoid occurred in Syria, Jordan, Turkey (Çikman et al. 2008), Iran (Ghahari et al. 2012), Morocco (Lahmer and Zeouienne1987), and Algeria (Papp 1982).

Parasitism rate.

Results regarding the parasitism rates of the braconid *O. monilicornis* during 2016 and 2017 were reported in Fig. 2. Results revealed that this species was very abundant in both sites (Beja and kef). The correspondent parasitism rates during 2016 were 23.2% in Beja and 21.05% in Kef for winter variety, while for the spring one parasitism rates were 18.5%, in Beja and 17.3% in Kef. During 2017, an increase in parasitism rates was observed during June. Indeed, the peaks

of parasitism rates were 35.8% for winter variety and 35.11% for spring variety in Beja. Regarding Kef site, parasitism rates were 21.4% for winter variety against 28% for spring variety.

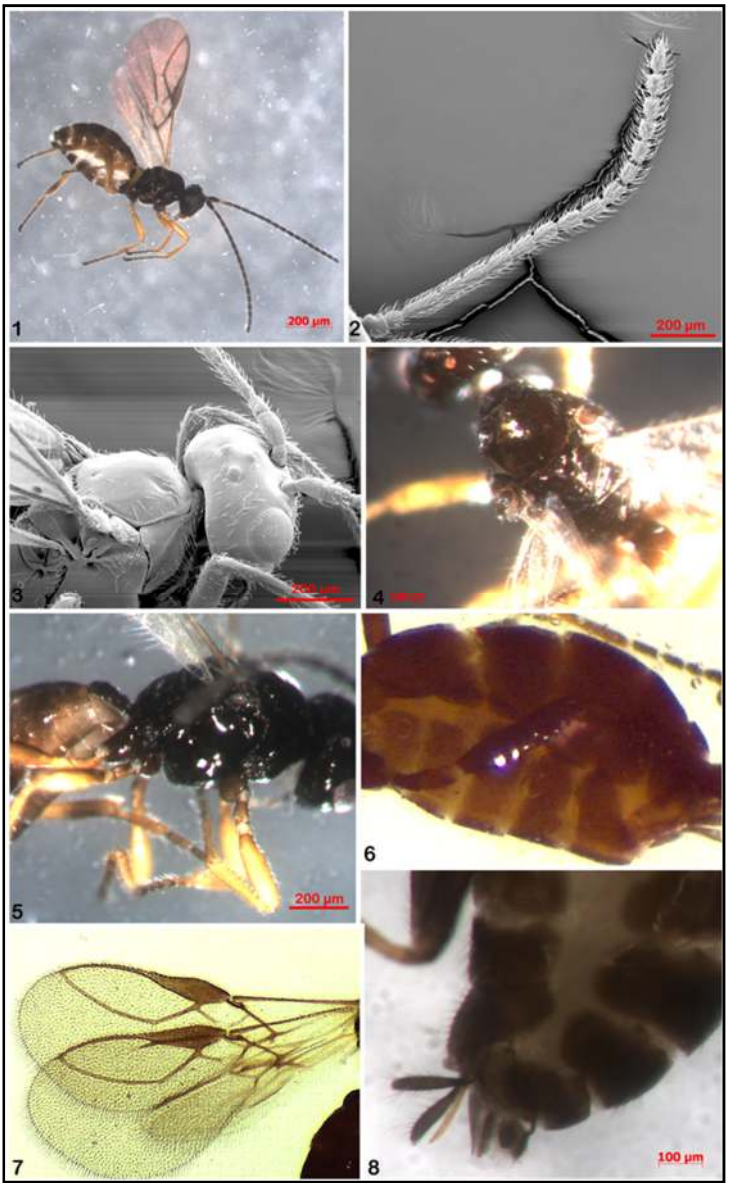

Fig. 1. (1) *Opius monilicornis* (adult ♂) (1) Antenna, (2) Mesoscutum (3) Mesosoma (4) Scutellum (5) Hind Femur (6) Lateral view of metasoma, and (7) Wings (8) Ovipositor.

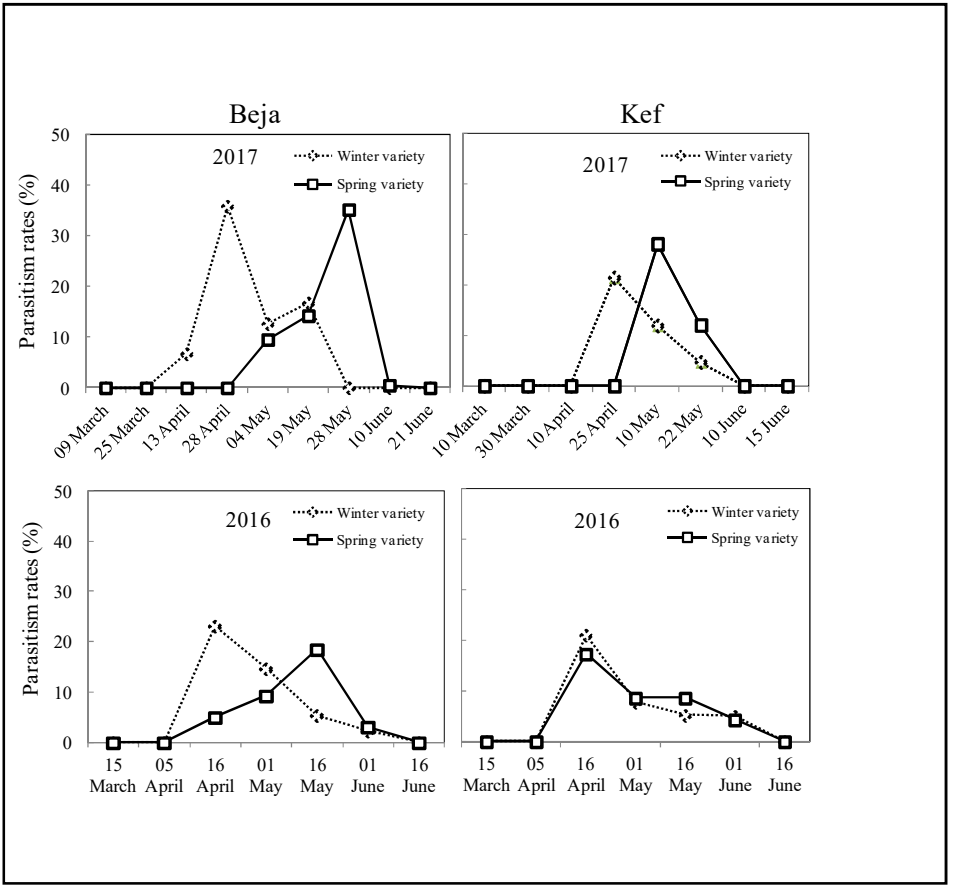

Fig. 2. Evolution of parasitism rates (%) *Opius monilicornis* during 2016 and 2017 in Beja and Kef sites.

Fig. 3 reported the emergence of *O. monilicornis* according to season-sown crops and sites. Results showed that the distribution depends on crops (winter or spring chickpea) and year. Statistical analysis showed the presence of high significant differences between the two sites ($F = 37.91$, $P < 0.01$). Additionally, statistical analysis revealed that in Beja site, *O. monilicornis* presented significant differences between winter and spring chickpea varieties for 2017, while no significant differences between winter and spring crops were observed for 2016.

The braconid *O. monilicornis* represented 44.09 and 43.2% of the total emerged insects in Beja and Kef, respectively, during 2016 and 52.5 and 44.9% during 2017. Furthermore, results indicated that Beja site differed from Kef by the number of individual parasitoids during both years for winter and spring varieties.

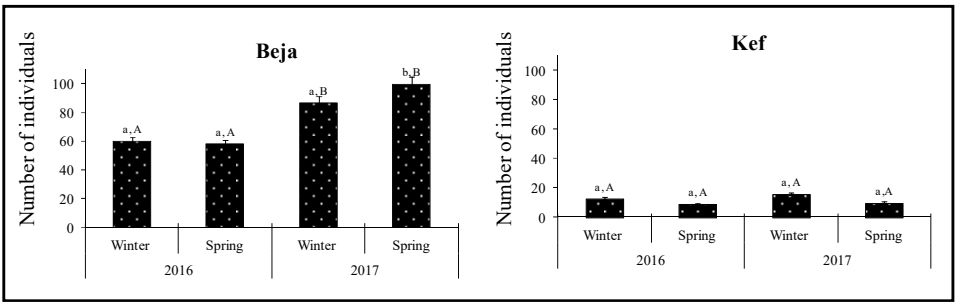

Fig. 3. Distribution of *Opius monilicornis* according to cropping season and years in Beja and Kef sites. Different letters indicated significant differences ($P < 0.05$) for each year among winter and spring chickpea varieties (lowercase letters); and among years for each winter or spring crops (uppercase letters). Each value is the mean ± SD of three replicates.

DISCUSSION

Agromyzid leaf miners are known to have rich natural enemy communities (Gençer, 2004). Numerous studies have been reported on the natural enemies of the Agromyzidae including *L. cicerina* in various countries (Asadi et al. 2006; Baideng 2016; Fathipour et al. 2006; Heinz and Parrella 1990; Lahmar and Zeouienne 1990; Sivapragasam et al. 1999). However, no previous studies were conducted on the natural enemies of the chickpea leaf miner in Tunisia. Thus, the present work carried out the first investigations on parasitoids occurring on *L. cicerina* in chickpea fields in Tunisia. This study reported the first occurrence of the braconid wasp *O. monilicornis* with an interesting parasitism rate of 19.77% in Beja during 2017. According to previous records, this parasitoid belongs to the superfamily of Ichneumonidea (Murphy and LaSalle 1999). Previous investigations on *L. cicerina* parasitoid fauna revealed that *O. monilicornis* is one of the parasitoid complex of chickpea leaf miner. In this regards, Çikman et al. (2008) reported that in Sanhurfa region in Turkey, the braconid *O. monilicornis* was one of the *L. cicerina* parasitoid fauna. Earlier, Hincal et al. (1996) and Gençer (2004) reported respectively *O. monilicornis* as parasitoid attacking *L. cicerina* in Izmir, Denizil, Uşakand Ankara province (Turkey). Besides, our results agreed with those obtained in Syria which revealed that the parasitoid *O. monilicornis* was found to be the most effective against the chickpea leaf miner, compared with *Diglyphusisaea*, as the parasitism reached about 70% (El-Bouhssini et al. 2008). In Morocco, Lahmar and Zeouienne (1990) identified *O. monilicornis* as the only parasitoid of *L. cicerina* achieving a parasitism rate of 20.35%. Such activity seems to be important compared to results accomplished in Turkey where *O. monilicornis* occurred with a low parasitism rate of 3.27% (Çikman et al. 2008). The relatively higher parasitism rate can suggest that this parasitoid is an important mortality factor in the dynamics of leaf miner populations. For this reason, it can be considered as a potential biocontrol agent against this pest.

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RESUME

Soltani A., Beyareslan A., Haouel-Hamdi S., Bousselmi A., Amri M. et Mediouni-Ben Jemâa J. 2018. Premier signalement du parasitoïde Braconidae *Opius monilicornis* sur la mineuse du pois chiche *Liriomyza cicerina* en Tunisie. Tunisian Journal of Plant Protection 13 (1): 93-100.

Des prospections ont été menées en 2016 et 2017 dans les cultures de pois chiche pour étudier les parasitoïdes de la mineuse (*Liriomyza cicerina*) du pois chiche dans les sites de Béja et du Kef (Nord-Ouest de la Tunisie). Une espèce de Braconidae *Opius monilicornis* a été enregistrée pour la première fois comme parasitoïde sur les larves de *L. cicerina*. Le parasitisme des larves a été observé à partir de la fin du mois de mars et a atteint son pic en avril, coïncidant avec la deuxième génération annuelle du ravageur. Le parasitisme n'a été observé que sur les larves des deuxième et troisième stades. L'abondance du parasitoïde était plus élevée dans le site de Béja que dans celui du Kef. Dans les cultures de pois chiche d'hiver, les taux de parasitisme durant 2016 et 2017 ont varié de 11,44 à 17,95% tandis que dans celles de printemps, ils ont fluctué de 11,96 à 19,77%.

Mots clés: Braconidae, mineuse des feuilles, *Opius monilicornis*, parasitisme, pois chiche

ملخص

سلطاني، عبير وأحمد بيرسلان وسمية حوال-حمدي وعربية بوسالمي ومعز عمري وجودة مديوني-بن جماعة. 2018. تقرير الأول عن وجود طفيل براكوندي *Opius monilicornis* على حشرة نافقة أوراق الحمص *Liriomyza cicerina* في تونس. Tunisian Journal of Plant Protection 13 (1): 93-100.

أجريت دراسة استقصائية سنتي 2016 و2017 في مزارع الحمص حول طفيليات الحشرة نافقة أوراق الحمص (*Liriomyza cicerina*) في موقعي باجة والكاف (الشمال الغربي لتونس). وقد تم تسجيل نوع من البراكوندي (Braconidae) (*Opius monilicornis*) لأول مرة كطفيل على يرقات *L. cicerina* . وقد لوحظ تطفل اليرقات من نهاية مارس ثم بلغ ذروته خلال شهر أفريل بالتزامن مع الجيل السنوي الثاني للآفة. وقد لوحظ التطفل فقط على يرقات الطّور الثاني والثالث للحشرة العائلة. وكانت وفرة الطفيل أعلى في موقع باجة مقارنة بموقع الكاف. وتراوحت معدلات التطفل في مزارع الحمص الشتوي خلال 2016 و2017 من 11,44 إلى 17,95% ومن 11,96 إلى 19,77% في مزارع الحمص الربيعي.

كلمات مفتاحية: تطفل، حمص، نافقة الأوراق، Braconidae، *Opius monilicornis*

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Management of the Tomato Leaf Miner *Tuta absoluta* in Tunisia: A Three Years' Survey

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ABSTARCT

Cherif, A., and Grissa-Lebdi, K. 2018. Management of the tomato leaf miner *Tuta absoluta* in Tunisia: A three years' survey. Tunisian Journal of Plant Protection 13 (1): 101-112.

Tuta absoluta is considered as one of the most devastating pest causing significant losses to tomato production worldwide. Thus, the knowledge of its biological characteristics makes its management less challenging. Here, the population dynamics of this pest was monitored in protected tomato crop in Takelsa region (Northeastern of Tunisia). Monitoring the flight activity of males by pheromone traps and leaf sampling revealed the presence of four generations. A maximum of 120 males / trap / week and 74.66 larvae / 40 leaves were recorded on May 13, 2016. Furthermore, there was a significant linear relationship between trapped adults and laid eggs ($R^2 = 0.81$; $P < 0.0001$), trapped adults and mines with larvae ($R^2 = 0.76$; $P < 0.0001$), and trapped adults and mines ($R^2 = 0.70$; $P < 0.05$). Likewise, mines with and without larvae were highly correlated ($R^2 = 0.72$; $P < 0.0001$).

Keywords: Population dynamics, tomato, traps, *Tuta absoluta*

Tomato (*Solanum lycopersicon*) is the second most commonly consumed vegetable in the world with a harvested area devoted to its production of about 5 million ha (Umpiérrez et al. 2012). In Tunisia, tomato cultivation and production resulted in a total of 29 000 ha/year and 1.2 million tons respectively (GIL 2016). This strategic crop may be threatened by many diseases and pests such as the tomato leaf miner *Tuta absoluta* (Cherif et al. 2017; 2018b; Ebdah et al. 2016; Ettaib et al. 2016a, 2016b; GIL 2016). This pest is noted as a major bio-aggressor of tomato cultivation originating from South America causing losses over than 80% in newly invaded areas (Bajracharya et al. 2016; Biondi et al. 2018; Cherif and Lebdi-Grissa 2013; Desneux et al. 2011; EPPO 2005). *T. absoluta* occurs in various cultivated solanaceous crops as well as wild species (Desneux et al. 2010, 2011; Notz 1992; Tropea Garzia et al. 2012) causing damages mainly to leaves, stems and fruits by creating mines (Bajracharya et al. 2016; Cocco et al. 2015; Salvo and Valladares 2007). The knowledge of *T. absoluta* population dynamics as well as its host range, voltinism overwintering and pest plant distribution is important to develop efficient control programs (Cherif and Lebdi-Grissa 2017; Cocco et

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al. 2015; Radcliffe et al. 2009). *T. absoluta* is not only a multivoltine non-diapausing species but has also a high population potential growth (Pereyra and Sánchez 2006). In the Mediterranean area, this pest was detected during the entire year and over winter in all life stages (Delrio et al. 2012; Tropea Garzia et al. 2012; EPPO 2005; Vercher Aznar et al. 2010). Life cycle of *T. absoluta* depends on climatic conditions as well as quality of available hosts (Cocco et al. 2015). Variable number of *T. absoluta* generations has been detected. In South America, up to 10-12 generations were detected (EPPO 2005) whereas, in Tunisia, population dynamics of this leaf miner was studied within single growing season (Abbes and Chermiti 2011; Cherif et al. 2013; Cherif and Lebdi-Grissa 2014; 2017; Harbi et al. 2012).

Few researchers have investigated the correlation between trap catches and infestation level (Abbes and Chermiti 2011; Caffarini et al. 2000; Mohamedova et al. 2016) and management strategies were based mainly on pesticides applications. At least, 12 classes of insecticides including organophosphates and pyrethroidsare were used to manage *T. absoluta* (Bueno et al. 2016). However, the excessive use of insecticides had strong side-effects not only on beneficial organisms, human health and biodiversity but also led, to the occurrence of resistant strains (Arno´ and Gabarra 2011; Biondi et al. 2012, 2013; Calvo et al. 2013; Campos et al. 2014; Cherif et al. 2018b; Roditakis et al. 2015, 2017; Siqueira et al. 2000) which requires new insecticides and also necessitates biological and biotechnological methods of control (Buenoet al.2016) including the use of natural enemies and resistant tomato varieties (de Oliveira et al. 2012; Desneux et al. 2010). A large number of wasps have been cited and tested against *T. absoluta* including parasitoids belonging mainly to the Trichogramatidae and Braconidae (*Trichogramma cacoeciae*, *Braconni gricans*) and predators such as mirids (*Nesidiocoruris tenuis*, *Macrolophus pygmaeus*) (Cherif and Lebdi-Grissa 2013; Cherif et al. 2018b; Desneux et al. 2010; Zappalà et al. 2013). Moreover, the use of sexual synthetic pheromones for mass trapping, mating disruption, or the sterile insect technique have been successfully tested against *T. absoluta* (Caparros-Megido et al. 2012; Cocco et al. 2013).

The main goal of the present work was to study the population dynamics of *T. absoluta* and understand the link between trapped adults, laid eggs and infestation level of tomato crop over three consecutive years in Takelsa.

MATERIALS AND METHODS

Population dynamics.

Experimental site. Population dynamics of *T. absoluta* was investigated in protected tomato crops (Takelsa region, Gouvernorate of Nabeul, Northeastern Tunisia) over three campaigns of autumn-spring growing season (2013/14, 2014/15, and 2015/16). Takelsa had a typical Mediterranean climate, with dry hot summers and cold rainy winters.

A total of 18 greenhouses were selected for this study. Six greenhouses were chosen each year for the survey of *T. absoluta*. Each greenhouse (500 m^2, 60 m length, 8 m wide and 3 m height) contained 1400 tomato plants.

Distance between rows and plants was 1.5 and 0.5 m, respectively. Three different cultivars chosen by farmers including Cenkara, Galaxy and Mirano, were grown separately in 2013/14, 2014/15 and 2015/16 periods of culture. Tomato plants were drip-irrigated under plastic mulch.

Lateral and upper openings of protected crops were equipped with insect-proof screens and were opened during times of high solar radiation to do not alter *T. absoluta* population dynamics inside the greenhouse (Cocco et al. 2015). Air should circulate from the outside to the inside of the greenhouse in order to remove excess heat and to avoid the risk of disease development. Basal leaves and lateral shoots were pruned by farmers when needed. For each study year, the surveyed greenhouses had the same calendula in terms of fertilization practices and insecticides sprays. Insecticides were applied against *T. absoluta* when the threshold fixed by the Tunisian ministry of agriculture, was reached (50 moth/sex pheromone trap).

In 2013/14 season, three insecticides (cyromazin (WP, Clave (75%), 30 g/hl), flubendiamid (WG, Takumi (20%), 30 g/hl) and azadirachtin (EC, Fortune aza (32 g/hl), 150 cc/hl)) were applied on 06/05/2014 only in three selected greenhouses. In 2014/15 season, no insecticide application was carried out. In 2015/16 season, two insecticide sprays were performed using azadirachtin (EC, Fortune aza (32 g/hl), 150 cc/hl)) on 24/03/2016 and indoxacarb (SC, Amiral (150 g/hl), 50 cc/hl) on 14/04/2016. Insecticides were sprayed by farmers until runoff at the recommended doses using a hydraulic knapsack hand sprayer of 10 liters**.** The seasonal phenology of the pest was less influenced by insecticides applications compared to its populations abundance (Cocco et al. 2015).

Monitoring of *T. absoluta*. *T. absoluta* population was monitored using two sex pheromone traps (Koppert Biological Systems, Pherodis®, 0.5 mg) per greenhouse set up regularly on 10/10/2013, 19/11/2014 and 24/11/2015. Visual inspections of tomato plants were also recommended to study its seasonal fluctuations as well as the population dynamics (Cocco et al. 2015; Witzgall et al. 2010**).** A distance of 25 m was kept between the pheromone traps placed at 40 cm above the ground.

Trapped adults were counted and removed from traps weekly. The contents of traps (water and a thin layer of vegetable oil) were replaced when needed. Sex pheromone capsules were renewed every 4 weeks.

The number of generations achieved by *T. absoluta* was evaluated through the developmental structure of immature stages (eggs, larvae and pupae). Insect presence was assessed weekly by random sampling of 40 tomato leaves from apical and middle parts in the upper area of plants in each greenhouse. Leaves were collected taking into account the increase in length of tomato plants during the growing period (Cherif et al. 2013).

The number of eggs, mines with or without alive larvae and pupae per leaf were recorded. Sampled leaves were inspected using a binocular microscope (Leica® Model MS5).

Statistical analysis.

Data of linear regressions and population dynamics were subjected to one-way ANOVA. Means of treatment were separated using Duncan's Multiple Range test at 5% level of probability (SPSS 21, 2012).

RESULTS

Population dynamics in protected crops.

Results of male flight activity indicated that, after three consecutive agricultural campaigns, *T. absoluta* performed up to four peaks under Takelsa greenhouse conditions. The first peak was recorded from October to December, the second was registered between January

and February. However, the third and fourth peaks were noted in March and May (Fig. 1). *T. absoluta* adults started to appear with low number in late autumn-early winter 20 days after plantation, whereas the highest catches were registered under spring at favorable climatic conditions. Trap catches during the autumn-winter period were low, of about 6.04, 2.27, and 8.36 males/trap/greenhouse, respectively, for the three surveyed growing season and then increased steadily in spring in parallel with the increasing of the temperatures (Fig. 1).

Finally, a significant difference between the number of laid eggs on the underside of tomato leaves (which correspond to 79 % of eggs) and those laid on the upper side ($F_{1, 95} = 11.386$; $P = 0.001 < 0.05$) was observed.

Relationship between trap catches, number of laid eggs and infestation levels.

Results from One-way ANOVA showed that there was a significant linear relationship between trapped adults and laid eggs, trapped adults and mines with larvae, trapped adults and total mines (with and without larvae) and mines with and without larvae which correspond to ($R^2 = 0.81$; $F_{1, 191} = 35.275$; $P < 0.0001$) (Fig. 3a) ($R^2 = 0.76$; $F_{1, 191} = 20.065$; $P < 0.0001$) (Fig. 3b) ($R^2 = 0.70$; $F_{1, 191} = 25.583$; $P < 0.0001$) (Fig. 3c) and ($R^2 = 0.72$; $F_{1,191} = 56.442$; $P < 0.0001$) (Fig. 3d) respectively.

DISCUSSION

Population dynamics in protected crops.

T. absoluta is a key pest of tomato cultivation worldwide (Biondi et al. 2018; Cherif et al. 2013; Cherif and Lebdi-Grissa 2017; Mohamadi et al. 2017). In Tunisia, tomato crop is grown all year round through main and late growing season which have great economic impact but represent optimal trophic conditions for occurrence of this pest. Moreover, Tunisia has Mediterranean climatic conditions which favor the development of this pest**.**

Here, population dynamics of *T. absoluta,* investigated through unheated greenhouses, indicated the occurrence of 4 flight peaks from October to May and 4 generations of eggs and larvae recorded on tomato leaves from February to May. Our findings are in accordance with other results from similar Mediterranean areas showing that *T. absoluta* was able to perform several generations per year (Abbes et al. 2012; Allache et al. 2012; Cherif et al. 2013, 2014; Cherif and Lebdi-Grissa 2017; Cocco et al.2013, 2015; Harbi et al. 2012; Lebdi-Grissa et al. 2010; Mahmoud et al. 2015). Pupae are present in a few number on leaves which confirm that pupation occurs usually in the soil (Cherif and Lebdi-Grissa 2017; Michereff and Vilela 2001). In protected tomato crops, initial infestation depends on residual populations from the previous cultivation while the pest density depends on length of the intercrop period (Cocco et al. 2015). Our study showed that trap catches increased in the spring, when temperature rose, with remarkable number compared to autumn-winter favoring the development of the pest. Our results are in line with those reported by many authors in Tunisia (Abbes et al. 2012; Cherif et al. 2013; Cherif and Lebdi-Grissa 2014; Cherif and Lebdi-Grissa 2018a; Harbi et al. 2012) and in other Mediterranean regions (Cocco et al. 2015; Mahmoud et al. 2015).

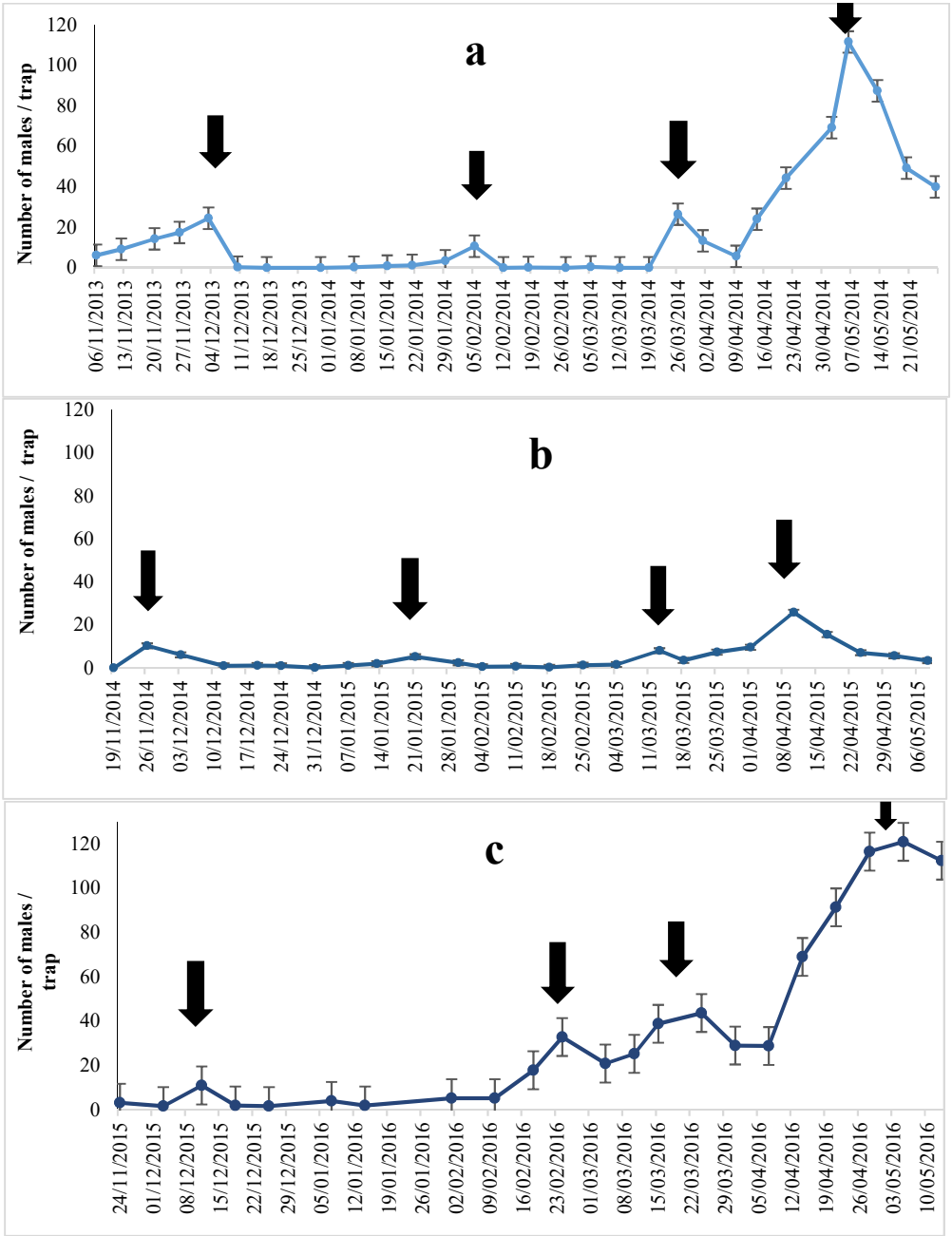

Fig. 1. Male flight activity of *Tuta absoluta* under tomato greenhouse conditions in Takelsa region. **a**: Campaign 2013/14. **b**: Campaign 2014/15. **c**: Campaign 2015/16).

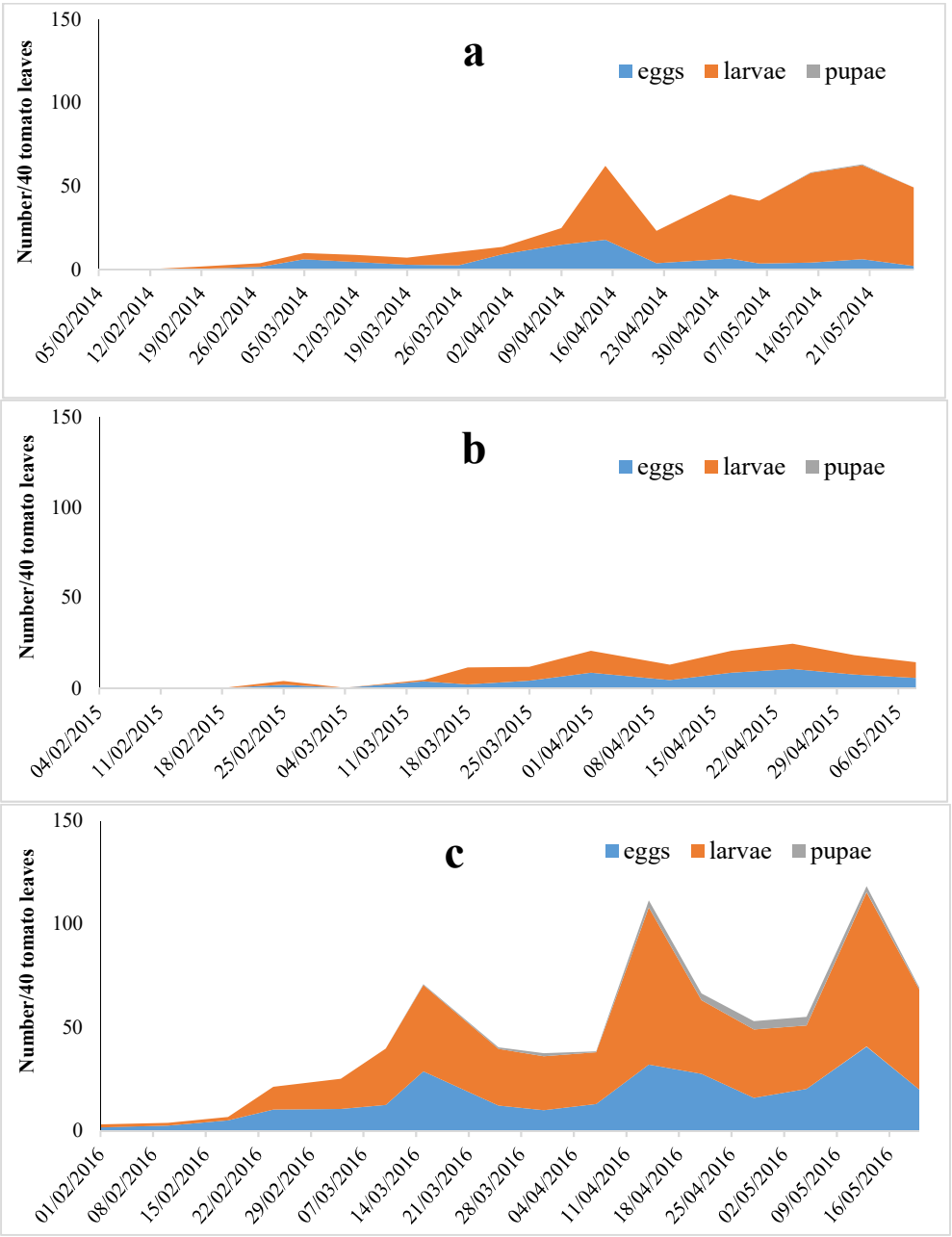

Fig. 2. Population dynamics of *Tuta absoluta* on tomato leaves under greenhouse conditions in Takelsa region. **a**: Year 2014. **b**: Year 2015. **c**: Year 2016

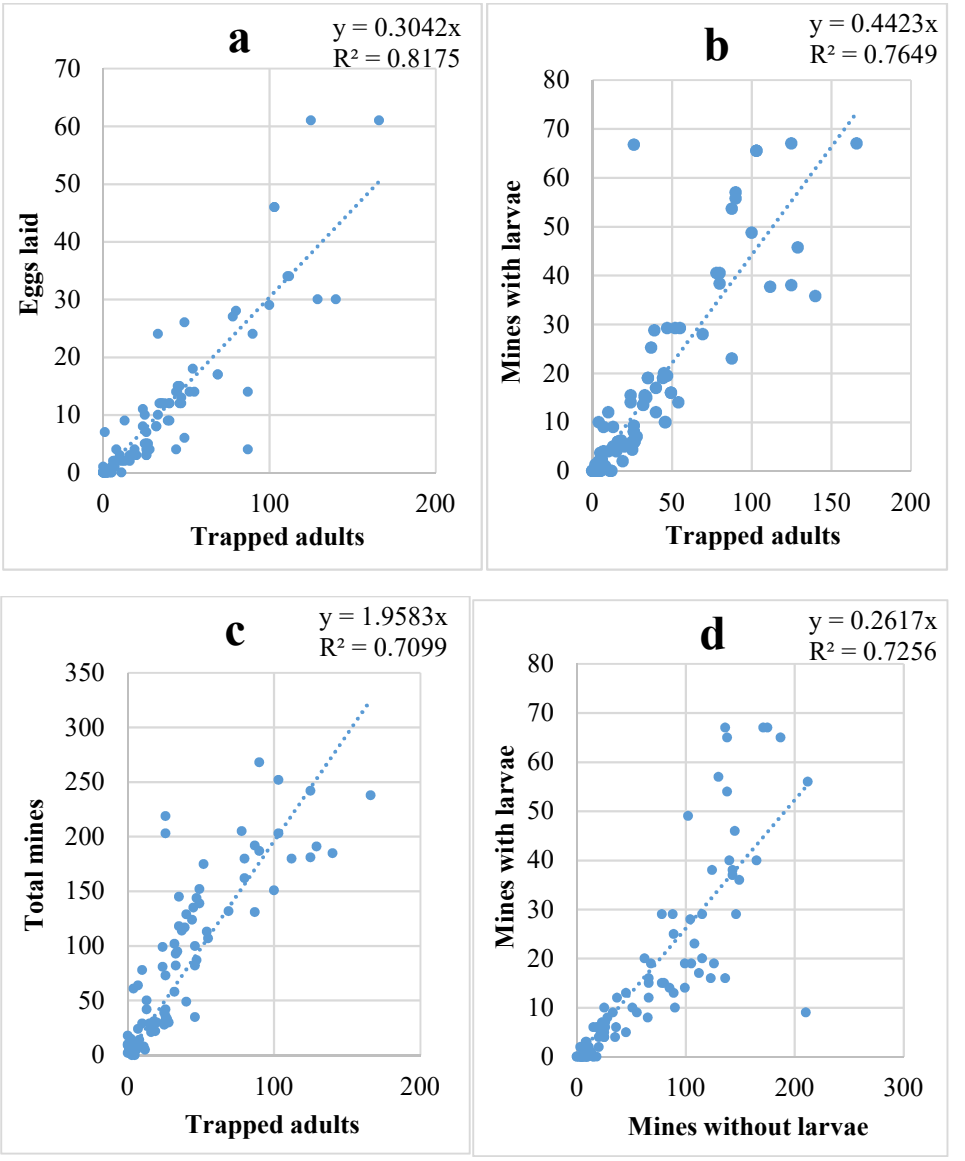

Fig. 3. Linear regression between trapped adults and laid eggs (**a**), trapped adults and mines with larvae (**b**), trapped adults and total mines (**c**), and mines with and without larvae (**d**) in Takelsa greenhouses (from 2014 to 2016).

Adults, eggs and larvae were simultaneously recorded during the tomato growing season from February to May for all study years. Our result confirms generations-overlapping which may be due firstly to the long oviposition period of *T. absoluta* (up to 24 days) and secondly to variable development time of individuals caused by change in microclimatic conditions (Cocco et al. 2015; Lee et al. 2014). In a previous study, Cocco et al. (2015) indicated that

the capture pattern in protected crops was largely unimodal with low catches in winter, increasing population density from the spring, in conjunction with highest temperature. The same authors demonstrated that the development time of *T. absoluta* is influenced by the presence of tomato plants known as its principal host as well as high temperatures values (Cocco et al. 2015).

Moreover, the present results show that *T. absoluta* prefer laying eggs on the underside than on the upper side of tomato leaves confirming Cherif and Lebdi-Grissa (2017); Torres et al. (2001). This finding may help farmers to choose appropriate insecticides that can reach effectively eggs and larvae on leaves.

Our study indicates that damages caused by *T. absoluta* differ between the study years. This finding may be attributed partly to differences in applied insecticides and used cultivars. In fact, Cherif et al. (2013) indicated in a previous study that, under open-field conditions, the tomato cultivars Shams and Chebli were shown to be the least suitable for *T. absoluta* egg-laying compared to the cultivar Firenze. Furthermore, Cherif et al. (2018a) demonstrated in a recent study that insecticides including flubendiamid and cyromazin can reduce significantly the number of *T. absoluta* larvae in tomato greenhouses.

Differences in *T. absoluta* attacks in our study may be linked also to differences in climatic conditions especially temperature as demonstrated by Cherif and Lebdi-Grissa (2014).

Despite the severity of *T. absoluta* attacks differ according to the factors discussed above, management of this pest is based mainly on applying insecticides when threshold was reached.

Relationship between trap catches, number laid of eggs and infestation levels.

In this study, we demonstrated a significant linear regression between trapped males and laid eggs, trapped males and active infestation and trapped males and total infestation which corroborate results obtained of Abbes and Chermiti (2011). However, on the opposite, Mohamedova et al. (2016) indicated that there was no correlation between the number of trapped moths and damaged leaves and fruits.

Our study demonstrated a significant relationship between active and total infestation, while Caffarini et al. (2000) found a non-significant regression between infested leaves and damaged fruits at low pest densities in protected crops. Obtained results of the possible linear regression are varying which may be related mainly to differences in the infestation rates found in the surveyed crops. Our study approved the decrease of damaged leaves at the end of growing season. Abbes and Chermiti (2011) explain these findings by the widespread devastation of tomato plant canopies due to the high number of feeding which may limit the presence of laid eggs as well juvenile larvae on leaves. In this case, females lay eggs on stems and sepal of fruits causing high yield losses (Abbes and Chermiti 2011).

Due to the real threat that *T. absoluta* may cause to the continued production of tomatoes in Tunisia, the knowledge of its seasonal population dynamics and its occurrence is a vital step to apply an appropriate management program. Nonetheless, the use of sexual pheromone lures is important for monitoring; it helps decision makers to apply chemical treatment when thresholds were reached, therefore to avoid repeated insecticides sprays that lead in some cases

to problems of resistance. The combined use of chemical insecticides based on different modes of action with other control strategies (mass trapping, cultural and biological control) might constitute an important improvement of insect pest management.

Further studies should focus on the development of management strategies based mainly on the use of parasitoids or predators mass releases, with or without insecticides sprays. Similarly, researches based on improving efficiency, stability as well as longevity of pheromone lures, are also needed.

In conclusion, the knowledge of *T. absoluta* population dynamics is mandatory to plan effective control methods. Control management strategies recommended against *T. absoluta* would be using sex pheromone traps for monitoring and planning insecticides applications according the thresholds taking into account the safety of tested active ingredients towards natural enemies present in the crop. Doses of selected insecticides used for the control of *T. absoluta* should be optimized in order to reduce the possibility of development of resistant populations. Laboratory trials in this issue should be performed. Moreover, the impact of climatic changes on the ecosystem level which may influence the efficacy of crop protection strategies should be taken into consideration.

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RESUME

Cherif A. et Grissa-Lebdi K. 2018. Gestion de la mineuse de la tomate *Tuta absoluta* en Tunisie: Trois ans d'étude. Tunisian Journal of Plant Protection 13 (1): 101-112.

Tuta absoluta est considéré parmi les ravageurs les plus nuisibles causant des pertes significatives à la production mondiale de la tomate. Ainsi, la connaissance de ses caractéristiques biologiques rend sa gestion moins difficile. Ici, la dynamique des populations de ce ravageur a été suivie sur la culture de tomate cultivée sous serre dans la région de Takelsa (Nord-est Tunisien) pendant trois campagnes agricoles. Les résultats obtenus ont mis montrer que cette mineuse est capable de présenter 4 générations. Un maximum de 120 mâles/piège/semaine et de 74,66 larves/40 feuilles de tomate a été obtenu le 13 mai 2016. Des corrélations linéaires significatives entre les adultes piégés et les œufs pondus ($R^2 = 0,81$; $P < 0,0001$), les adultes piégés et les mines avec larves ($R^2 = 0,76$; $P < 0,0001$) et les adultes piégés et les mines ($R^2 = 0,70$; $P < 0,05$), sont enregistrées. De même, les mines avec et sans larves sont fortement corrélées ($R^2 = 0,72$; $P < 0,0001$).

Mots clés: Dynamique des populations, pièges à phéromones, tomate, *Tuta absoluta*

ملخص

شريف، أسماء وكوثر قريسة لبدي. 2018. مقاومة حافرة طماطم *Tuta absoluta* في تونس : دراسة ثلاث سنوات. Tunisian Journal of PlantProtection 13 (1): 101-112.

تعتبر حشرة حافرة الطماطم *Tuta absoluta* من الآفات الأكثر خطورة حيث تتسبب في خسائر فادحة لإنتاج الطماطم على المستوى العالمي. لذلك التعرف على الخصائص البيولوجية لهذه الحشرة يجعل مقاومتها أقل صعوبة. تمت دراسة ديناميكية مجموعات الحشرة في البيوت المحمية (تاكلسة، الشمال الشرقي لتونس). وفقا لنتائجنا، لهذه الحشرة 4 فترات طيران رئيسية. بينت النتائج أن عدد الذكور في المصائد وصل إلى 120 كرا/مصيدة/الأسبوع و74.66 يرقات/40 ورقة طماطم في 13 ماي 2015. بالإضافة إلى لك، هنا ترابط إيجابي بين الذكور في المصائد والبيض ($P < 0,0001$

،($R^2 = 0,081$ ، بين الذكور في المصائد واليرقات ($P < 0,0001$ ، $R^2 = 0,76$) ، بين الذكور في المصائد والأنفاق ($P < 0,0001$ ، $R^2 = 0,72$) وبين الذكور في المصائد والأنفاق باليرقات أو بدونها ($P < 0,0001$ ، $R^2 = 0,70$).

كلمات مفتاحية: ديناميكية المجموعات، طماطم، مصائد، *Tuta absoluta*

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Effect of Benzothiadiazole and Salicylic Acid Resistance Inducers on *Orobanche foetida* Infestation in *Vicia faba*

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ABSTRACT

Triki, E., Trabelsi, I., Amri, M., Nefzi, F., Kharrat, M., and Abbes, Z. 2018. Effect of benzothiadiazole and salicylic acid resistance inducers on *Orobanche foetida* infestation in *Vicia faba*. Tunisian Journal of Plant Protection 13 (1): 113-125.

The broomrape or orobanche (*Orobanche foetida*) is considered as an important agricultural problem of faba bean (*Vicia faba* var. *minor*) production in Tunisia. The effect of salicylic acid (SA) and benzothiadiazole (BTH) on the induction of faba bean resistance to *O. foetida* was studied. Three application methods (seed soaking, foliar spraying and watering) were used. SA and BTH treatments reduced broomrape infestation under controlled conditions in pot and Petri dish experiments. In pot experiment, SA and BTH treatments reduced broomrape total number. Seed soaking treatments were more effective than foliar spraying and watering. In Petri dish experiment, *O. foetida* seed germination and the number of orobanche tubercles were reduced. The most efficient method was watering for SA and BTH treatments. This reduction was associated to a delay in the tubercle formation. The different application methods of SA and BTH treatment attest that the induced systemic resistance to *O. foetida* can be used in integrated management of broomrapes.

Keywords: Benzothiadiazole, *Orobanche foetida*, resistance inducers, salicylic acid, systemic acquired resistance, *Vicia faba* var. *minor*

Faba bean (*Vicia faba*) is among the most cultivated crop grain legumes in the world. It plays important agronomic and socio-economic roles. However, production, yield and growing areas are variable from year to year (Sillero et al. 2010). In Tunisia, faba bean represents the most important grain legume occupying around 84% of the total grain legume area (DGPA 2016). However, its average yield (about 0.7 t/ha) is below its potential yield in favorable zones of the North, where the average rainfall is higher than 400 mm. The low yield is mainly due to low inputs used, climatic variation, diseases and pests. Broomrapes or orobanches (*Orobanche* spp.) are one of the most important factors reducing faba bean yields in Tunisia (Kharrat and Souissi 2004). These root parasitic plants threaten several crops in many parts of the world. They completely depend on their hosts for their nutritional

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requirements. The most harmful species are *O. crenata*, *O. cumana*, *Phelipanche aegyptiaca*, *P. ramosa* and *O. minor* (Parker 2009). *O. foetida* is considered an important agricultural problem of faba bean production in Tunisia, causing yield losses between 66 to 90% in Beja region (Abbes et al. 2007a). Kharrat and Souissi (2004) indicated that in highly infested areas, farmers generally avoid growing faba bean or other susceptible crops, resulting in substantial reductions of cultivated areas and food legume production. Damages caused in faba bean crops were reported only in Tunisia till now. However, the risk of spread of this parasite to other areas, displaying similar growth conditions, is probable (Abbes et al. 2007a).

Several methods (cultural, chemical, biological, genetic control) have been used for controlling broomrapes but without complete success. In Tunisia, research activities on orobanche were intensified and faba bean resistant cultivars were identified (Abbes et al. 2007a, 2010a, 2011; Kharrat et al. 2010; Trabelsi et al. 2015, 2017). Some chemicals (glyphosate, imazaquin, imazapyr, imazethapyr and sulfosate) were tested and succeeded to induce reduction in *O. foetida* infestation (Kharrat et al. 2002). Late sowing could be an interesting cultural method to reduce significantly the number and the dry weight of emerged *O. foetida* shoots and to significantly increase faba bean seed yield (Abbes et al. 2010b). Bouraoui et al. (2012) indicate that *Rhizobium leguminosarum* reduced *O. foetida* infestation and increased faba bean shoot dry weight. All these methods and strategies resulted to incomplete protection. Thus, alternative or supplementary methods should be considered to prevent or reduce infestation.

Chemically induced resistance enhanced natural defenses of the plant to control pathogens (Hafez et al. 2014; Somaya and El-Sharkawi 2014). Induced resistance of host plants can be used for the control of agronomical important orobanche species (Abbes et al. 2014; Pérez-de-Luque et al. 2004; Sauerborn et al. 2002). It can be activated by exogenous application of salicylic acid (SA) or its synthetic functional analog benzo (1,2,5) thiadiazole - 7 - carbothioic acid S-methyl ester (BTH) (Pérez-de-Luque et al. 2004; Perez et al. 2003).

Systemic Acquired Resistance (SAR) has proven to be effective as a tool for controlling plant pathogens, including fungi, bacteria, viruses, and parasitic weeds. Several studies on different plant species such as clover (Kusumoto et al. 2007), pea (Pérez-de-Luque et al. 2004), tobacco, hemp (Gonsior et al. 2004), sunflower (Buschmann et al. 2005; Muller-Stover et al. 2005; Sauerborn et al. 2002) and oilseed rape (Véronési et al. 2009) indicate that BTH and SA applications also triggers SAR against broomrapes. However, there is a little knowledge about the effects of SAR on faba bean in order to reduce *O. foetida* infestation. Thus, the aim of this study was to evaluate the effect of chemical resistance inducers on *O. foetida* infestation in faba bean.

MATERIALS AND METHODS

Plant material.

The improved Tunisian variety (cv. Bachaar) of faba bean (*Vicia faba* var. *minor*) known for its high productivity in orobanche-free soils and its high susceptibility to *O. foetida* and *O. crenata* was used (Abbes et al. 2007a; 2007b; JORT 2004). Bachaar seeds were provided by the food legume program, Field Crops Research Laboratory of National Agricultural Research Institute

in Tunisia (INRAT). Faba bean seeds were surface-disinfected with 5% calcium hypochlorite for 15 min and then rinsed five times with sterile distilled-water. Disinfected seeds were placed in Petri dishes on a sterile filter paper imbibed with sterile distilled-water and allowed to germinate at 21±2°C in the dark for seven days. *O. foetida* seeds were collected from flowering spikes in infested faba bean fields from Beja (Tunisia) in 2013. Washed seeds were sterilized in 2% calcium hypochlorite for 5 min and rinsed five times with sterile distilled water.

Pot experiment.

Pot experiment was performed under natural conditions at INRAT. Two liters plastic pots were filled up to 2/3 of their height with sterilized soil and then artificially inoculated with 20 mg of *O. foetida* seeds/kg of soil. Plants were watered when necessary.

Chemical inducers salicylic acid (SA, Sigma-Aldrich, purity: ≥ 99.0%) and BTH in the form of acibenzolar acid (Sigma-Aldrich, Pestanal®, analytical standard) were applied to seeds and plants in three different treatments: (i) seed soaking, (ii) foliar spray and (iii) watering. SA and BTH concentrations were chosen because they were frequently reported in the literature as concentrations inducing plant resistance to various pathogens (Abbes et al. 2014; Borges et al. 2003; Daniel and Guest 2006; Elmer 2006; Jackson et al. 2000; Lawton et al. 1996; Pajot et al. 2001; Pérez-de-Luque et al. 2004; Saindrenan et al. 1988; Sauerborn et al. 2002; Véronési et al. 2009).

- Seed soaking: Faba bean seeds were germinated in Petri dishes containing 1 mM water solution of SA or 0.05 g/l of BTH. Control seeds were placed in distilled water for germination.
- Foliar spray: 20-day-old seedlings were sprayed with a hand held sprayer (capacity of 500 ml), with a 1 mM solution of SA or 0.05 g/l of BTH to which Tween 20 was added as wetting agent (3 drops per liter). Each seedling received 10 ml of the solution. Three additional sprays were performed at 35, 50 and 65 days after planting (dap). Control plants received distilled water plus Tween 20.
- Watering: this treatment was used only for SA. Plants (20-day-old) were watered with a 1 mM solution of SA. Each seedling received 100 ml of the solution. Three applications were given at 35, 50 and 65 dap. Control seedlings were watered with distilled water.

Plants were biweekly treated through watering and foliar spraying. The necessity for a repetitive application of low doses of these plant activators was demonstrated by several authors (Pajot et al. 2001; Pérez-de-Luque et al. 2004) in order to increase the efficacy of SAR.

At maturity stage (four months after planting), roots of infected plants were gently removed from the substrate, washed with water, and the orobanche attachments were carefully harvested. Orobanche attachments were sorted according to their developmental stage as reported by Labrousse et al. (2001):

- S1: parasite attachment to the host root,
- S2: small tubercles without root development,
- S3: growing tubercles with crown roots without shoot formation,
- S4: tubercles carrying underground growing shoots,
- S5: emerged parasites.

The first stage (S1) was not observed in the pot experiment. The dry weight of tubercles per plant was recorded. Five replicates per individual treatment were considered.

Petri dish experiment.

The Petri dish experiment was used to evaluate the underground development of root parasitic weeds such as germination and further growth stages since such evaluation is impossible in pot experiments.

Faba bean and orobanche seeds were surface sterilized as described above. Plastic Petri dishes (120 × 120 ×17 mm, Greiner) were filled with autoclaved sand then covered with glass fiber filter paper imbibed with water. Three perforations were made in each Petri dish: the big one was made in the highest board, to allow the shoot out of the dish, and the others were made on the opposite sides to allow root feeding in culture medium (Vincent 1970). Sterilized orobanche seeds (20 mg) were spread between the dish cover and the glass fiber filter paper. Pre-germinated faba bean seeds were placed on the glass fiber filter paper. Petri dishes were closed and vertically stored in a sterile polypropylene tray containing sterile distilled water. The co-culture system was kept in the greenhouse at a temperature above 22±3°C, natural light and in relative humidity above 70%.

As described in pot experiment, the same concentrations and chemical inducers (BTH and SA) were differently applied to seeds and seedlings: seed soaking, foliar spray and watering.

- Seed soaking: Faba bean seeds were germinated in Petri dishes containing the chemical inducers.
- Foliar spray: 20-day-old seedlings were sprayed, with the chemical inducers using a hand held sprayer. Each seedling received 10 ml of the solution. Two additional sprays were performed at 27 and 34 dap.
- Watering: Seedlings were watered with the chemical inducers. Each seedling received 100 ml of the solution. Three applications were given at 27 and 34 dap.

Seed germination was determined by a binocular microscope. Four squares of 1 cm^2 near infested faba bean roots per Petri dish were observed and the number of germinated seeds was counted and expressed as percentage of total seeds. Estimations of percent germination were performed weekly between 21 and 77 dap. In addition, the total number of tubercles was counted weekly between 52 and 101 dap and classified according to their developmental stage (Labrousse et al. 2001).

Statistical analysis.

ANOVA was performed using the SPSS statistical program v.15 (IBM Corporation, Armonk, New York, U.S.A). Tukey's test at P=0.05 was used.

RESULTS

Pot experiment.

SA and BTH foliar spray reduced parasite number by 39 and 32%, respectively (Table 1). Watering with SA reduced *O. foetida* number by 42%. Seed soaking in both SA and BTH were slightly more effective by reducing *O. foetida* number by 45 and 42%, respectively. All treatments reduced significantly the number of underground tubercles but not the number of tubercles reaching the stage S5. Regarding orobanche dry weight, no significant differences were observed between treated and untreated plants (Table 1).

Table 1. Effect of salicylic acid (SA) and benzothiadiazole (BTH) on the total number and dry weight of *Orobanche foetida* in pot experiment

Treatment	Total orobanche number	Orobanche dry weight (g)	Number of underground tubercles (S2+S3+S4)*	Number of emerged orobanche (S5)	Percentage of emerged orobanche (S5)
Control	120.1±14.0b*	6.7±0.5ab	116.5±14.2b	3.5±0.9ab	2.9
Foliar spray SA	73.5±7.0a	8.9±1.2b	68.6±7.3ab	4.8±0.7ab	6.5
Watering SA	69.5±9.0a	7.5±1.1ab	66±9.5a	3.5±1.4ab	5.0
Seeds soaking SA	66.1±11.2a	6.2±1.2ab	62±10.3a	4.1±2.1ab	6.2
Foliar spray BTH	82.2±11.1a	7.4±0.7ab	76±10.8a	6.2±0.8b	7.5
Watering BTH	70±8.1a	4.4±0.8a	68.3±9.8ab	1.6±0.4a	2.2

* S2, S3, S4 and S5 are the stages of orobanche development. Data with the same letter per column are not significantly different (n=5. *P*=0.05, Tukey's test). Rate of S5 is the percentage of emerged orobanche compared to the total number of orobanche.

Petri dish experiment.

SA and BTH treatments strongly reduced the germination percentage of orobanche seeds on faba bean roots (Figs. 1 and 2). The significant reduction was observed with watering method for SA (31% at 42 dap and 38% at 77 dap) and seed soaking method for BTH treatments (51% at 42 dap and 60% at 77 dap).

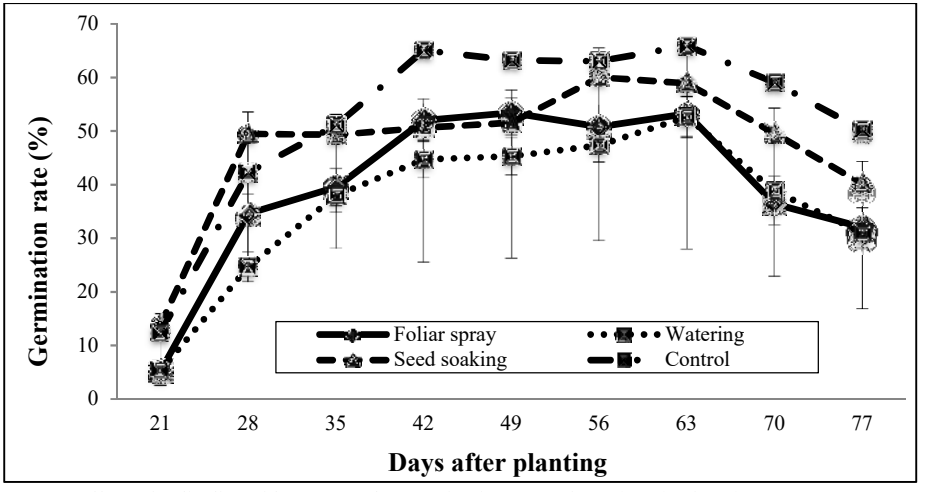

Fig. 1. Effect of salicylic acid (SA) on the germination rate of *Orobanche foetida*. Data are means ± Standard Error (SE).

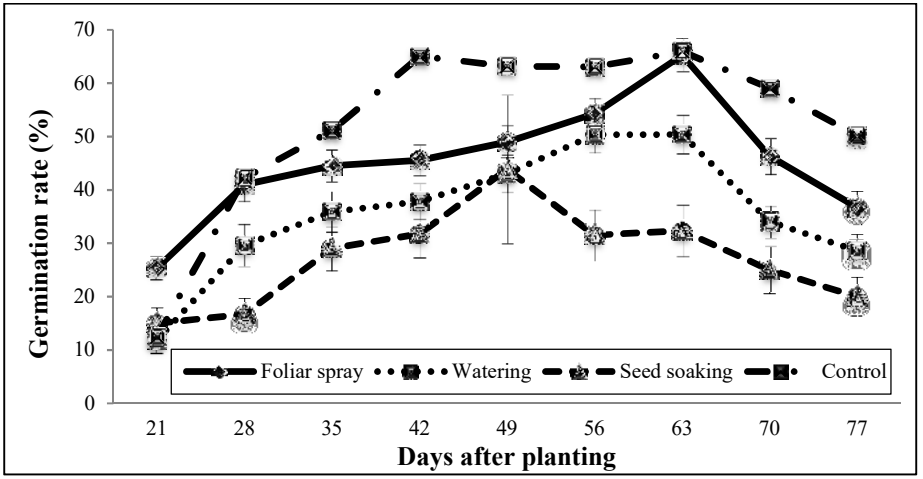

Fig. 2. Effect of benzothiadiazole (BTH) on the germination rate of *Orobanche foetida.* Data are means ± SE.

SA reduced the number of *O. foetida* tubercles on faba bean roots at 80 dap by 52, 55 and 30% with foliar spray, watering and soaking seeds, respectively (Figs. 3 and 4). Watering with SA not only reduced the total number of broomrape attachments but also retarded by one week their formation.

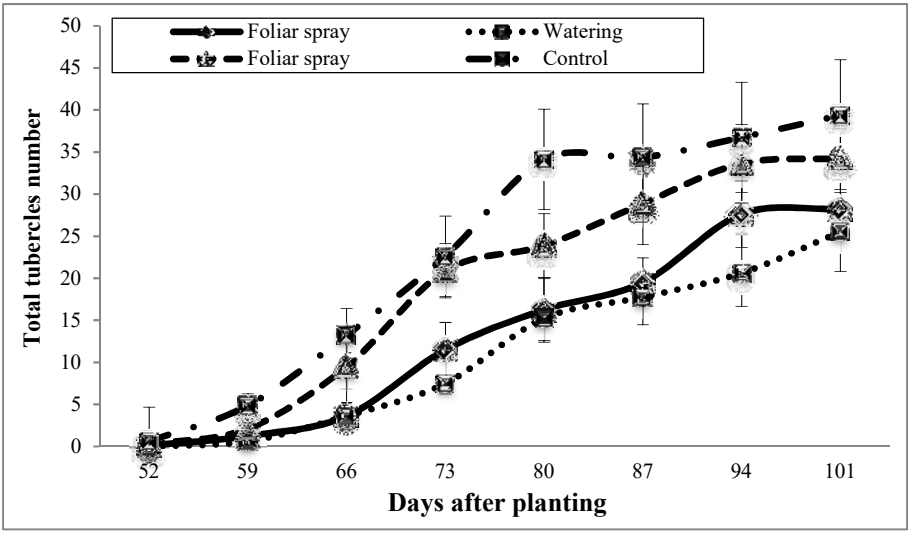

Fig. 3. Effect of salicylic acid (SA) on the total number of *Orobanche foetida* tubercles. Data are means ± SE.

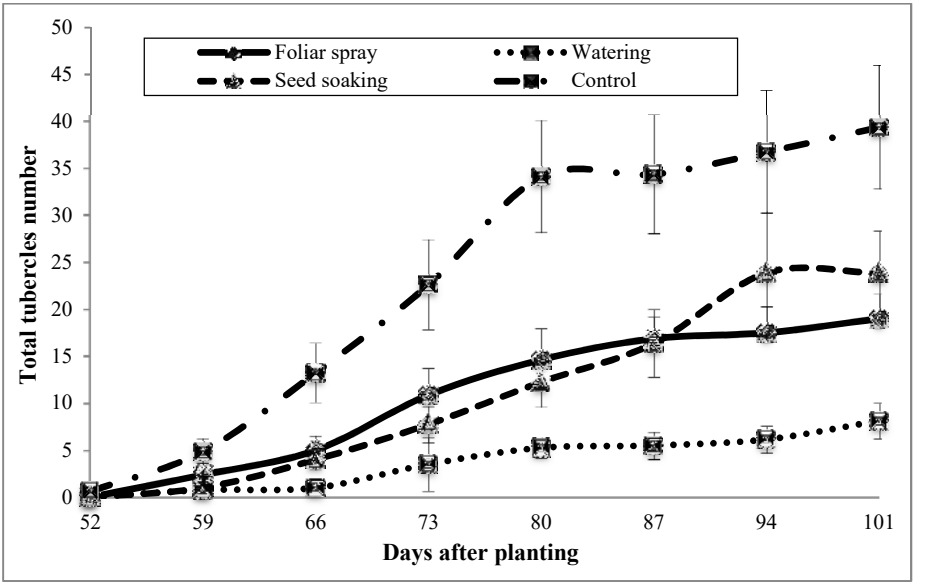

Fig. 4. Effect of benzothiadiazole (BTH) on the total number of *Orobanche foetida* tubercles. Data are means ± SE.

BTH treatments strongly reduced the number of broomrape attachments on faba bean roots. A significant reduction in the number of attachments was observed in BTH-watered plants with less than six attachments per plant. Significant decrease in broomrape infestation was also observed after BTH seed soaking or BTH foliar spray (Figs. 3 and 4). Watering with BTH was the most efficient treatment. It has reduced broomrape infestation by 84% at 80 dap. This reduction was also associated to a delay in the tubercle formation with all BTH treatments (delay of one week). As observed in the pot experiments, no tubercle necrosis was observed following SA and BTH treatments.

DISCUSSION

In pot experiment, the reduction of orobanche number is in agreement with previous studies (Abbes et al. 2014; Buschmann et al. 2005; Fan et al. 2007; Pérez-de-Luque et al. 2004; Véronési et al. 2009). This reduction was characterized by a reduced number of underground tubercles without significant changes in orobanche dry weight or number of tubercles reaching Stage 5. In the present work, the untreated control presented the highest number of orobanche but not necessarily the highest dry weight of orobanche. This can be explained by the high competition level between tubercles for water and nutrients, thus they remain small (Aalders and Pieters 1987; Rubiales et al. 2006; Ter Borg et al. 1994; Trabelsi et al. 2016; Zeid et al. 2013). According to Ter Borg et al. (1994), the major indicator of resistance to broomrape is the tubercle number per plant. Sillero et al. (1996) reported also that indices based only on size and weight of broomrapes can be misleading.

Several authors (Abbes et al. 2014; Bigirimana and Hofte 2002; Lopez and Lucas 2002; Perez et al. 2003) mentioned that, in addition to the reduction of orobanche number, SA and BTH treatments induced observable growth

reduction in hosts plants. Heil et al. (2000) explain the biomass reduction as allocation cost of plants treated with resistance-inducing agents which is a result of a metabolic competition between biomass production and defense. These plants use energy and assimilates for defense reactions and not for biomass production. In order to reduce the negative effects of BTH on plant biomass and to increase the efficacy of orobanche control, the timing of the first treatment and the number of treatments should be studied as suggested by Buschmann et al. (2005).

The germination of broomrape seeds in Petri dish experiment was significantly affected by SA and BTH treatments in comparison with the untreated control. The most effective reduction was observed with the watering method for SA and seed soaking method for BTH. Chemicals seemed to induce low production of stimulant substances or increase the release of inhibitory substances by the host. In order to determine whether BTH or SA effects on parasitism were mediated through host responses or via direct impacts on the parasite, several authors treated orobanche seeds with activator chemicals. These authors did not show a significant reduction of germination of treated orobanche seeds, demonstrating that there are no toxic effects of chemicals on orobanche seeds (Kusumoto et al. 2007; Pérez-de-Luque et al. 2004; Sauerborn et al. 2002; Véronési et al. 2009). Buschmann et al. (2005) showed that BTH applied as a root chamber drench did not interfere with *O. cumana* seed germination. Our study demonstrated that BTH or SA foliar applications (no direct contact with orobanche seeds) reduced significantly orobanche germination percentage. Based on these data, we can strongly suggest that BTH and SA do not act via an herbicidal activity but via induction in faba bean roots of the SAR pathway. Foliar applications demonstrate that the induced resistance to orobanche is systemic.

In addition, the three methods of applications of SA and BTH resulted in a reduction of *O. foetida* infestation, but the most efficient was watering for both SA and BTH treatments. This reduction was associated to a delay in the tubercle formation with these two treatments (delay of one week). No necrosis of developing parasite tubercles was observed. Similar data were reported in tobacco and hemp infected by *O. ramosa* (Gonsior et al. 2004), in pea infested with *O. crenata* (Pérez-de-Luque et al. 2004), in sunflower attacked by *O. cumana* (Buschmann et al. 2005; Fan et al. 2007; Muller-Stover et al. 2005), in oilseed rape infected with *O. ramosa* (Véronési et al. 2009) and in faba bean infected by *O. foetida* (Abbes et al. 2014). The reduction in the number of established parasites by SA and BTH can be related to the reduction in orobanche germination. Kusumoto et al. (2007) show that the reduction of *O. minor* tubercles in red clover caused by SA and BTH was due to the inhibited elongation of *O. minor* radicles and the activation of defense responses in the host root including lignification of the endodermis. Sauerborn et al. (2002) indicated that the total number of *O. cumana* shoots was reduced with BTH treatment, and this was due to synthesis of scopoletin (coumarin phytoalexin) and of hydrogen peroxide in the BTH-treated sunflower roots, but with no increase in lignification. Other studies showed that lignification has been reported as a defense reaction against *Orobanche* spp. penetration, connection to the vascular system and/or tubercle development (Barandiaran et al. 1999;

Goldwasser et al. 1999; Stermer et al. 1987).

Other mechanisms such as an increased synthesis of the scopoletin and ayapin in sunflower (Prats et al. 2002; Sauerborn et al. 2002; Serghini et al. 2001), or the accumulation of reactive oxygen species and hydroxyl coumarin phytoalexins (Buschmann et al. 2002) can be induced by BTH or SA application. Those mechanisms of action of SA and BTH against orobanche infestation were also reported in the control of some plant diseases. *Fusarium oxysorum* f.sp. *ciceris* incidence on chickpea reduction was found to be associated with phytoalexin, pathogenesis-related proteins and chitinase and β-1,3-glucanase induction (Kuc 2006; Sarwar *et al.* 2005). Same results were reported for infections of potato by *Rhizoctonia solani* (Hadi and Balali 2010), faba bean by chocolate spot (Hassan et al. 2006; Mbazia et al. 2016), cucumber and bean by *Botrytis cinerea*, pepper by Fusarium wilt/ root rot causal agent (Abdel-Monaim et al. 2010) and tobacco by TMV (Achuo et al. 2004).

These studies suggested that these chemical defense-inducers could prevent orobanche infestation by activating the SA dependent pathway in host plants. The present study suggests that SAR could be an important method to control broomrapes. This control method should be confirmed under field conditions and could be a useful tool for an integrated control program leading to reduced soil infestation by orobanche. More researches and studies by varying rates, treatment methods and application times are necessary in order to reduce the danger of biomass reduction in faba bean and to increase the efficacy of *O. foetida* control.

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RESUME

Triki E., Trabelsi I., Amri M., Nefzi F., Kharrat M. et Abbes Z. 2018. Effet de l'acide salicylique et du benzothiadiazole comme inducteurs de la résistance, sur l'infestation de *Vicia faba* par *Orobanche foetida.* Tunisian Journal of Plant Protection 13 (1): 113-125.

L'orobanche (*Orobanche foetida*) est considérée comme étant un problème majeur entravant la production de féverole (*Vicia faba* var. *minor*) en Tunisie. L'effet de l'acide salicylique (SA) et du benzothiadiazole (BTH) sur l'induction de la résistance à *O. foetida* chez la féverole a été étudié. Trois méthodes d'application (trempage des graines, pulvérisation foliaire et arrosage) ont été testées. Les traitements ont réduit l'infestation par l'orobanche en pots et en boîtes de Pétri. En pots, SA et BTH ont réduit le nombre total d'orobanches. Le trempage des graines était généralement plus efficace que l'arrosage et la pulvérisation foliaire. En boîtes de Pétri, la germination des graines d'*O. foetida* et le nombre de tubercules d'orobanche ont été réduits. La méthode la plus efficace était l'apport de SA et BTH par arrosage. Cette réduction est associée à un retard de la formation des tubercules. Les différentes méthodes d'application de SA et BTH montrent que la résistance induite à *O. foetida* est systémique. Ces résultats démontrent que la résistance systémique acquise est une méthode à utiliser dans les programmes de lutte intégrée contre l'orobanche.

Mots clés: Acide salicylique, benzothiadiazole, inducteurs de résistance, *Orobanche foetida*, résistance systémique acquise, *Vicia faba* var. *minor*

ملخص

تريكي، آمنة وإيمان طرابلسي ومعز عامري وفاطمة نفزي ومحمد خراط وزهير عباس. 2018. تأثير محفزات المقاومة، حامض الساليسيليك ومستحضر البانزوثياديازول) على الإصابة بالهالوك (*Orobanche foetida*) على الفول المصري (*Vicia faba*). Tunisian Journal of Plant Protection13 (1): 113-125.

يعتبر الطفيل الهالوك (*Orobanche foetida*) مشكلة رئيسية حد من إنتاج الفول المصري (*Vicia faba* var. *minor*) في ونس مت راسة أثير امض الساليسيليك ومستحضر البانزوثيا يازول لتحفيز المقاومة د الهالوك لدى الفول المصري م استعمال ثلاثة طرق طبيق: غمس البذور، رش الأوراق والري. وقد خفض استعمال امض الساليسيليك ومستحضر البانزوثيا يازول من الإصابة بالهالوك في الأصص وفي أطباق بتري. فيما يخص جربة الأصص، خفضت ميع طرق استعمال امض الساليسيليك ومستحضر البانزوثيا يازول العد الإ مالي لدرنات الهالوك، ولكن غمس البذور عموما كان أكثر فاعلية للحد من عد رنات الهالوك. أما مخبريا، فإن إنبات ب الهالوك وعد رنا ه انخفض أيضا. كانت الطريقة الأكثر فعالية هي الري. يرتبط هذا الانخفاض بتأخير في كون الدرنات. ميع الطرق المستعملة مع امض الساليسيليك ومستحضر البانزوثيا يازول بين أن مقاومة الطفيل هي مقاومة هازية مكتسبة. وتظهر هذه النتائج أن المقاومة الجهازية المكتسبة يمكن أن كون عنصر من عناصر المكافحة المتكاملة د الهالوك.

كلما مفتا ية: امض الساليسيليك، محفزات المقاومة، مستحضر البانزوثيا يازول، مقاومة هازية مكتسبة، *Orobanche foetida*، *Vicia faba var. minor*

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Risk Assessment of Tunisian Consumers and Farm Workers Exposed to Residues after Pesticide Application in Chili Peppers and Tomatoes

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ABSTRACT

Toumi, K., Joly, L., Tarchoun, N., Souabni, L., Bouaziz, M., Vleminckx, C., and Schiffers, B. 2018. Risk assessment of Tunisian consumers and farm workers exposed to residues after pesticide application in chili peppers and tomatoes. Tunisian Journal of Plant Protection 13 (1): 127-143.

In Tunisia, to prevent and control pests and diseases during cultivation under greenhouses, chili pepper and tomato require the use of a wide range of pesticides potentially toxic and thus presenting a possible risk for farm operators, workers or consumers. A study has been carried out in the Sahel region of Tunisia to assess the risk for farm operators and workers exposed, by contact during harvest tasks, to possible pesticide residues remaining in tomato and chili pepper crops, and for the Tunisian consumers (adults and children) after intake. A questionnaire was addressed to a group of 73 market gardeners to better understand the local professional practices and to determine the main route of exposure to pesticide. Twenty samples of cotton gloves (2 pairs / sample) were distributed to 20 volunteers who worn them for two consecutive half-days during the harvest of chili peppers or tomatoes before analysis of the dislodgeable pesticide residues which could be transferred from crops to hands. Using models, predictive exposures values were calculated for consumers and farm workers. The highest exposure of consumers was observed for chlorpyrifos residues on tomatoes (with 82% and 312% of the Acute Reference Dose (ARfD), for adults and children respectively). The systemic exposure (SE) of farm workers was estimated for the median, the 90th percentile and the maximum concentration. At the highest observed concentrations, 15 pesticide residues (active ingredients and metabolites) used in pepper greenhouses, and 9 in tomato crops, exceeded the Acceptable Operator Exposure Level (AOEL). Exposure appeared to be particularly critical for chlorothalonil sprayed in chili pepper greenhouses with SE_{MAX} values 113 times higher than the AOEL (11285%). Long task duration (8 h/day) after re-entry in greenhouse, limited access to personal protective equipment (PPE), lack of hygiene and bad habits (eating, drinking, or smoking at work) have also been observed and discussed as risk factors.

Keywords: Consumers, dermal exposure, farm workers, pesticide residues, risk assessment, Tunisia

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In Tunisia, horticulture is an important, dynamic and vast agricultural sector. Today the total area of vegetable crops (field, tunnel and greenhouse) exceeds 160,000 ha. Tunisia produces around 3.2 million tons of vegetable crops (GIL 2015), mainly tomato (39%), watermelon (15%), onion (12%), potato (11.5%) and chili pepper (10%) (APIA 2015; GIL 2015). Tomato and green pepper are a basic component of the Tunisian diet and are used almost on a daily basis as part of raw or home cooked preparations (Jeder et al. 2017).

During cultivation, chili pepper and tomato require the use of a broad range of pesticides to prevent and control pests and diseases. Fruits and vegetables are sprayed several times and up to the final harvest. Pesticides are considered necessary by farmers to provide high crop yields ensuring food security, high agriculture productivity and good quality products. Despite their popularity, pesticides are potentially toxic to humans (farm operators, workers or consumers) and can generate both acute and chronic health effects (WHO 2005), mostly in developing countries (Ortiz et al. 2002). Harvested products are often put onto the markets without consideration of the pre-harvest interval (PHI). As a consequence, the pesticide residues left on fruits can generate a potential health hazard for consumers (Chourasiya et al. 2015; Darko and Akoto 2008; Elgueta et al. 2017; Nougadère et al. 2012).

Many pesticides sprayed on tomatoes and chili peppers leave persistent, fat-soluble pesticide residues which can be dislodged from the two-sided foliar surface of a plant through contact. Workers who enter treated areas for pruning or who handle products during harvesting can easily absorb residues through their skin (EFSA 2014), are potentially exposed daily and thereby possibly endangering their health. Potential exposure of workers through contact with treated foliage is a significant concern in greenhouse production. High humidity, temperature and poor ventilation in greenhouses promote dermal exposure during working (Hanke et al. 2004). Several studies have assessed dermal exposure to pesticides during re-entry of workers in greenhouses for various crops: cucumbers (Caffareli et al. 2004; Jurewicz et al. 2009), strawberries (Caffareli et al. 2004), tomatoes (Caffarelli et al. 2004; Kasiotis et al. 2017; Kittas et al. 2013; Ramos et al. 2010) and peppers (Kasiotis et al. 2017). Health problems have been reported for workers exposed to pesticides during re-entry activities, including reproductive problems (Abell et al. 2000a-b), genetic damage (Lander et al. 2000), neurological disorders (Baldi et al. 2011, increases in bladder cancer (Boulanger et al. 2016) and even breast cancer (Lemarchand et al. 2016).

In this context, a study has been carried out in the Sahel region of Tunisia to estimate the concentrations of pesticide residues on tomatoes and chili peppers collected in greenhouses in order to assess the potential exposure for some consumers groups and farm workers during the harvest tasks through models.

MATERIALS AND METHODS

Fruit sampling for residue analysis.

A random sampling of tomatoes (10 samples) and chili peppers (10 samples) was carried out from 25 to 28 April 2017 in Sousse governorate, according to the guidelines of the European Directive 2002/63/EC (EU Commission 2002) (sampling at the precise time of harvest and sample size of about 1 kg). Samples of 1.00 kg ± 0.04 kg

(with at least 10 units) were collected in 20 greenhouses and weighed. The average unit weight (U, the smallest discrete portion in each lot, Directive 2002/63/EC) was about 106 g for tomatoes and 70 g for chili peppers. All samples were labeled and all useful information was collected for each sample (sample number, origin, sampling date, plant protection product applied, etc.).

Analytical procedures.

The residual pesticide deposits were analyzed by PRIMORIS (formerly FYTOLAB, Technologiepark 2/3, 9052 Zwijnaarde, Belgium) laboratory holding a BELAC (Belgian Accreditation Council) accreditation to ISO/CEI 17025 for pesticide residues on vegetables and herbal products in general. Food and glove samples were analyzed with a multiple-residue Quick Easy Cheap Effective Rugged Safe (QuEChERS) method validated by the laboratory for analysis of residues in foodstuffs, which will detect approximately 500 different pesticide residues (active ingredients and metabolites) in a single analysis thanks to a combination of gas chromatography (GC) and liquid chromatography (LC) according to the active ingredients to be determined (GC-MS/MS for small, thermally stable, volatile, non-polar molecules or LC-MS/MS for larger, thermolabile, non-volatile, and polar molecules). For almost all active ingredients, the quantification limit (LOQ) was ≤ 0.01 mg/kg. The extraction procedure is based on the AOAC Official Method (Lehotay 2007). Briefly, a homogenous 10.0 g sub-sample (crushed fruits or small pieces of gloves) was weighted into a 50 ml polypropylene tube. Then, 10 ml of acidified acetonitrile (1% acetic acid), 4 g of anhydrous magnesium sulfate (MgSO4), and 1 g of sodium acetate (NaOAc) were added. After shaking and sonication in an ultrasonic bath, the polypropylene tube was centrifuged. A portion of the acetonitrile phase (upper layer) was transferred to vials and further analyzed (Toumi et al. 2016a-b; Toumi et al. 2017a-b). The analytical results were corrected when necessary with the previously determined recovery rates (Toumi et al. 2017a).

Consumer risk assessment.

The human health risk was evaluated based on the concentration of pesticide residues in chili peppers and tomatoes at harvest. To evaluate the acute risk for child and adult consumers, we used a Predicted Short Term Intake (PSTI) values calculated with the general following formula: PSTI= (LP × OR × v) / bw, where **LP** is the 97.5th percentile of the portion size taken by people consuming tomatoes or chili peppers, in kg food per day, **OR** is the observed residue level the sample (in mg/kg), **bw** is the mean body weight for the target population subgroup (in kg) and **v** variability factor, the factor applied to the composite residue to approximate the residue level in a high-residue single unit.

For samples with pesticide residues, PSTI values were calculated with the EFSA Primo Model (RASFF, 2016; excel file version 11, 17/04/2017) and the European food consumption database was used since Tunisia has no national data for large portions. The risk level for each active ingredient was established by comparison to the Acute Reference Dose (ARfD): according to FAO (2002) a risk exists when the PSTI > ARfD. When no ARfD value was available, no calculation of the acute risk was performed.

Exposure scenario of farm operators and farm workers.

To have a better understanding of the route of exposure and professional practices, a survey through questionnaires was carried out among 73 farmers which were randomly chosen from professionals located in the Sahel region of Tunisia, more precisely in governorates of Sousse (59 market gardening farmers, i.e., 81%) and Monastir (14 market gardening farmers, i.e., 19%). Farmers were contacted with the help of the heads of Extension Territorial Cells (ETC) and met individually. The size of the group was considered large enough to be representative as all of the participants have the same activities.

The survey was conducted between February and April 2017. It consisted on face-to-face interviews with farm operators and farm workers in rural areas in the Sahel region where horticultural crops (vegetables, fruits) were mainly cultivated. A questionnaire was addressed to two professional categories: operators who are directly exposed to plant protection products (PPP) and workers who are indirectly exposed to PPP during re-entry activities (pruning, tying, leaf pulling, harvesting, etc.). They were asked to answer a detailed questionnaire (fourteen pages) on their socio-demographic data (identity, age, sex, level of education, etc.), their horticultural production, their estimated working hours, the personal protective equipment (PPE) they worn, their hygiene rules, and their perception of health problems linked to their occupation, their management of PPP, their knowledge of pesticide residues and their suggestions and recommendations related to this subject.

Assessment of workers hands exposure using cotton gloves.

Dermal exposure was determined according to a previously published procedure (Toumi et al. 2017a-b). Twenty volunteers working in tomato or chili pepper greenhouses located in Sousse governorate were chosen at random to measure the transfer of pesticide residues from treated fruits to hands and to evaluate their potential dermal exposure (PDE). Two pairs of 100% cotton gloves were distributed to each worker and worn during two consecutive half days during harvesting fruits in 10 tomato and 10 chili pepper greenhouses (from min 2 to max 3 h/day). The two pairs were collected as a single sample (4 gloves/sample), weighed, cut in small pieces, and stored in freezing bags at -18°C until transport and analysis.

Based on the determination of pesticide residues detected on gloves, the potential dermal exposure values were estimated. For each substance, a PDE value was calculated as follows: PDE (mg/kg bw per day) = (C_T (mg/kg) × GW (kg) × 4)/bw (kg), where **C** is the concentration of the substance in the sub-sample (5 g), **GW** is the average weight of the cotton gloves samples (61 ± 3.27 g), **T** is the task duration (2 h during the trial; 8 h/day), and **bw** is the body weight (conventionally, 60 kg). The duration of the task used to evaluate the dermal exposure of workers is 8 h/day (EFSA 2014) and the local survey showed an average harvesting time close to 8 h.

The PDE values were then converted into systemic exposure values (SE) using an appropriate dermal absorption percentage of 75% (default value) (EFSA 2012) as follows: SE (mg/kg bw per day) = PDE (mg/kg bw per day) × 0.75. The risk characterization is obtained as the ratio of the systemic

exposure level to the reference value of each active ingredient, the AOEL (Acceptable Operator Exposure Level; in mg a.s./kg bw per day). It should not be exceeded to avoid any adverse effect to farm operators' and workers' health. To assess the risk, several prediction levels of the SE were considered: the median, 90th percentile, and the maximum (in mg/kg bw per day). Therefore, the SE values were expressed as percentage of the AOEL. It has been assumed that the most appropriate level to cover and assess the risk is the maximum value of the SE (SE_{MAX} or worst case).

RESULTS

Lessons learnt from observation of practices and interviews.

According to the survey, the majority of the 73 interviewed people were plot owners (86%), predominantly adult male aged from 20 to 78 years (mean age: 47 ± 12 years). A vast majority of the respondents (77%) can be considered as workers (people who enter in treated areas or who handle treated crops) as well as operators (people involved in mixing, loading, spraying or emptying/cleaning operations) (categories defined by EFSA, 2014). Sixteen respondents should only be considered as workers and one as an operator applying PPP. The main crops in the Tunisian Sahel region were tomatoes (26%), chili peppers (28%) and potatoes (25%), evenly distributed between greenhouses (45%) and open fields (44%). A small part of the production is carried out under shelter (8%) or in tunnels (3%). Tomatoes and chili peppers are grown in greenhouses and are exposed to various pests (*Tuta absoluta*, whiteflies, soil nematodes, mites, psyllids and thrips) and diseases (*Phytophthora* sp. and *Botrytis* sp.). Almost all preventive and/or curative treatments are systematic, with PPP obtained from the local authorized suppliers.

The majority of respondents (53%) have a rather low level of education but an average working experience of 30 years. Bad habits (smoking) and lack of hygiene rules (36% workers eat and 60% drink while working) observed during the survey contribute to increase the risk of exposure of operators and farm workers to pesticide residues through direct or indirect contact.

Behavioral observations of operators made during the survey showed that 42% of them do not read the labels on PPP packaging and 9% do not understand the instructions of use. More than 20% have no idea about the recommended dosage of the PPPs and use them based on their experience or according to their supplier indications. The majority harvest their products without respect of the PHI, sometimes the day after treatment. Regarding security, 57 operators never wear Plant Protection Equipment (PPE) during mixing and loading or cleaning of the spray equipment. During application, some of them wear a protective coverall (2%), gloves (23%), masks (16%), boots (12%), goggles (11%) or blouse (14%). After application, 47% wash only their hands; 19% wash their hands and arms; 28% their hands, arms, and faces. A relatively high percentage of operators (84%) take a shower when they return to home.

The survey indicates that working time in greenhouses can vary according to the season and the crop. Activities extend over the entire week, with an average daily duration of 8 ± 1 h (n = 73). The observed contact duration of workers with crops is 5 ± 2 h/day (n = 72 workers). Most workers (65%) return to plots or greenhouses immediately or a few hours

after treatment. The majority of them wear long (68%) or short (39%) sleeve shirts and long (68%) or short (14%) leg trousers, but very few wear appropriate protective equipment such as gloves (8.3%), aprons (15%) or special clothing (22%). After working, 44% of workers wash only their hands; 18% wash their hands and arms, 38% their hands, arms, and faces. Nevertheless 74% of workers made full body toilet (shower) at home.

Operators report various health problems such as: eye problems (21); respiratory problems (13); skin problems: irritation (13) and dry (5) and other symptoms: stomach cramps (5); nosebleeds (4); nausea (4); dizziness (3); headaches (2); and sweating (2); repetitive fatigue (2); fevers (1) and dry mouth (1) sneezing (1). Farm workers complain about: eye problems (8); respiratory problems (5); skin problems: irritation (7) and dry (3) and other symptoms: nausea (3); stomach cramps (2); repeated strain (1); nosebleeds (1); headaches (1) and dizziness (1). Despite all reported problems, the majority of respondents have a passive attitude regarding pesticide use and no proposal for improvement was formulated from the survey. Problems are mainly linked to regulation weaknesses, the lack of awareness and monitoring, but also to the inefficacy of some PPP leading them to increase the dosage or application frequency. Workers have proposed that PPE (including gloves) be distributed, or offered with purchased PPP, to encourage them to wear protective equipment and to improve their behavior.

Results of analysis of residual deposits in fruit samples.

Pesticide residues have been detected in almost all tomato and chili pepper samples. Only two samples (one of tomato and one of chili pepper) were free from detectable residues (concentrations below the analytical limit of quantification). Eighteen active ingredients have been detected on 10 chili pepper samples (average: 2.9 a.i./sample), with an average total pesticide load of 0.41 mg a.i./kg. Two fungicides, proquinazide and benomyl (and its metabolite, carbendazim), had the highest detection frequency (30%) (Table 1).

Fifteen different active ingredients have been detected in 10 tomato samples (average: 2.4 a.i/sample), with an average total pesticide load of 0.38 mg a.i./kg. The most frequently detected residue on tomatoes is the fungicide propamocarb (6 samples out of 10) (Table 2).

Consumer risk assessment.

Tables 1 and 2 summarize the detected active ingredients and their concentrations (in mg/kg) in the fruit samples, the concentration expressed as percentage of MRL (Maximum Residue Limit), the PSTI (in mg/kg bw/day) and the PSTI value expressed as a percentage of ARfD for both adults and children. Seven MRL exceedances were reported: six exceedances in chili pepper (Table 1) and one exceedance for chlorpyrifos ethyl (insecticide) in tomatoes (Table 2). The MRL exceedances appeared particularly critical for propargite (chili pepper) and chlorpyrifos-ethyl (tomato) with concentration values respectively 20 (2000% of MRL) and 26 (2685% of MRL) times higher than the MRL values.

Table 1. Results of 10 chili pepper analyzed samples: detected active ingredients; concentrations expressed as a percentage of MRL; PSTI values; PSTI expressed as a percentage of ARfD, for adults and children

Chili pepper sample	Active ingredient	Concen-tration (mg/kg)	Concen-tration (% MRL)	PSTI (mg/kg bw/day)		ARfD (%)	
				Adults	Children	Adults	Children
Sample 1	Bifenazate	0.1800	6.0	0.00025	0.00029	n.a	n.a
	Proquinazid	0.0100	50.0	0.00001	0.00002	0.01	0.01
Sample 2	Acetamiprid	0.0649	21.6	0.00009	0.00011	0.09	0.11
	Carbendazim and benomyl	0.0121	12.1	0.000017	0.00002	0.08	0.10
	Indoxacarb	0.0453	15.1	0.00006	0.00007	0.05	0.06
	Proquinazid	0.0682	341.0	0.00009	0.00011	0.05	0.06
	Thiophanate methyl	0.2983	298.3	0.00004	0.00005	0.21	0.24
Sample 3	Spiromesifen	0.0139	2.8	0.00002	0.00002	0.00	0.00
	Carbendazim and benomyl	0.0455	45.5	0.00006	0.00007	0.32	0.37
	Fluopicolide	0.0349	3.5	0.00005	0.00006	0.03	0.03
	Myclobutanil	0.4776	95.5	0.00066	0.00078	0.21	0.25
	Propamocarb	0.1548	5.2	0.00021	0.00025	0.02	0.03
	Proquinazid	0.0901	450.5	0.00013	0.00015	0.06	0.07
	Thiophanate methyl	0.3235	323.5	0.00004	0.00005	0.22	0.26
Sample 4	Imidacloprid	0.0549	5.5	0.00008	0.00009	0.10	0.11
	Tebuconazole	0.0809	13.5	0.00011	0.00013	0.37	0.44
	Tebufenpyrad	0.1865	37.3	0.00026	0.00030	1.29	1.52
Sample 5	-	-	-	-	-	-	-
Sample 6	Acetamiprid	0.4668	155.6	0.00065	0.00076	0.65	0.76
Sample 7	Carbendazim and benomyl	0.0655	65.5	0.00009	0.00011	0.45	0.53
	Indoxacarb	0.0103	3.4	0.00001	0.00002	0.01	0.01
	Spirotetramat	0.0170	0.9	0.00002	0.00003	0.00	0.00
Sample 8	Tebuconazole	0.3706	61.8	0.00051	0.00060	1.71	2.01
Sample 9	Cyproconazole	0.0218	43.6	0.00003	0.00004	0.15	0.18
	Spinosad	0.0630	3.2	0.00009	0.00010	n.a	n.a
Sample 10	Propagite	0.2000	2000.0	0.00028	0.00033	0.93	1.08
	Bupirimate	0.3411	17.1	0.00047	0.00055	n.a	n.a

n.a.: not available; MRL and ARfD values from EU Pesticides database.

Table 2. Results of 10 tomato analyzed samples: detected active ingredients; concentrations expressed as a percentage of MRL; PSTI values; PSTI expressed as a percentage of ARfD, for adults and children

Tomato sample	Active ingredient	Concen-tration (mg/kg)	Concen-tration (% MRL)	PSTI (mg/kg bw/day)		ARfD (%)	
				Adults	Children	Adults	Children
Sample 1	Chlorantraniliprole	0.0147	2.5	0.00022	0.00085	n.a	n.a
	Propamocarb	0.2629	6.6	0.00400	0.01529	0.40	1.53
Sample 2	Propamocarb	0.0475	1.2	0.00072	0.00276	0.07	0.28
Sample 3	Propamocarb	0.1003	2.5	0.00153	0.00583	0.15	0.58
Sample 4	Indoxacarb	0.0295	5.9	0.00045	0.00172	0.36	1.37
Sample 5	Acetamiprid	0.1058	21.2	0.00161	0.00615	1.61	6.15
	Flubendiamide	0.0260	13.0	0.00040	0.00151	0.40	1.51
	Myclobutanil	0.0141	4.7	0.00021	0.00082	0.07	0.26
	Pirimicarb	0.0232	4.6	0.00035	0.00135	0.35	1.35
	Propamocarb	0.0135	0.3	0.00021	0.00078	0.02	0.08
Sample 6	Propamocarb	0.5206	13.0	0.00793	0.03027	0.79	3.03
Sample 7	-	-	-	-	-	-	-
Sample 8	Azoxystrobin	0.0226	0.8	0.00034	0.00131	n.a	n.a
	Chlorantrraniliprole	0.0311	5.2	0.00047	0.00181	n.a	n.a
	Difenoconazole	0.1231	6.2	0.00187	0.00716	1.17	4.47
	Indoxacarb	0.1020	20.4	0.00155	0.00593	1.24	4.74
Sample 9	Chlorpyrifos-ethyl	0.2685	2685.0	0.00409	0.01561	81.75	312.24
	Carbendazim and benomyl	0.0649	21.6	0.00099	0.00377	4.94	18.87
	Propamocarb	0.0326	0.8	0.00050	0.00190	0.05	0.19
	Pyrimethanil	0.5877	58.8	0.00895	0.03417	n.a	n.a
	Thiophanate methyl	0.9763	97.	0,00149	0.00568	7.43	28.38
Sample 10	Boscalid	0.0242	0.8	0.00037	0.00141	n.a	n.a
	Spinosad	0.0360	5.1	0.00055	0.00209	n.a	n.a

n.a.: not available; MRL and ARfD values from EU Pesticides database.

Results of analyses of residual deposits in glove samples.

All active ingredients detected on vegetables were also measured at rather high concentrations on cotton gloves. For people working in chili pepper greenhouses, 63 a.i. were identified (average: 18 a.i./sample), with an average total concentration of 148 ± 285 mg/kg. Four main active ingredients were identified: thiophanate-methyl (100%), benomyl (and its metabolite carbendazim) (90%), acetamiprid (70%) and propamocarb (70%)). A total of 57 a.i. were detected on all the gloves worn by people working in tomato greenhouses

(average: 18 a.i./sample), with an average total concentration of 111 ± 193 mg/kg. Propamocarb was detected in all samples, followed by diafenthiuron (90%) and thiophanate methyl (80%). DEET (N, N-diethyl-3-methylbenzamide) was also detected on all glove samples as it is used as a biocide in textile sector/industry.

Risk characterization for farm operators and farm workers.

Tables 3 and 4 present the systemic exposure values (SE median, 90th percentile, and maximum values, in mg/kg bw per day) and the systemic exposure expressed as a percentage of the AOEL for all active ingredients detected on the cotton gloves worn by workers in chili pepper (Table 3) and tomato greenhouses (Table 4) and having a SE exceeding their respective AOEL value.

DISCUSSION

Among all vegetable analyzed samples, only two (one tomato sample and one chili pepper sample) have residue levels below the limit of quantification (0.01 mg/kg). Most often multiple residues were detected in the samples (up to seven pesticides). These results are a direct consequence of local poor practices and bad pest management, as reported for many other countries over the world (Arias et al. 2014; Murcia and Stashenko 2008).

Chili peppers appear to be slightly more contaminated than tomatoes (higher number of different residues and more MRL exceedances). Even though the two vegetables belong to the Solanaceae and are produced according to similar practices, the difference may result from the physiological characteristics of each species and the difference in composition of each cuticle. It is known that the lipophilicity of the cuticle can help some pesticides to enter into the plant (Trapp 2004). Stronger and thicker cuticle of chili peppers could better retain the residues, and the bigger surface area could intercept more pesticide drift than tomatoes fruits (Riederer and Schönherr 1984). However, it is difficult to predict the cuticle absorption and the degradation of chemical ingredient as they depend on many factors such as the physicochemical characteristics of the chemical, the contact area, the cuticle composition and its surface (Bonmatin et al. 2015). Four samples of chili peppers (40%) had pesticide residues above the maximum residue limits (MRLs). A total of 6 MRL exceedances were observed for a single collection of 10 samples. Residues of proquinazid, thiophanate-methyl, acetamiprid and propargite exceeded dramatically the MRLs (for 156% up to 2000%). A study conducted in Egypt in 2015 showed that only one of 31 pepper samples had acetamiprid residue levels higher than the MRL value (Alla et al. 2015).

Only one sample of tomato reported a MRL violation for the insecticide chlorpyrifos-ethyl by 2685%. Similar trends in results was also reported by Bojacà et al. (2011) that conducted a monitoring study for tomatoes in Colombia and indicated that almost all the samples of greenhouse tomatoes positive for acephate, cymoxanil, hexaconazole or thiocyclam exceeded the MRLs, on average, by 356, 525, 606 and 1375%, respectively. Chlorpyrifos-ethyl in tomatoes has been detected in different countries around the world including India and Ghana (Essumang et al. 2008; Singh 2012). In contrast, any pesticide residue exceeded the MRL on 19 tomato samples (Alla et al. 2015).

Table 3. Active ingredients detected on gloves worn by workers in chili pepper greenhouses and having a SE exceeding their AOEL values, the corresponding systemic exposure (median, 90th percentile, and maximum values) in mg/kg bw per day, the systemic exposure as a percentage of the AOEL and their toxicological properties (AOEL values, and CLP classification according the EU Pesticides database)

Active ingredient		AOEL (mg/kg bw/day)	SE (Median) (mg/kg bw per day) (AOEL%)	SE (90th P) (mg/kg bw per day) (AOEL%)	SE (Maximum) (mg/kg bw per day) (SE in AOEL%)	CLP classification
Acetamiprid		0.07	0.0006 (1%)	0.0307 (44%)	0.0700 (100%)	H302
Bifenazate		0.0028	0.0044 (159%)	0.0163 (583%)	0.0195 (697%)	H317, H373
Benomyl and carbendazim		0.02	0.0055 (27%)	0.0939 (470%)	0.2745 (1373%)	H315, H317, H335, H340, H360FD
Chlorothalonil		0.009	0.5079 (5644%)	0.9141 (10157%)	1.0157 (11285%)	H317, H318, H330, H335, H351
Cyhalothrin*	**Gamma**	0.0003	0.0005 (181%)	0.0005 (181%)	0.0005 (181%)	-
	Lambda	0.00063	0.0005 (86%)	0.0005 (86%)	0.0005 (86%)	H301, H312, H330
Cypermethrin		0.06	0.0002 (0%)	0.0572 (95%)	0.0949 (158%)	H302, H332, H335
Dimethoate		0.001	0.0046 (456%)	0.0082 (817%)	0.0091 (907%)	H302, H312
Flubendiamide		0.006	0.0342 (569%)	0.0614 (1024%)	0.0683 (1138%)	-
Indoxacarb		0.004	0.0005 (13%)	0.0679 (1699%)	0.0883 (2208%)	H301,H317, H332, H372
Omethoate		0.0003	0.0004 (131 %)	0.0004 (131%)	0.0004 (131%)	H301, H312
Proquinazid		0.02	0.0043 (21%)	0.0241 (120%)	0.0315 (158%)	H351
Spiromesifen		0.015	0.0092 (61%)	0.0996 (664%)	0.1551 (1034%)	-
Tebuconazole		0.03	0.0053(18%)	0.0326 (109%)	0.0421 (140%)	H302, H361d
Tebufenpyrad		0.01	0.0038 (38%)	0.0592 (592%)	0.0961 (961%)	H301, H317, H332,H373
Thiophanate-methyl		0.08	0.0044 (6%)	0.4392 (549%)	1.6470 (2059%)	H317, H332, H341

H301: Toxic if swallowed; H302: Harmful if swallowed; H312: Harmful in contact with skin; H315: Causes skin irritation; H317: May cause an allergic skin reaction; H318: Causes serious eye damage; H330: Fatal if inhaled; H332: Harmful if inhaled; H335: May cause respiratory irritation; H340: May cause genetic defects; H341: Suspected of causing genetic defects; H351: Suspected of causing cancer; H360FD: May damage fertility; May damage the unborn child; H361d: Suspected of damaging the unborn child; H372: Causes damage to organs through prolonged or repeated exposure; H373: May cause damage to organs through prolonged or repeated exposure.* The analytical method is unable to identify cyhalothrin (lambda or gamma), therefore the risk assessment was performed for both cases.

Table 4. Active ingredients detected on gloves worn by workers in tomato greenhouses and having a SE exceeding their AOEL values, the corresponding systemic exposure (median, 90th percentile, and maximum values) in mg/kg bw per day, the systemic exposure as a percentage of the AOEL and their toxicological properties (AOEL values, and CLP classification according the EU Pesticides database)

Active ingredient	AOEL (mg/kg bw/day)	SE (Median) (mg/kg bw per day) (AOEL%)	SE (90th P) (mg/kg bw per day) (AOEL%)	SE (Maximum) (mg/kg bw per day) (SE in AOEL%)	CLP classification

Bifenazate		0.0028	0.0060 (215%)	0.0097 (348%)	0.0107 (381%)	H317, H373
Benomyl and carbendazim		0.02	0.0005 (2%)	0.0621 (311%)	0.1220 (610%)	H315, H317, H335, H340, H360FD
Chlorothalonil		0.009	0.0150 (167%)	0.0431 (479%)	0.0488 (542%)	H317, H318, H330, H335, H351
Chlorpyrifos-ethyl		0.001	0.0001 (8%)	0.0052 (524%)	0.0104 (1036%)	H301
Cyhalothrin*	**Gamma**	0.0003	0.0001 (40%)	0.0009 (291%)	0.0012 (398%)	-
	Lambda	0.00063	0.0001 (19%)	0.0009 (138%)	0.0012 (189%)	H301, H312, H330
Flubendiamide		0.006	0.0390 (651%)	0.0702 (1170%)	0.0780 (1300%)	-
Indoxacarb		0.004	0.0163 (408%)	0.0938 (2344%)	0.1019 (2548%)	H301, H317, H332, H372
Spinosad		0.012	0.0106 (88%)	0.0182 (152%)	0.0201 (168%)	-
Thiophanate-methyl		0.08	0.0005 (1%)	0.5716 (714%)	1.6989 (2124%)	H317, H332, H341

H301: Toxic if swallowed; H312: Harmful in contact with skin; H315: Causes skin irritation; H317: May cause an allergic skin reaction; H318: Causes serious eye damage; H330: Fatal if inhaled; H331:Toxic if inhaled; H332: Harmful if inhaled; H335: May cause respiratory irritation; H340: May cause genetic defects; H341: Suspected of causing genetic defects; H351: Suspected of causing cancer; H372: Causes damage to organs through prolonged or repeated exposure; H373: May cause damage to organs through prolonged or repeated exposure.

* The analytical method is unable to identify cyhalothrin (lambda or gamma), therefore the risk assessment was performed for both cases.

The active ingredients detected on vegetables, with higher concentrations above their respective MRL values (e.g. chlorpyrifos-ethyl, acetamiprid, thiophanate-methyl, propagite or proquinazid), are known for their potential detrimental effects to health; therefore vegetables should be considered as non-compliant for the market. Nevertheless, PSTI calculation consists to estimate the actual risk to consumer group (adults and children) and whether an observed violation of an MRL can lead to a risk to the consumers (Łozowicka 2012). As shown in Table 1, no values were above the ARfD for chili pepper samples. The PSTI values in chili-pepper samples were in the range of 0.00-1.71% and 0.00-2.01% ARfD for adults and children, respectively (Table 1). Only in one tomato sample, the PSTI of the insecticide chlorpyrifos-ethyl exceeds the ARfD with a factor of 3.1 times (312%). This exceedance of the ARfD was observed for children but not confirmed for adults. The PSTI values in tomato samples were in the range of 0.02-82% and 0.08-312%ARfD for adults and children, respectively (Table 2).

These results demonstrate that despite the high level of residues in some samples of vegetables, the Tunisian consumers do not face a serious acute risk, except with insecticides such as chlorpyrifos-ethyl. Nevertheless considering the number of detected residues, their chronic exposure through the consumption of raw vegetables could be associated with a health risk. Moreover, it should be borne in mind that dietary pesticide exposure estimated in this study, considered only exposures through consumption of chili peppers and tomatoes, and did not include other food

products such as fruits, other vegetables, grains, dairy products, fish and meat. Furthermore, the estimated risk assessment is based on toxicological evaluation of single compounds and not based on an evaluation of cumulative exposure to multiple pesticide residues in crops. In addition, these horticultural commodities are also essential ingredients in Tunisian diet, more consumed than in Europe. As a result, the global consumer exposure should be higher than in the present evaluation.

Workers who come into contact with the crop or handle treated products will be contaminated through contact with pesticides that are still available on the crop after application (Dong and Beauvais 2013; Krol et al. 2005). Previous studies (Nigg et al. 1984; Zweig et al. 1985) demonstrated the relationship between the levels of residues on the crops and the dermal exposure of workers during harvesting activities. Similarly, in this study, all pesticide residues measured in chili pepper and tomato samples were also detected in glove samples worn by farm workers during harvesting. Contact with contaminated vegetable samples resulted in the transfer of pesticide residues to gloves worn by the workers allowing their measurement. All glove samples appeared to be highly contaminated by many different pesticide residues (63 active ingredients detected with an average of about 18 a.i. per sample and an average total concentration per glove sample of 148 mg/kg for chili peppers and 57 active ingredients detected with an average of about 18 a.i. per sample and an average total concentration per glove sample of 111 mg/kg for tomato). These concentrations are 1000 times higher than the concentrations which are usually detected on foodstuffs. The systemic exposures of workers were estimated for the median, for P90, and for the maximum concentration of residues in samples (Tables 3 and 4).

For chili pepper samples, six, thirteen and fifteen active ingredients exceed the AOEL respectively at the median, the P90 and maximum values of SE indicating risk situations. However, for tomato samples, four active ingredients exceed the AOEL at the SE median values. At P90 and the maximum (or worst case), nine active ingredients exceed the AOEL indicating potential risk situations.

A recent study was conducted in Greece to assess a worker dermal exposure during re-entry activities in greenhouses reported that the total worker PDE levels ranged from 0.16 to 0.72 mg/kg bw per day and from 0.09 to 0.17 mg/kg bw per day for tomato and chili pepper crops, respectively (Kasiotis et al. 2017).

Exposure could be particularly critical for chlorothalonil, with SE_{MAX} values 113 times higher than the AOEL (11285%) for chili peppers, followed by indoxacarb and thiophanate methyl which are above 20 times higher than the AOEL: 2208 and 2059%, for chili peppers and 2548% and 2124% for tomatoes, respectively. At SE_{MAX}, five active ingredients are above 10 times higher than the AOEL: benomyl and carbendazim (1373%), flubendiamide (1138%) and spiromesifen (1034%) for chili peppers and chlorpyrifos-ethyl (1036%) and flubendiamide (1300%) for tomatoes. Even when wearing personal protection equipment that will minimize exposure by 90%, SE values will always exceed the AOEL at the worst case for these active ingredients. The systemic exposure values are in accordance with the results of a study conducted in Italy to

evaluate the risk of pesticide dermal exposure where the highest absorbed doses for workers re-entering in tomato greenhouse in % of AOEL are 288 and 7959% for azoxystrobin and chlorpyrifos-ethyl, respectively (Cafferli et al. 2004). Several reasons are behind these violations of the health based guidance values including, applying higher dose than the recommended ones, not respecting the pre-harvest interval, etc.

According to the CLP classification (Tables 3 and 4), the majority of the active ingredients detected in chili pepper and tomato samples and having a SE exceeding their AOEL value, have potential hazardous acute and/or chronic effects. The results of the observed levels of dermal exposure after re-entry of greenhouses led to the conclusion that a health hazard may exist, especially after application of high rates of relatively toxic pesticides which easily penetrate the skin.

From the survey of 73 farmers, it is concluded that workers and operators may be exposed during usual pesticide handling and re-entry activities. The task duration for harvesting for farm workers, which is an important factor to consider when building exposure scenarios for a group of workers, is equal to the default value of 8 h proposed in the EFSA Guidance Document 2014 (EFSA 2014). A considerable number of the farmers reported not using protective equipment on a regular basis. The obtained results were agreeing with those reported in Nepal (Shrestha et al. 2010), Palestine (Sa'ed et al. 2010), Lesotho (Mokhele et al. 2011), Iran (Hashemi et al. 2012), Tanzania (Lekei et al. 2014), Uganda (Oesterlund et al. 2014), Indonesia (Yuantari et al. 2015), Ghana (Okoffo et al. 2016), Gambia (Idowu et al. 2017), and Burkina Faso (Son et al. 2017). Bad personal behavioral habits (eating, drinking, or smoking at work) were reported by many farmers (operators and workers). Thus, oral exposure may occur secondarily to dermal exposure, through hand to mouth transfer. Health risks can be due to mishandling and habits exhibited during pesticide application and re-entry activities. According to their answers in the survey, workers seem to be affected by many health problems, while it was not possible to conclude only on the basis of personal feelings and declarations, analytical results and the estimations of exposure confirmed that Tunisian farm operators and workers in the study area are at high risk.

In conclusion, observations completed by analytical results indicate multiple pesticide applications leading to MRL exceedances and probable acute risk for Tunisian consumers. It is a pity that exposure was assessed using a European food consumption database while chili peppers and tomatoes are among the staple foods in Tunisia with a consumption significantly higher than in Europe. Thus, these results stress the need for a national consumption survey and continuous monitoring programs that cover all food commodities consumed locally, especially fruits and vegetables. According to systemic exposure values, workers who spend several hours on a daily basis in greenhouses are at risk during re-entry activities, with potential effects on their health. It appears that lack of awareness, bad habits and absence of personal protective equipment increase their exposure level and their health risks. There is an urgent need for awareness raising amongst professionals' and training on good practices and hygiene rules to avoid their excessive exposure. This survey should be completed later by a bio-monitoring of the operators during

spraying and workers during re-entry activities, with analysis of blood, urine and hair samples. Moreover, considering that the concentration of pesticides in the air is of high concern in greenhouses, the evaluation of the inhalation exposure is highly recommended in the future.

RESUME

Toumi K., Joly L., Tarchoun N., Souabni L., Bouaziz M., Vleminckx C. et Schiffers B. 2018. Evaluation des risques pour les consommateurs et les travailleurs agricoles tunisiens exposés aux résidus après l'application de pesticides sur les piments et les tomates. Tunisian Journal of Plant Protection 13 (1): 127-143.

En Tunisie, pour prévenir et contrôler les ravageurs et les maladies sur les cultures sous serres, le piment et la tomate nécessitent l'emploi d'une large gamme de pesticides potentiellement toxiques et pouvant donc présenter un risque pour les exploitants agricoles, travailleurs ou consommateurs. Une étude a été menée en Tunisie dans la région du Sahel pour évaluer le risque pour les exploitants agricoles et les travailleurs exposés, lors des récoltes, aux éventuels résidus de pesticides restant dans les cultures de tomates et de piments et pour les consommateurs tunisiens (adultes et enfants) après ingestion de ces légumes. Un questionnaire a été adressé à un groupe de 73 maraîchers pour mieux comprendre les pratiques professionnelles locales et déterminer la principale voie d'exposition. Vingt échantillons de gants en coton (2 paires / échantillon) ont été distribués à 20 volontaires qui les ont portés pendant deux demi-journées consécutives lors de la récolte de piments ou de tomates avant l'analyse des résidus de pesticides délogeables qui pourraient être transférés des cultures aux mains. En utilisant des modèles d'exposition prédictive, les valeurs ont été calculées pour les consommateurs et les travailleurs agricoles. L'exposition la plus élevée des consommateurs a été observée pour les résidus de chlorpyrifos-éthyl dans les tomates (avec 82% et 312% de l'ARfD (dose de référence aiguë), respectivement pour les adultes et les enfants). L'exposition systémique (SE) des travailleurs agricoles a été estimée pour la médiane, le 90ème centile et la concentration maximum. Aux concentrations observées les plus élevées, 15 résidus de pesticides (substances actives et métabolites) utilisées dans les serres de piment, et 9 dans les cultures de tomates, dépassent le niveau d'exposition acceptable pour l'opérateur (AOEL). L'exposition semble particulièrement critique pour le chlorothalonil pulvérisé dans des serres de piment avec des valeurs de SE_{MAX} 113 fois plus élevées que l'AOEL (11285%). La durée prolongée du travail (8 h/ jour) après rentrée dans la serre, l'accès limité aux équipements de protection individuels (EPI), le manque d'hygiène et les mauvaises habitudes (manger, boire ou fumer au travail) ont également été observés et discutés en tant que facteurs de risque.

Mots clés: Consommateurs, évaluation des risques, exposition cutanée, résidus de pesticides, travailleurs agricoles, Tunisie

ملخص

توومي، ك.، جولي، ل.، طرشون، ن.، ناجي، ل.، صوابني، ل.، مروى بوعزيز، م.، وكريستاين فيلمانكس، س. وبرونو شيفارس، ب. 2018. تقييم مخاطر على المستهلكين وعمال المزارع التونسيين لبقايا بعد استخدام مبيدات في زراعتيْ الفلفل والطماطم. Tunisian Journal of Plant Protection 13 (1): 127-143.

في تونس، بهدف منع مكافحة الآفات الأمراض عند الزراعة في البيوت المحمية، يتطلب الفلفل الطماطم استخدام مجموعة اسعة من المبيدات التي يحتمل أن تكون سامة، مما يشكل خطرا محتملا على المزارعين العمال أ المستهلكين. قد أجريت دراسة في منطقة الساحل بتونس لتقييم المخاطر التي يتعرض لها المزارعون العمال المعرضون لمخلفات مبيدات الآفات عن طريق اللمس خلال مهام جني الطماطم الفلفل، المستهلكين التونسيين (البالغين الأطفال) بعد الاستهلاك. تم توجيه استبيان إلى مجموعة متكونة من 73 مزارع خضر لفهم أفضل الممارسات المهنية

المحلية□تحديد الطريق الرئيسي للتعرض. تم توزيع عشرين عينة من القفازات القطنية (ز□جين / عينة) على 20 متطوعا كانوا يلبسونها لمدة نصفيْ يومين متتاليين خلال موسم جني الفلفل أ□ الطماطم قبل تحليل بقايا مبيدات الآفات القابلة للتنقل من المحا□يل إلى اليدين باستخدام نماذج التعرض التنبئية، تم حساب قيم المستهلكين□العمال الزراعيين. لوحظ أن أعلى تعرض المستهلكين لمخلفات كلوربيريفو□ إتيل في الطماطم (82%□312% من الجرعة المرجعية الحادة على التوالي للبالغين□الأطفال). تم تقدير التعرض المنهجي (SE) للعمال الزراعيين للمتوسط، 90 مئوي□أقصى التركيز.□في أعلى التركيزات الملحوظة، تستخدم15 بقايا مبيدات الآفات (المادة الفعالة□المستقلبات) في البيوت المحمية للفلفل□ 9 بقايا مبيدات الآفات (المادة الفعالة□المستقلبات) للطماطم تجا□ز□ا مستوى التعرض المقبول للمشغل (AOEL). يبد□ التعرض حاسما بشكل خاص لكلور□ثالونيل المرشوش على الفلفل مع قيمة SE 113 مرة أعلى من مستوى التعرض المقبول للمشغل (11285٪). لوحظت□نوقشت عوامل الخطر مثل ساعات العمل الطويلة (8 ساعات / يوم) بعد العودة إلى البيوت المحمية□محد□دية فرص الحصول على معدات الحماية الشخصية (PPE)□انعدام النظافة□العادات السيئة (الأكل □الشرب أ□ التدخين في العمل) أيضا.

كلمات مفتاحية: بقايا مبيدات الآفات، تعرض الجلد، تقييم المخاطر، عمال المزارع، مستهلكون

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Announcement

of

The 3rd Africa-International Allelopathy Congress AIAC-2018

Blida, Algeria, 20-22 November, 2018

Organized by:
Laboratory of Research on Aromatic and Medicinal Plants,
Nature and Life Sciences Faculty,
University of Saad Dahlab, Blida 1, Algeria

Preamble

Allelopathy is a solution for the development of agriculture, forestry and the environment. It aims to improve the productivity of field crops, vegetables and fruit trees, as well as their conservation. The allelochemicals, natural products and phytochemicals scientists work together to reduce pollution and maintain ecological balance. Those products are currently widely used in production and crop protection, as well as in agroforestry and horticulture. In this concept, allelopathy is for sustainable agriculture.

Invitation

The Laboratory of Research on Aromatic and Medicinal Plants with the collaboration of Nature and Life Sciences Faculty of Blida 1 University, have the great pleasure to invite scientists to participate to the 3rd Africa-International Allelopathy Congress (AIAC-2018) which will be organized for the first time in Algeria, at Blida 1 University, from 20 to 22 November, 2018. This congress is organized every two years and constitutes an important meeting place for researchers, industrial exhibitors and PhD students in all fields of allelopathy in African countries and worldwide. This congress is organized with collaboration of Allelopathy Journal and Tunisian Journal of Plant Protection (TJPP). The communications presented in this lecture will be peer reviewed and those judged appropriate will be published in the Special Issues of Allelopathy Journal and TJPP.

Objectives

This congress aims to bring together active researchers, PhD students and industrialists working in the field of

allelopathy in Africa and internationally, highlighting the current state of research for innovation and the development of the socio-economic sector, to review progress, identify constraints and project future prospects for basic and applied research in Allelopathy.

This congress will be held in English and French, according to the following themes:
Theme 1: Allelopathy in Sustainable and Organic Agriculture,
Theme 2: Allelopathy in Natural Ecosystems & Invasive Plants,
Theme 3: Allelopathy in Soil Sickness,
Theme 4: Chemistry of Allelochemicals,
Theme 5: Physiology, Biochemistry and Molecular Biology of Allelopathy,
Theme 6: Allelopathy Mechanisms and Interactions.

Honorary Presidents of the Congress
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-Pr Atika Benrima (Dean of the Nature and Life Sciences Faculty).

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Recent Doctorate Theses in Plant Protection (2017/18)

Anissa, Lahdhiri. 2017. Weed Flora Diagnosis of crop systems. Doctorate Thesis in Sustainable Agriculture, ISA Chott-Meriem, University of Sousse, Sousse, Tunisia, 189 pp. *(Public Defense: 30 September 2017)*

The agronomic diagnosis of the cropping systems (CS) is a crucial step, allowing their evaluation and innovation. This study is a contribution to reflection to enhance sustainability of CS in the irrigated public area of Chott-Meriem (IPA). The objective is to analyze the weed flora as an assessment indicator of CS in the Agro-ecosystem in this IPA. Firstly, we studied weed densities detected throughout the long-term experiment on cereal CS at the INRA of Dijon-Epoisses farm. Principal component analyzes (PCA) concluded that the weed flora composition is correlated with the cultural practices characterizing each CS. This result confirms our hypothesis that weeds can be a relevant indicator of CS durability.

Involving weed flora in the diagnosis requires a specific protocol, especially concerning the choice of optimal sampling unit size. Among the five studied sizes (0.05 m^2, 0.1 m^2, 0.2 m^2, 0.4 m^2 and 0.8 m^2), we concluded that the quadrat of 0.2 m^2 provides reliable results at a lower cost. Monitoring the evolution of weeds in three forage crops (berseem (*Trifolium alexandrinum*), ryegrass (*Lolium multiflorum*) and oats (*Avena sativa*)) showed that berseem was the least weeded with 57, 37 and 24 weeds/m^2 in the first three surveys due to its post-emergent growth rate and its ability to fix atmospheric nitrogen. Weeds accounted for 43 and 48% of the total vegetation in oats respectively at the second and third surveys. This weed competition had a negative impact on oat biomass, which is shown by Pearson's negative correlation between weed density and oat biomass ($r = -0.664$, $P = 0.003$). The high weeding of the three crops is correlated with their weakening caused by their low establishment and neglect of their cultural preferences.

In a third step, we lead off the agronomic diagnosis of zucchini (*Cucurbita pepo*) crop management in the IPA of Chott-Meriem. Through regular monitoring of farmers, we recorded seven descriptors that are: previous crop, planting, irrigation, fertilization, weed control, crop control and yield. The unsuitable plantation date, the random irrigation and crop control, the low fertilizer inputs (11-68% N, 2-126% of P_2O_5 and >11% K_2O) and the inadequate rotation explain the low yields (<33 t/ha). As for the agroecological diagnosis, it was based on periodic and stratified weed surveys whose analysis shows the dominance of the Therophytes species characteristic of disturbed areas. The Shannon Diversity Index has been low (<2) reflecting undiversified communities. Weed densities were high with maximums of 157, 58 and 77 plants/m^2 respectively on the edge, the row and the inter-row reflecting the failure of current crop management to control these communities. The results of the agronomic and agroecological diagnoses converge to show the crop management fragility causing the CS failure in the IPA. The rational planning of crop management, at the plot scale, is a basic step to innovate these CS and promote their sustainability.

Ultimately, this study, with methods and obtained results, presents an interesting methodological reflection that deserves to be extrapolated at the succession scale and at the level of other IPA for innovation.

Cherif, Asma. 2018. The tomato leafminer *Tuta absoluta* (Meyrick, 1917) (Lepidoptera: Gelechiidae): Biological, ecological parameters and alternative control methods. Doctorate thesis in Agronomic Sciences (Phytiatry), INAT, University of Carthage, Tunis, Tunisia, 182 pp. *(Public Defense: 11 January 2018)*

Tuta absoluta (Lepidoptera: Gelechiidae) is an important pest of tomato crops causing significant yield losses up to 80% in newly invaded area. Obtained results of this study indicated a high genetic conformity of this pest and its susceptibility to develop in tested artificial diets. Our data showed that this pest was able to develop under different levels of temperature (21, 25 and 28°C) and relative humidity (32, 52 and 72%) as well as various host plants (tomato, potato and eggplants). This pest was able to achieve up to 4-5 flight peaks and 3-4 generations of eggs and larvae under greenhouse and field conditions. A positive correlation between some specific parameters (captured males and laid eggs, captured males and mines with larvae, captured males and total mines and mines with larvae and mines without larvae) was emphasized. The combined use of mass trapping and insecticides gave encouraging results given that problems of resistances were not found. Dose of twenty *Trichogramma cocoeciae* (Hymenoptera: Trichogrammatidae) tested in protected and open filed crops was the most effective in reducing the pest population. This study shows the toxicity of some insecticides (indoxacarb, spinosad…), widely used in Tunisia tomato crops, on all *T. cocoeciae* development stages and highlights the safety of others (azadirachtin, *Bacillus thuringiensis* and virus HaNPV). This study allows a better understanding of *T. absoluta* issue in terms of biology, population dynamics and genetic characterization. It proposes efficient control strategies when using effective insecticides with less side effects on parasitoid. Also, this research promotes a better biological control using *Trichogramma* mass releases. However, additional studies, are still required to test new control strategies and propose new ones in order to avoid problems of resistance as reported for example in many countries such as in Brazil.

Cherif, Amira. 2018. Study of the bio-ecology of cereal midges and assessment of some control methods. Doctorate Thesis in Agronomic Sciences (Phytiatry), INAT, University of Carthage, Tunis, Tunisia, 140 pp. *(Public Defense: 28 February 2018)*

Cereal midges are considered among the most destructive pests of wheat, barley and oat in most cereal areas of the world. These pests cause significant economic losses to cereal yields. During the present work, a survey was carried out, in north Tunisia, in order to identify and study the distribution of the different gall midge species in this area. The study

of the bio-ecology of these insect pests, the impact of their damages and the evaluation of some pest control methods against these midges were also the objectives of this work.

Through a molecular characterization, using two sequences of the mitochondrial DNA the cytochrome oxidase c and the 16S rDNA genes, three species of gall midges were recognized as infesting cereals in north Tunisia: wheat gall midge or Hessian fly *Mayetiola destructor*, barley stem gall midge *Mayetiola hordei* and oat gall midge *Mayetiol aavenae*.

These species of midges are widely distributed in all surveyed cereal areas of north Tunisia: Bizerte, Beja, Zaghouan, Siliana and Kef, but with frequencies, infestation percentages and incidence rates higher in semi-arid regions than in sub-humid ones. During the 3 years study, highest infestations were observed in the region of Zaghouan. On the other hand, *M. hordei* has proved to be the most predominant gall midge in north Tunisia.

Populations' dynamic of the 3 gall midge species showed that adults' emergence, population densities and the duration of each developmental stage differed depending on species, years, and regions. These differences are highly dependent on climatic factors, mainly temperature and rainfall. Indeed, heavy rainfalls (more than 70 mm) could reduce gall midges' density. Likewise, low and high temperatures may decrease the emergence density of gall midge adults as observed with *M. destructor*. However, the number of generations and the life cycle of these insects remain basically the same. Indeed, for the three gall midge species *M. destructor*, *M. hordei* and *M. avenae*, 3 generations were detected annually on wheat, barley and oat crops. The first and second generations are complete and the last one is incomplete since no emerged adults were observed. Indeed, once temperatures become high (from 23°C), cereal gall midges enter in diapause and spend the summer as pupae

Assessment of gall midges impact on cereal's yield parameters has shown that for all cereal species, plant height was the most affected parameter followed by tillers number and shoot dry weight. Study of the co-occurrence of *M. hordei* and *M. destructor* on barley crops showed that *M. hordei* is present during all the year on barley, whereas *M. destructor* is only observed from March month. In a final part, the assessment of some control methods to minimize populations of these insect pests was performed.

Trials to identify sources of resistance showed that varieties containing H22, H25 and H26 genes are resistant to the Tunisian biotype of Hessian fly. On the other hand, the barley variety Kounouz was the least attacked by *M. hordei* compared to the other tested varieties. For crop control, our study showed that seasonal seeding of wheat and barley is recommended to minimize midge populations. Indeed, the later is the planting date, the greater are infestation percentages, incidence rates and damages of gall midges on cereal plants. On the other hand, a biennial rotation with a no-till sowing of wheat was a good combination to reduce infestation percentages of durum wheat by midge. The results obtained in the present study could be used to develop an integrated pest management program against cereal midges in Tunisia.

Nefzi, Ahlem. 2018. Valorization of two wild *Solanaceae* (*Solanum linnaeanum* and *Lycium arabicum*) as potential sources of bioactive molecules and biocontrol agents against *Fusarium oxysporum* f. sp. *radicis-lycopersici* infecting tomato. Doctorate Thesis in Biological Sciences, Faculty of Sciences

of Bizerte, University of Carthage, Tunisia, 171 pp. *(Public Defense: 17 April 2018)*

As a part of the research for alternatives to the industrial products, the present work aims the valuation of endophytic fungi and plant extracts from two Solanaceae species (*Solanum linnaeanum* and *Lycium arabicum*) on tomato cv. Rio Grande plants to control *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) and to enhance tomato growth. The approach consists in the collection of fungal isolates from both species and the study of their endophytic capacity in tomato. Fungal isolates (used as conidial suspensions or cell-free culture filtrates), were shown able to colonize tomato tissues, as well as aqueous (15 and 30% w/v) and organic (chloroform, ethyl acetate and butanol) extracts used at 1, 2, 3 and 4% v/v were screened for their growth-promoting effect in tomato and their in vivo and in vitro antifungal potential against FORL. Results showed that, among 115 fungal isolates, recovered from the sterilized surfaces of leaves, stems, flowers and fruits from both species, 15 isolates presented similar morphological traits as the wild types and were considered as endophytes. Tested under greenhouse conditions, the isolates I74, I92, I15 and I18 were found to be the most active in improving root elongation by 83.8-91.8%, plant height by 80.5-94%, roots fresh weight by 91.3%, and aerial part fresh weight by 84-91.6% in treated tomato plants as compared to the untreated controls. These endophytes were the most bioactive in the suppression of disease severity by reducing the leaf and root alteration index by 80-90%, the vascular browning extent by 84-97.2 and FORL colony diameter by 77.2-82.1% compared to FORL-inoculated and hymexazol-treated controls. The molecular identification of the most bioactive isolates allowed their affiliation to four fungal species namely *Penicillium crustosum* (MF188258), *Fusarium proliferatum* (MF188256), *Alternaria alternata* (MF693801), and *F. proliferatum* (MF693802). Leaf aqueous extracts from both species, applied at 30% w/v (LC2 treatment), were found to be the most effective treatments in enhancing seed germination and tomato growth. In addition, they reduced disease severity by 84.6-97.5%, relative to FORL-inoculated and untreated control, and by 36.2-89.6% compared to pathogen-infected and hymexazol-treated control. FORL mycelial growth on PDA medium amended with aqueous and organic extracts of *S. linnaeanum* and *L. arabicum* varied depending on plant organs, the nature of extracts and the tested concentrations. Aqueous, chloroformic, ethyl acetate and butanol extracts were all active against FORL. The most important antifungal activity (expressed as reduction by 41.4-60.8% compared to control) was displayed by leaf butanolic extracts of both species applied at 4% (v/v). High performance liquid chromatography (HPLC) analysis performed for *S. linnaeanum* butanolic leaf extract revealed the presence of thirteen phenolic compounds including seven phenolic acids, four flavonoids and two stilbenes. The present study is the first one that demonstrates the possibility of valuation of these two wild Solanaceae as sources of fungal biocontrol agents and biomolecules active against FORL with bio-fertilizing and disease-suppressive effects on tomato plants.

Abdellatif, Emna. 2018. *Pseudomonas syringae* as pathogen of Citrus: Insights into phenotypic and genotypic characterization. Comparative genomics and biological control. Doctorate Thesis in Phytiatry, INAT, University of Carthage, Tunisia, 200 pp. (*Public Defense: 14 May 2018*)

Pseudomonas syringae pv. *syringae* (Pss) is the most polyphagous plant bacterium belonging to the *P. syringae* species which has become recognized as a phylogenetic complex of strains from terrestrial and aquatic habitats. In the light of this and a recent outbreak of so-called *Citrus* blast and *Citrus* black pit disease in Tunisia where Pss-like bacteria were constantly isolated, the aim of our study was to investigate if Pss caused this *Citrus* disease, and if so, to study the disease and its causal agent in depth. The emergence of *Citrus* blast and black pit in the Tunisian *Citrus* orchards was observed as early as the spring of 2011. A collection of bacteria with colony morphology similar to type strain of Pss was obtained and characterized. Samples were collected mostly from fields located in the North of Tunisia where disease symptoms were expanding and less from the Center where the disease was virtually absent through 2013-2015.

Based on phenotypic tests (LOPAT and GATTa) and genetic studies (16S rRNA, rep-PCR, PCR-MP) the causal agent was confirmed to be Pss. As more recently Multi Locus Sequence Analysis (MLSA) was described as a reliable technique for species delineation and strain identification in *Pseudomonas*, we used it for a better species classification in comparison to strains from various countries and hosts. Overall, fingerprinting techniques and housekeeping genes diversity analyses showed that Pss strains from *Citrus* in Tunisia are homogenous without any geographic distinction and that they are closely related to the LMG5694 Pss strain isolated from *Citrus sinensis* from Greece in 1962. An eventual diversity was noticed based on the host of isolation like almond. Whole-genome sequences of two Pss strains from *Citrus* EC33 (from Tunisia) and LMG5496 (from Greece), is providing valuable information on the taxonomic relationships between the *Citrus* strains and *Pseudomonas* strains from various hosts. Average nucleotide identity showed a strong correlation with MLSA. Furthermore, in planta pathogenicity testing allowed fulfilling Koch's postulates and to determine *Citrus* spp. cultivar susceptibility among the most cultivated ones.

Additionally done, characterization of strains showed that our strains produce the bacterial toxin syringomycin in vitro and they also have the genes *syrB* and *syrD* needed for this toxin production. Studies of the population structure using a principal component analyses based on copper sensitivity in vitro and detection of resistance and effector protein genes revealed a remarkable degree of congruence with the previous genetic and genomic clustering.

The detection of a Pss *Citrus* population resistant to copper during our study led to the search for an alternative sustainable control strategy using epiphytic antagonistic bacteria from the same phyllosphere as the pathogenic ones. Potential antagonistic bacteria belonging to *Bacillus* sp. and *Pseudomonas* spp. (*P. moraviensis*, *P. korensis* and *P. fluorescens*) were identified.

Ben Amira, Maroua. 2018. Study of mycoparasitic relationship between *Trichoderma harzianum* and *Fusarium solani* in Olive trees: Molecular and functional characterization of aquaporins from *Trichoderma harzianum*. Doctorate Thesis in Biological Sciences, Faculty of Sciences of Bizerte, University de Carthage, Tunis, Tunisia; Clermont Auvergne University, Clermont Ferrand, France, 96 pages. *(Public Defense: 24 May 2018)*

Biological disease control through the use of microorganisms has a great potential for future use in integrated pest management. In a multidisciplinary and fundamental context of molecular physio-phytopathology and to provide solutions for the actors in the olive profession and consumers, we have been studying the activity of a fungal biocontrol agent, *Trichoderma harzianum* (strain *Ths97*) against the olive tree pathogen *Fusarium solani* (strain *Fso14*), which causes major problems for olive production in Tunisia and elsewhere. The study consists of two parts. In the first part, we have demonstrated that *Ths97*is a biocontrol agent effective against *F. solani Fso14 pathogen*. Induction of plant defense responses by *Ths97 was shown to be partly responsible for the biocontrol effect*. I*n vitro* tests further showed that *Ths97* develops mycoparasitic activities towards *F. solani Fso14*, by forming infection structures such as hyphae windings and wedges, appressoria and papillae. In the second part of the study, we investigated the Major Intrinsic Proteins (MIP) superfamily in the *Trichoderma* genus. This multigenic family has never been investigated in a hyperparasitic fungal species. Seven MIP members are present in *T. harzianum* and are classified into 3 subgroups: AQP, AQGP and XIP. Their three-dimensional structures and their putative involvement in transport of water and certain polyols have been examined. Finally, their transcription profiles were monitored in *Ths97 in planta* in antagonistic situations and *in vitro* in a parasitic situation with *Fso14* and show that 4 MIP are expressed and regulated differentially during the interaction. Our work has shown that *Ths97* must be considered as a biological control agent and biostimulator of plant defenses, and that MIPs are involved in the trophic relationships between *T. harzianum* and the environment. These data contribute to the further development of *T. harzianum* as an efficient biocontrol agent for sustainable protection of olive trees in Tunisia and around the world.

El Hajji, Lobna. 2018. Pathogenicity, identification of *Meloidogyne* population *fungal* mycoflora infecting tomato and management method. Doctorate Thesis in Agronomic Sciences (Plant Protection and Environment), ISA Chott-Mariem, University of Sousse, Chott-Mariem, Tunisia, 213 pp. *(Public Defense: 27 June 2018)*

The field investigations carried out in different areas in Tunisia, producing tomato infested by root-knot nematodes revealed the occurrence of three species of *Meloidogyne*. *M. incognita* (40%) and *M. javanica* (37%) were the most prevalent species in different study areas. *M. arenaria* (6,63%) was found only on three sites; Monastir (12%), Sousse (33,33%) and Kebili (21,43%). The morphological identification of fungi associated with *Meloidogyne* infestation showed the occurrence of *Fusarium* genus with an average frequency of 14,2%. The specific diversity showed that *F. oxysporum* commonly isolated in soil and root matrices with an average frequency of 11,3% on all tomato plots. However, *F. solani* (6%) was less abundant. The screening of fungal microbiote revealed the prevalence of *Aspergillus*, *Trichoderma* and *Penicillium* genera. The fungal diversity analysis including Shannon-Wienner's and Simpson index showed that mycoflora varied within sampling seasons and prospection localities. The multi-variate analysis (NMDS and CCA) showed the strict correlation between environmental factors in particular temperature, humidity and the fungal community distribution.

The interaction tests between *Meloidogyne javanica* and *Fusarium oxysporum lycopersici* (FORL and FOL), characterized morphologically and by molecular tools, confirmed the synergetic relation between the two pathogens on susceptible and resistant tomato cultivars. The root-knot nematodes infection predisposed resistant host plant to ulterior infection by *Fusarium.* The simultaneous inoculation by tow pathogens or the nematode inoculated before the fungi caused wilting and browning vascular, symptoms not shown with single inoculation by fungi. The root knot nematode incidence on plant host was greater in concomitant inoculation than sequential one. The co-inoculation of susceptible cultivars by two pathogens increased significantly the *Fusarium* wilt severity. The interaction mechanism involved several factors as the resistance/susceptibility of plant host, inoculation time of each pathogen, *Fusarium* race and *Meloidogyne* virulence.

The disease complex of root-knot nematode-*Fusarium* evaluated on three tomato cultivars with different resistance levels (Riogrande, Colibri and Firenze) showed differential response between cultivars and synergetic relation occurred independently of resistance degree. After the co-infection by RKN and FOL, the enzymatic activities of peroxidase (POX), catalase (CAT), polyphenol-oxidase (PPO) and superoxide dismutase (SOD) were greater in resistant cultivar than susceptible one. The quantification of proteins content differed with pathogens and resistance degree of tomato cultivars. The co-infection enhanced significantly proteins, total phenol and total sugar contents in roots. At late stage, this co-infection reduced significantly mineral nutrients concentrations (Cu, Zn, Mn, and Fe) in roots of all tested tomato cultivars (susceptible, moderate resistant and resistant). Although, the individual inoculation by root knot nematode increased the Cu concentration on roots and the individual inoculation by FORL enhanced significantly Zn contents on roots compared with healthy control. After cytological sections of tomato roots co-infected by two pathogens, results showed that plant host defense may be started at early stage, 7 days post-inoculation. The defense could be provided either by hypersensitivity reaction or mechanical barrier following suberin and structural protein synthesis. The co-infection by both pathogens promoted the colonization of cortex cells by fungal hyphae which invaded epidermal cells. The comparative qualitative proteomic approach showed the modification of root proteome at early infection stage reflecting induced response of plant host towards separated or associated infection by pathogens.

The fungal diversity richness was explored for researching new biological control candidates. Ten fungi genera tested for their antagonistic activities either against *M. javanica* and/or *F. oxysporum* f. sp. *lycoperisici*. The results demonstrated that five genera (*Paecilomyces*, *Lecanicillium*, *Penicillium*, *Pochonia* and *Trichoderma*) exhibited a notable nematicide activity. However, the fungicide potential was limited to *Trichoderma* and *Penicillium*. The scanning electronic microscopic observations (SEM) showed that *Trichoderma* and *Lecanicillium* could broke the nematode cell wall and penetrate inside egg of *M. javanica*. Furthermore, the *Trichoderma* isolates coiled along *F. oxysporum* mycelium and dissolved cells wall. These isolates were identified by molecular analysis as *T. harzianum*, *T. viride* and *T. asperellum*. These species were tested for nematicide and fungicide effect in vitro and in vivo under controlled conditions. The biocide effect in vitro of filtrate culture of these three species differed between different concentrations. The highest culture filtrate concentrations (50, 75 et 100 %) of *Trichoderma* species inhibited the mycelium growth of *F. oxysporum* and egg hatching and caused the considerable mortality of *M. javanica* after 72 h of exposition. The treatment of tomato plant by three *Trichoderma* species improved the plant growth and decreased the disease incidence caused by nematode

and/or pathogen fungus. The mycelium proteome sequencing of three *Trichoderma* species revealed that several proteins responsible to their action mode like effect proteins (PR proteins) responsible of stimulation of plant host resistance, stress oxidative enzymes, hydrolytic enzymes and antibiotics. The *Trichoderma* fungus exhibited the potential of combining several modes of action like competition, antibiosis and myco-parasitism proving its use as promoting biological control candidate.

Ihem Guesmi-Mzoughi 2018. Characterization and pathogenicity of nematofauna associated to olive in Tunisian orchards. Doctorate Thesis in Agronomic Sciences (Plant Protection and Environment), ISA Chott-Mariem, University of Sousse, Chott-Mariem, Tunisia, 217 pp. *(Public Defense: 4 July 2018)*

In a new view, studies were conducted to determine the structure and diversity of nematode communities associated to olive trees in Tunisia. Surveys have interested 123 olive orchards, in the mainly known regions for olive culture, located from the North to the Center of Tunisia. In these studies, 17 genus of plant parasitic nematodes were identified which are *Meloidogyne*, *Heterodera*, *Rotylenchulus*, *Pratylenchus*, *Zygotylenchus*, *Longidorus*, *Xiphinema*, *Trichodorus*, *Criconemoides*, *Paratylenchus*, *Helicotylenchus*, *Rotylenchus*, *Amplimerlinius*, *Merlinius*, *Tylenchorhynchus*, *Neodolichorhynchus* and *Tylenchus*. Free nematodes associated to olive were Rhabditidae, Cephalobidae (bacterivores), Aphelenchidae, Aphelenchoididae, *Filenchus* spp. (fungivores), Aporcelaimidae (omnivores) and Mononchidae (predators).

Ecological studies showed that the structure and diversity of nematode communities depends on olive orchard modalities and intensification of soils with the irrigation and the presence of cover crops. The intensification of olive orchards enhanced the multiplication of *Meloidogyne* and *Pratylenchus*. Soil physico-chemical properties have influenced the nematode community composition. The abundance of *Pratylenchus* is positively related to the conductivity, clay and silt contents and exchangeable K. However, the abundance of *Meloidogyne* is positively related to sand content and negatively related to silt content.

Plant parasitic nematode community structure was variable among the regions. It was more diversified in the Center with the intensification of soils. This diversity was studied with the identification of nematode species by morpho-biometric and molecular analysis. In total, 19 species of plant parasitic nematodes were identified which are *Meloidogyne javanica*, *M. incognita*, *M. arenaria*, *Heterodera mediterranea*, *Pratylenchus oleae, Zygotylenchus guevarai*, *Helicotylenchus oleae*, *Rotylenchus goodeyi*, *R. incultus*, *R. eximius*, *Tylenchorhynchus mediterraneus*, *Longidorus africanus*, *L. euonymus*, *L. glycines*, *Xiphinema conurum*, *X. italiae*, *X. meridianum*, *X. pachtaicum* and *X. robbinsi*. *P. oleae* is a new species identified and 8 species are identified for the first time on cultivated olive which are *Rotylenchus goodeyi*, *R. incultus*, *R. eximius*, *Longidorus euonymus*, *L. glycines*, *Xiphinema conurum*, *X. meridianum* and *X. robbinsi*.

Meloidogyne was prevalent in olive orchards visited. Therefore, a pathogenicity experiment was conducted under controlled conditions to evaluate the reaction of 4 olive cultivars, the most cultivated in Tunisia, Chemlali, Chetoui, Koroneiki and Arbequina against *M. incognita*. The inoculation rates tested are 10, 15 and 20 eggs and J2/ml of soil. No disease symptoms on aboveground plant parts were observed with the three inoculation

rates. Although, the presence of root galls, the reproduction factor values were less than 1.0. The cultivars tested, except Koroneiki, seem to be resistant to *M. incognita*.

In order to control the most important and prevalent plant parasitic nematodes, two experiments were conducted in two olive orchards which one is infested with *P. oleae* and the other with *M. javanica*. The biological products tested are *Verticillium leptobactrum* (60 X 10^7 de propagules/tree), Vertimec (0,5 ml diluted 2 liters of water/tree) and Novibiotec 7996 (30 ml diluted in 30 liters of water/tree) compared to a non-treated control. These products were not efficient against *P. oleae*. However, Novibiotec and *V. leptobactrum* have significantly reduced the population of *M. javanica* compared to the control.

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Photo of the cover page ***Heterodera mediterranea*** (Courtesy *Francesca De Luca*)

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