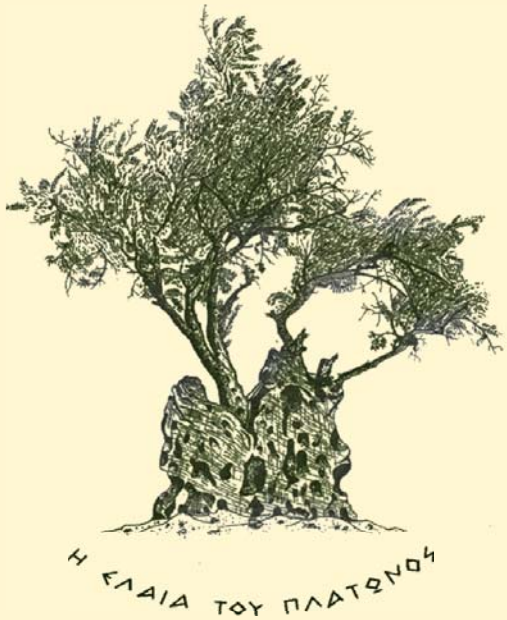
Volume 13, Issue 1, January 2020
ISSN 1791-3691

Hellenic Plant Protection Journal



A semiannual scientific publication of the
BENAKI PHYTOPATHOLOGICAL INSTITUTE

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Hellenic Plant Protection Journal **13:** 1-12, 2020
DOI 10.2478/hppj-2020-0001

Prototype Spatio-temporal Predictive System of pest development of the codling moth, *Cydia pomonella*, in Kazakhstan

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Summary A prototype for pest development stages forecasting is developed in Kazakhstan exploiting data from the geoinformation technologies and using codling moth as a model pest in apples. The basic methodology involved operational thermal map retrieving based on MODIS land surface temperature products and weather stations data, their recalculation into accumulated degree days maps and then into maps of the phases of the codling moth population dynamics. The validation of the predicted dates of the development stages according to the in-situ data gathered in the apple orchards showed a good predictivity of the forecast maps. Predictivity of the prototype can be improved by using daily satellite sensor datasets and their calibration with data received from a network of weather stations installed in the orchards.

Additional keywords: Codling Moth, day degrees, meteorological stations, land surface temperature, plant protection, remote sensing

Introduction

According to the data of the Ministry of Agriculture of the Republic of Kazakhstan (MoA RK), inadequate implementation of plant protection measures leads to an increase in infestation of agricultural lands by pests, diseases and weeds, resulting in gross harvest losses of 2.2 million tons or 191 million U.S. dollars annually in 80% of the acreage areas (The Ministry of Agriculture, 2017). Accurate forecasts allow to conduct the most effective actions during the phases of the greatest vulnerability of pests.

Pest forecasting models linking the dynamics of the pest development stages with agro-climatic factors (e.g. accumulated temperature, precipitation) have been developed over the past century (Zlatanova and Pastukhova, 1975; Riedl *et al*., 1976; Zlatanova, 1978; Welch *et al*., 1978; Boldyrev, 1981; Boldyrev, 1991; Knight, 2007; Jones *et al*., 2013; Drozda and Sagitov, 2017). The usual pest development forecast is based on the data of the closest meteorological stations or according to the interpolated data of meteorological stations which are extremely sparse (the average distance between the nearest meteorological stations in the Republic of Kazakhstan (RK) is more than 100 km). In this regard, farms that are remote from meteorological stations often receive distorted weather information and erroneous forecasts since at a complex terrain the meteorological conditions in the area between weather stations can differ largely - for example, the average daily temperatures can vary up to tens of degrees.

Today, remote sensing data (land surface temperature – LST – from satellite sensors) can supplement meteorological data in the intervals between weather stations and make the forecast more precise in space.

However, due to the fact that previously developed methods of agro-climatic forecasts for pest development are based on

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meteorological data, it is necessary to convert the LST data to match the data from weather stations (2 m above the ground). Conversion of such data is widely discussed in the scientific literature, but a uniform approach has not been developed. The majority of studies discuss regional problems of modelling temperatures of surface layer of the atmosphere from space thermal imagery data, focusing on the features of local landscapes (Fu *et al*., 2011; Benali *et al*., 2012; Williamson *et al*, 2014), while in other works algorithms, usually less successful, for recalculations in continental scales (Vancutsem *et al*., 2010; Shen and Leptoukh, 2011; Meyer *et al*., 2016) are proposed.

Land surface temperature (LST) is used in studies on argoclimatology and Integrated Pest management (IPM). Sepulcre-Canto *et al.* (2007) demonstrated the applicability of LST, derived from Airborne Hyperspectral Scanner (AHS) and ASTER satellite, in olive and peach orchards parameters indicating quality. Sona *et al.* (2012) applied MODIS LST data in order to calculate temperature vegetation dryness index (TVDI) in the Lower Mekong Basin. Raw LST data in this study was used along with Normalized Difference Vegetation Index (NDVI). As already noted, LST is also used in monitoring the risks in agricultural production such as pest's infestation. Lensky, I.M. and U. Dayan (2011) outlined the advantage of MODIS LST data in providing more precise predictions about the timing of *Heliothis* spp. population expansion in comparasion to weather stations data. LST was used in defining the spatial differences of sweet corn growth and in order to demonstrate the influence of topoclimate on the estimating the dates of the emergence of adult pest from its eclosion. Marques da Silva *et al.* (2015) studied temporal and spatial distribution of South American tomato moth, *Tuta absoluta* (Lepidoptera: Gelechiidae) in Portugal comparing LST MSG satellite data and *in-situ* meteorological data. And revealed a linear regression between Accumulated Degree-Days (ADD) calculated from *in-situ* and LST data. Blum *et al.* (2013; 2015) compared MODIS LST data and weather stations data for olive grove canopy in the East Mediterranean and claimed LST data to be more accurate than data gathered from meteorological stations and improve the monitoring of the olive fruit fly *(Bactrocera oleae)* (Diptera: Tephritidae).

Blum *et al.* (2018) compared MODIS LST data and weather stations data in terms of computing thermal thresholds for cotton bollworm (*Helicoverpa armigera*), taking also into account parameters such as migration patterns and pesticide use. Yones *et al*. (2012) used the thermograph and NOAA satellite imagery data in calculating the expected stages of the cotton leafworm *Spodoptera littoralis* (Boisd.), which were further compared with *in-situ* data results and produced correction factors to improve the predictability of their model. Blum *et al*. (2013) built a correction function for LST based on mean differences between LST and *in-situ* temperatures, included in Fourier series. For areas where *in-situ* measurements were not available, these parameters were estimated with the use of NDVI data.

Thus, despite the fact that the use of raw LST data shows good forecasting results, conversion of LST to meteorological data is still relevant to the present discussion, because the majority of models for forecasting pest development stages are based on meteorological data. The purpose of this study was the development of a prototype National pest forecasting system in the Republic of Kazakhstan based on exploitation of both LST and meteorological data. In accordance with the intended aim, codling moth IPM forecasting models were adopted in a spatial modeling system using remote sensing temperature data, which should improve the spatial accuracy of the forecasts. The work was conducted within the framework of the Project «GIS forecasting technology for the development of codling moth and apple scab in the southeast of Kazakhstan» during 2015-2017.

Materials and methods

Our predictive system was designed to define the spatio-temporal development of

vulnerable stages of the codling moth (*Cydia pomonella* L.). This prototype system uses average meteorological data from stations and data from the MODIS/Terra sensor of the satellite product MOD11A2.

Study area: The study area is the territory of Southeastern Kazakhstan including more than 600 apple orchards (Figure 1).

Data collection and temperature maps: Temperature maps were made with the use of weather stations' data and data derived from Modis/Terra satellite (MODIS/Terra Land Surface Temperature (LST), 2017). Temperatures from the archive of seven meteorological stations with known coordinates were used for calibration and approximation of the remote sensing data to the stations' data (Bulygina *et al.*, 2018). The MOD11A2 satellite provides an average 8-day, per-pixel, LST data in a 1200 x 1200 kilometer grid, spatial resolution 1 km (Wan *et al.*, 2015).

Phenological model used for ADD calculation:

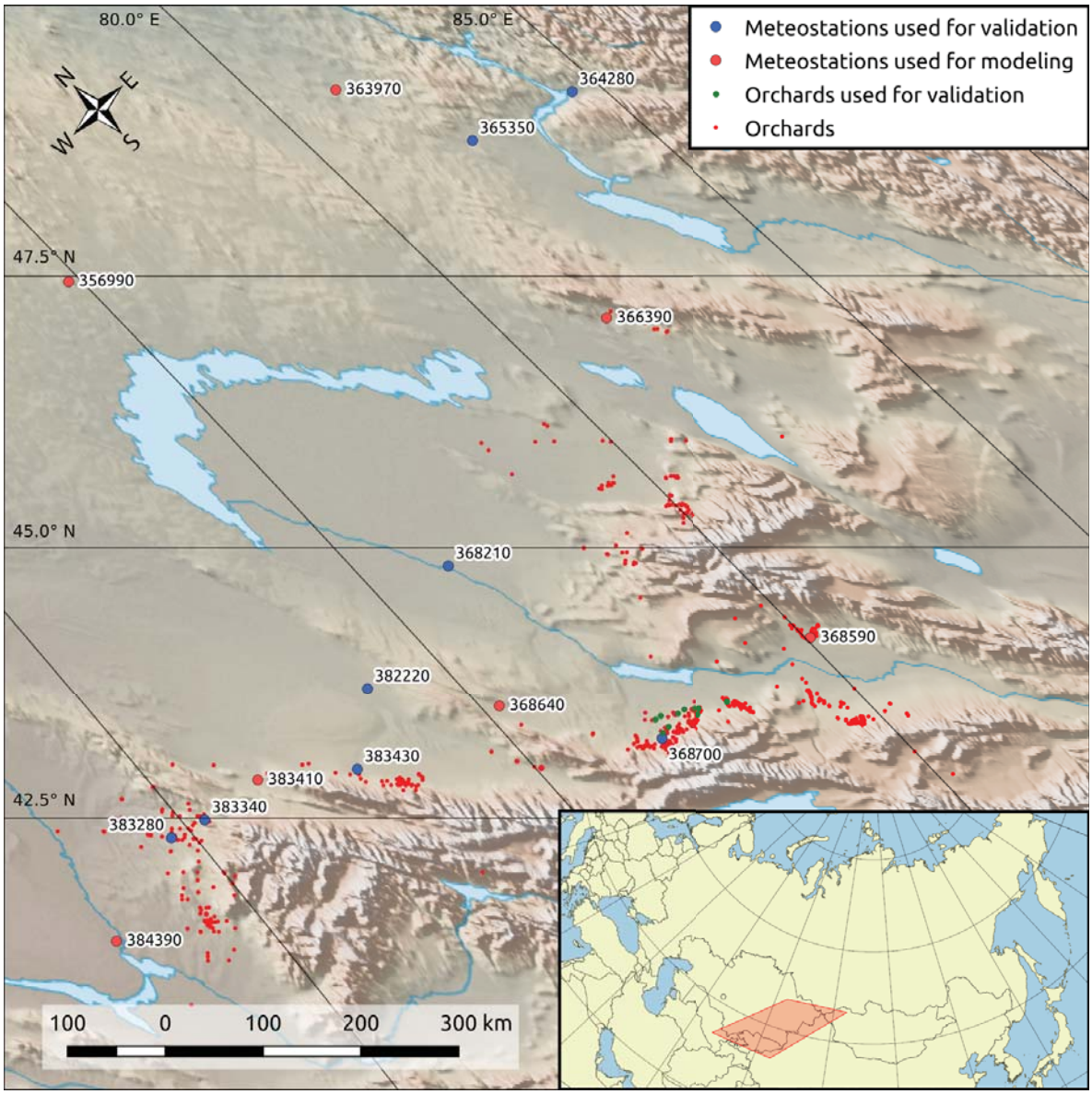

Figure 1. Southeastern territory of Kazakhstan (sinusoidal projection). Numbers on the map show World Meteorological Organization IDs of the stations.

Calculation of ADD and phenological parameters of codling moth were based on the bioclimatic model by Pralya (2013) (Table 1), in which the lower developmental threshold of the pest is 10°C. Designed for the orchards of the European territory of Russia, the model of Pralya (2013) predicts the stages of the South Kazakhstan population of the codling moth quite well. Nevertheless, the ADD values for the beginning of flight can vary widely for different geographic populations of the codling moth (Jones *et al*., 2013). Our observation makes it possible to make an assumption about some displacement of flight start date in the orchards of South Kazakhstan in comparison with the common Pralya model. Therefore, at this stage of the study, 140 ADD (above 10°C) was used as flight start date ADD. In Kazakhstan orchards pheromone traps are not consistently used, so we used dates of transition over 140 ADD as flight start date for the entire territory of the study. These ADD in accordance with the data of the model were used to calculate other dates of the most vulnerable stages of the codling moth and to build maps of phenological dates. The ADDs matrix was calculated using an image calculator module in Idrisi software (Eastman, 2012).

Temperature maps and ADD calculation: Since the average 8-day remote sensing LST data were used, the weather stations' mean daily temperatures were also previously recalculated to the average temperatures for fifteen 8-day periods (from 14 March until 11 July 2017). The average 8-day LST was calculated as the mean temperature measurement for night and daytime images (LST_{day}; LST_{night}). The LST values were taken from the LST maps from the raster cells where seven meteorological stations are located. A generalized regression model for converting LST into weather stations' data was calculated in the Statistica program (Hill and Lewicki, 2007). Then ADD maps were produced with the cumulative total (Figure 2) and ADD maps were recalculated into maps of phenological dates. The modified Pralya model was used to transfer the ADD to phenological dates.

According to the modified Pralya model we have determined the fact that the codling moth's flight start date occurs on the date of transition through the threshold of 140 ADD. The final map of phenological dates was created with the use of raster recalculation. Fifteen 8-day period ADD maps were used as input data. On each of fifteen 8-day period ADD maps we identified pixels, where transition through the threshold of 140 ADD occured for the first time during the relevant 8-day periods. These pixels are highlighted in green in the lower four maps of Figure 3. During the reclassification, the values of Julian dates of the relevant 8-day period, in which the transition through 140 ADD occurs, were assigned to these pixels. Such operation of reclassification was applied to all 15 layers, then these layers were summed up. Thus, after summing the reclassification results, a single raster was obtained, where each pixel contains a date of transition over 140 ADD. This map is presented in Figure 3 (upper map).

Model validation: A two-stage verification of

Table 1. The dependence of the codling moth number dynamic phases from Accumulated Degree Days (ADD) above 10° C according to Pralya (2013).

Stage	Codling moth first flight, %	Larva hatching, %	ADD	
			1st generation	2nd generation
Emergence	5	0	75-120	600-620
Rise in number	25	1-2	170-190	680-700
Peak period	50	25-30	260-270	960-980
Decline in number	75	45-50	370-380	1140-1160
End of development	95	70-75	480-520	1300-1350

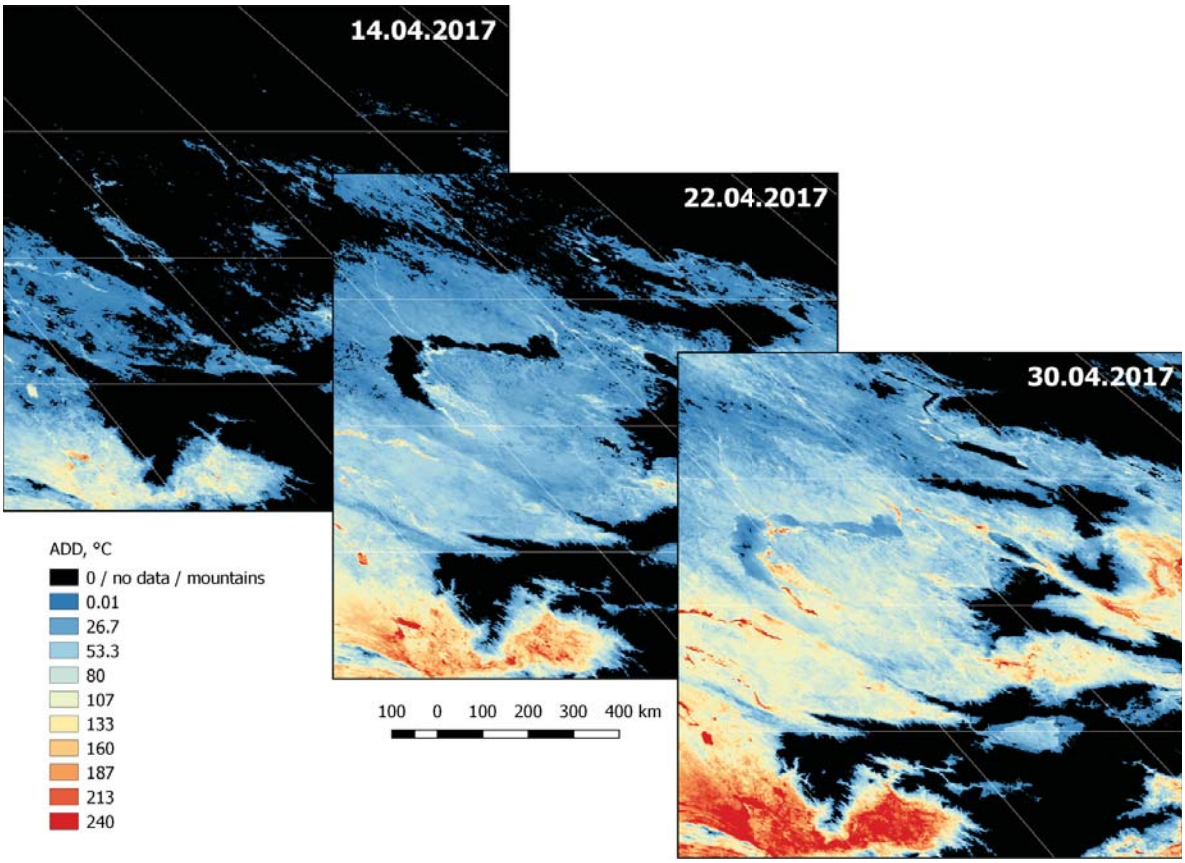

Figure 2. Accumulated degree days above 10°C for specific days in 2017 in the region of Southeastern Kazakhstan.

the accuracy of the created maps was carried out. The accuracy of the temperature recalculation of the LST values into meteorological temperature values was checked using data from eight weather stations, other than the seven weather stations where the original data came from. From LST maps and maps of conversion of LST data to meteorological stations data (LST_{meteo}), temperature values were extracted from the raster cells corresponding to the locations of weather stations. Temperature values for similar 8-day periods, calculated from meteorological data (T_{meteo}) were taken as the reference values. To validate the model, phenological data which were obtained from apple orchards with pheromone traps were also used.

Results and Discussion

The relation between temperature and the dates of the development stages of the apple trees as well as the codling moth was calculated from temperature data of the weather stations and LST received from satellite sensors.

The difference in average temperatures, and therefore the ADD, calculated from meteorological and remote sensing data is quite significant. However, the correlation between the temperature values is very high (r=0.98-0.99), which makes it possible to convert the LST to the values received from weather stations with a high degree of accuracy (Figure 4). In Table 2 for each 8-day period, temperature values averaged across the data of the seven weather stations are presented in 3 variants: 1) average values for meteorological data (T_{meteo}); 2) average values for LST data (raw LST data); 3) average values for LST converted by using a regression model (LST_{meteo}).

The following generalized regression formula was derived: $LST_{meteo} = 4.6 + 0.056$

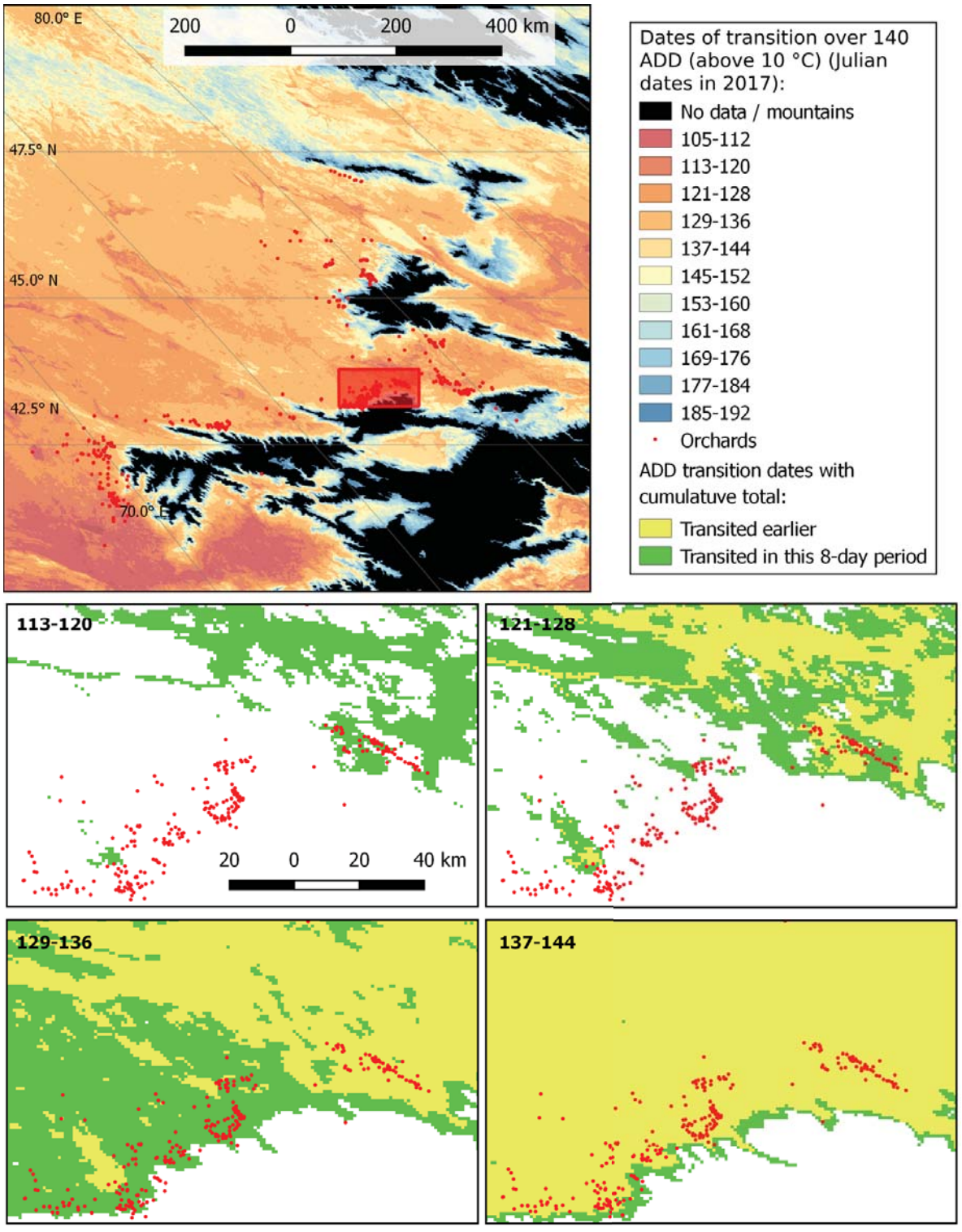

Figure 3. Dates of transition over 140 ADD (above 10°C) (Julian dates in 2017) in Southeastern Kazakhstan and the variant of presentation of codling moth first fligh 8-day prediction. Red dots show the apple orchards location.

* LST_{day} + 0.982 * LST_{night}. Figure 4a shows a comparison between the mean 8-day temperatures on the meteorological data (T_{meteo}), raw LST data and values calculated by the regression formula (LST_{meteo}). The average 8-day temperatures were recalculated into ADD with a threshold value of 10°C (Figure 4b).

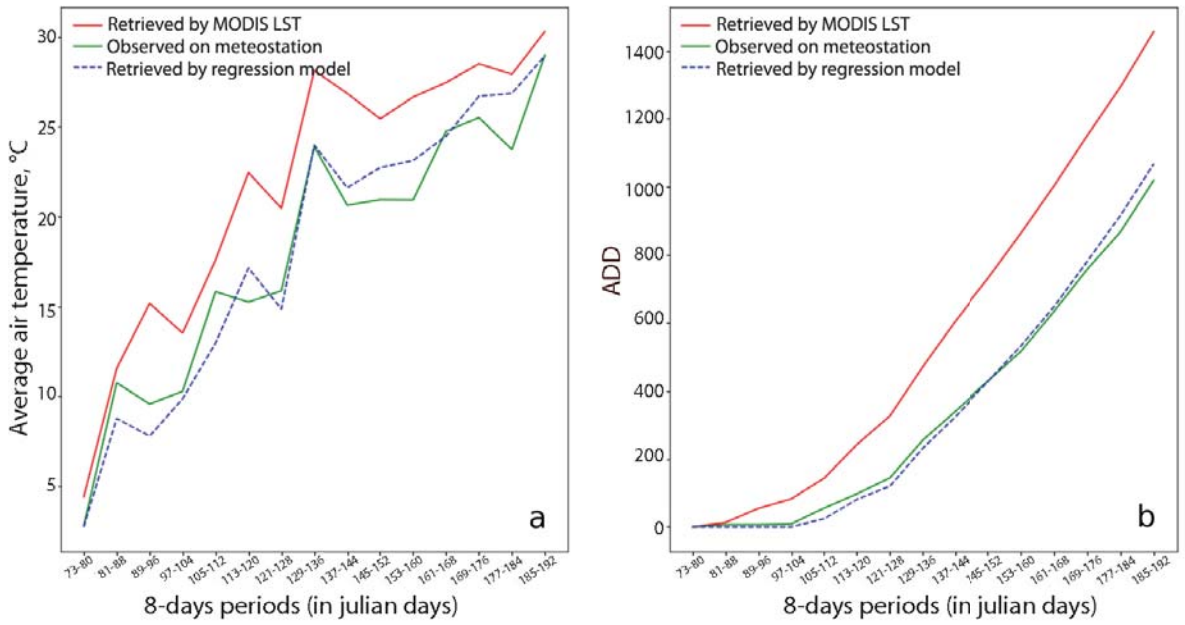

Figure 4. The dynamics of the average temperatures (a) and the ADD (b) calculated from the meteorological data (T_{meteo}), raw LST data and by using regressiom formula (LST_{meteo}) for meteorological station Zharkent (368590).

Table 2. Temperature data, based on meteorological, LST and regression data averaged across the data of seven model weather stations.

Date (Julian)	Average temperatures calculated according to weather stations data (T_{meteo}), (°C)	Average temperatures calculated according to MODIS LST data (raw LST data), (°C)	Average temperatures calculated by using regression models (LST_{meteo}), (°C)
2017_073-80	-2.25	-0.85	-1.98
2017_081-88	5.57	6.49	5.49
2017_089-96	4.37	6.94	4.84
2017_097-104	9.83	11.82	8.79
2017_105-112	14.45	16.16	13.55
2017_113-120	14.30	19.77	16.00
2017_121-128	14.96	18.39	14.59
2017_129-136	21.80	24.73	21.84
2017_137-144	18.70	22.56	19.05
2017_145-152	20.43	23.94	19.93
2017_153-160	20.74	25.27	20.80
2017_161-168	24.12	27.58	23.99
2017_169-176	26.37	29.15	26.12
2017_177-184	23.94	27.71	24.81
2017_185-192	27.88	30.91	27.48
RMSE		3.24*	0.66**

* Root Mean Square Error (RMSE) of average temperatures calculated according to weather stations data and average temperatures calculated according to MODIS LST data comparison.
** Root Mean Square Error (RMSE) of average temperatures calculated according to weather stations data and average temperatures calculated by using regression models comparison.

For each weather station, the differences between T_{meteo} and raw LST data; T_{meteo} and LST_{meteo} were calculated for the fifteen 8-day periods. For each variant out of 15 differences, RSME (Root Mean Square Error) was calculated. For example, for Kokpekty weather station RMSE by raw LST was 4.69, and by LST_{meteo} was 1.20. The average values of RMSE were: 3.46 for the T_{meteo} approximation variant by raw LST data, 1.62 by LST_{meteo} for model stations, and 1.65 for validating stations (Table 3).

Thus, the use of the regression model in the weather temperature maps made it possible to improve the accuracy of the meteorological temperature approximation significantly in comparasion to raw LST data. The mean values of Root Mean Square Errors (calculated by using regression model to validate stations) are quite equal, allowing the assumption that the recalculation error of conversion LST to T_{meteo} is approximately 1.7°C, while the error of using raw LST data is 3.5°C.

Conversion of temperature maps based on remote sensing data into maps of weather stations temperature values allowed cartographic forecasts with previously developed methods using weather station data.

The map of the Julian dates of transition through the threshold of 140 ADD, corresponding to the expected dates at the beginning of the spring flight of the codling moth in July 2017 is presented in Figure 3. Phenological maps, in contrast to pheromone traps, allow us to forecast the dates of essential pesticide treatment not only for separate orchards, but for each site (pixel) of the region. This is especially relevant for Kazakhstan due to the fact that monitoring traps are installed only in few orchards.

In the second stage of validation, actual dates of the first spring flight of codling moth, obtained from pheromone traps in 9 orchards in the Almaty region were compared with dates of transition over 140 ADD

Table 3. Accuracy of meteorological temperatures approximation by model and validating stations data.

Name of the station (WMO ID)	Accuracy of meteorological temperatures approximation (RMSE)		
	By LST_{meteo}*	By LST_{meteo} **	By raw LST data ***
Bektauata (356990)	1.10	–	2.24
Zhangiztobe (363970)	1.63	–	5.50
Bolshe Narymskoe (364280)	–	1.51	2.84
Kokpekty (365350)	–	1.20	4.69
Urdzhar (366390)	1.10	–	3.63
Bakanas (368210)	–	1.96	2.94
Zharkent (368590)	1.64	–	4.22
Otar (368640)	1.33	–	4.11
Almaty (368700)	–	2.52	3.89
Tole bi (382220)	–	0.89	3.18
Shymkent (383280)	–	1.99	2.19
Aul Turara Ryskulov (383340)	–	1.43	3.10
Taraz (383410)	1.78	–	3.69
Koolan (383430)	–	1.68	3.42
Cardara (384390)	2.76	–	2.32
Mean Values	1.62	1.65	3.46

* Root Mean Square Error of average temperatures calculated according to weather stations data and average temperatures calculated by using regression model comparison (for model stations).
** Root Mean Square Error of average temperatures calculated according to weather stations data and average temperatures calculated by using regression model comparison (for validating stations).
*** Root Mean Square Error of average temperatures calculated according to weather stations data and average temperatures calculated according to MODIS LST data comparison.

(above 10°C) at the locations of the orchards on the produced maps. Since our phenological maps were 8-day, daily details were achieved by interpolation of the values of the forecasted transition dates into the 8-day interval. The differences between the actual and calculated dates were for different orchards from 0 to 3 days, while the average difference for 9 orchards was 1.7 days (Figure 5).

The validation of the calculated dates of transition over 140 ADD (above 10°C) was also carried out by comparing the dates calculated for 15 studied stations by T_{meteo}, the dates calculated by raw LST data and the dates calculated by LST_{meteo} (Table 4). Table 4 is devoted to the results of the dates of transition over 140 ADD validation for meteorological stations. It was calculated similarly to validation of actual dates of the first spring flight of codling moth, obtained from pheromone traps in 9 orchards in the Almaty region. The difference is that in this case data of meteorological stations was used to calculate the 140 ADD transition dates and was taken as the reference data. The differences between meteorological and LST transition dates were in average 3.98 days for model stations (seven stations used for building the regression formula) and 4.11 days – for

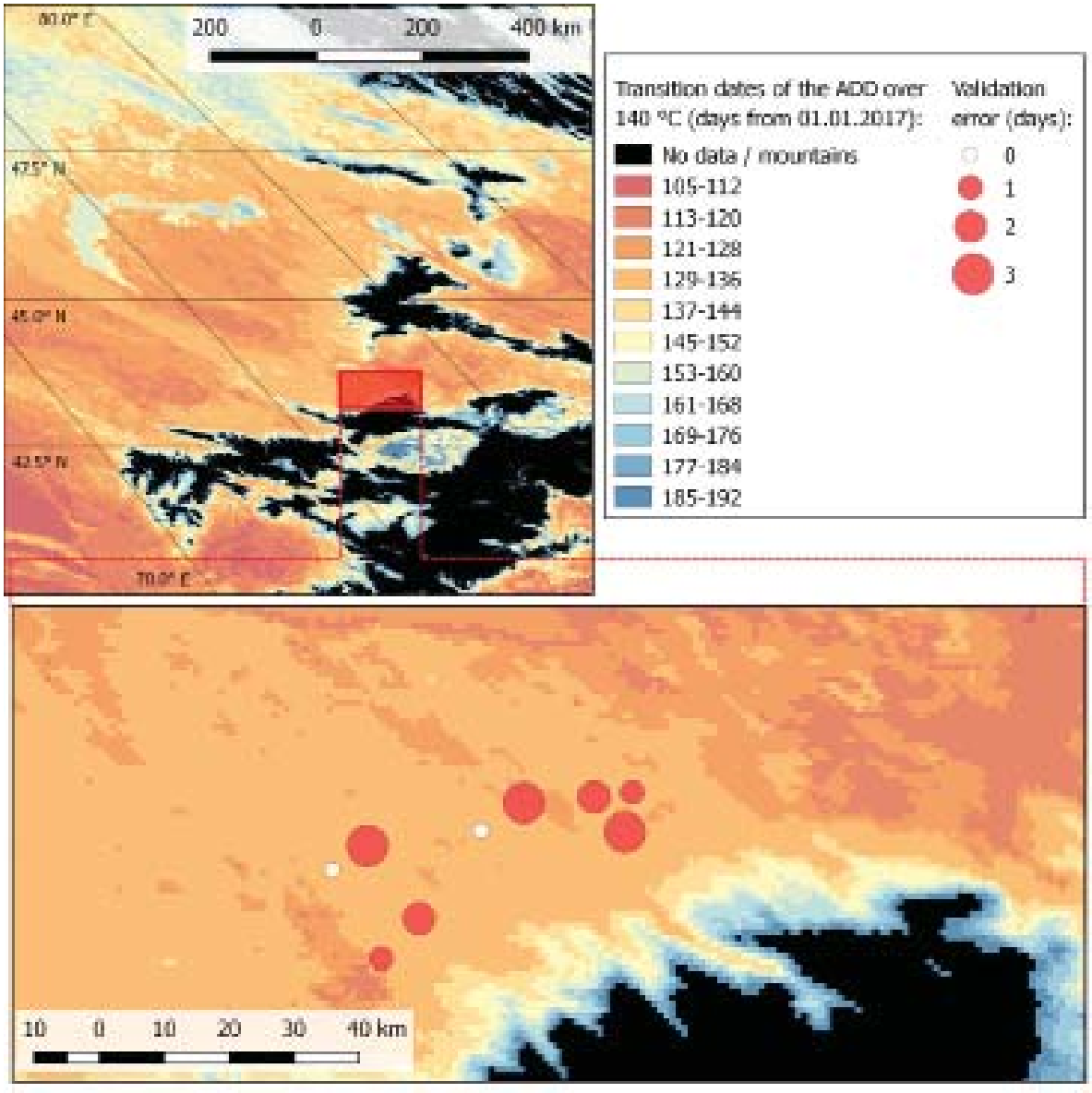

Figure 5. Validation of the expected dates of codling moth first flight by actual data.

Table 4. Validation of the expected dates of codling moth flight beginning by meteorological data.

Name of the station (WMO ID)	Accuracy of approximation of dates of transition over 140 ADD (above 10°C) calculated according to weather stations data (error in days)		
	By raw LST data	By LST_{meteo} data (for model stations)	By LST_{meteo} data (for validating stations)
Bektauata (356990)	3	2	–
Zhangiztobe (363970)	18	4	–
Bolshe Narymskoe (364280)	16	–	-2
Kokpekty (365350)	17	–	-2
Urdzhar (366390)	12	-3	–
Bakanas (368210)	10	–	-3
Zharkent (368590)	16	-2	–
Otar (368640)	13	2	–
Almaty (368700)	18	–	10
Tole bi (382220)	14	–	2
Shymkent (383280)	10	–	-3
Aul Turara Ryskulov (383340)	8	–	1
Taraz (383410)	16	7	–
Koolan (383430)	17	–	2
Cardara (384390)	9	5	–
RMSE	13.80	3.98	4.11

RMSE: Root Mean Square Error.

validating stations (eight stations used for validating the model).

It should be noted that the accumulation of degree days in the orchards lags behind the accumulation on the arid non-irrigated territory, which occupies a significant part of the area of 1 pixel (spatial resolution 1x1 km) in the southeast of Kazakhstan. Therefore, in the future, to enable the use of the developed model in the Kazakhstan National system for pest development forecasting in real time, layers of average air temperatures of 1km spatial resolution will be created at one day interval. The temperature data received from the temperature sensors of the Modis/Terra will be recalculated into the weather stations' data using the current established calibration regression models. The study territory will be devided into meteorological zones, where each pixel will refer to the area of one weather station and each zone will have its own conversion formula. The layers will be summed up and map of the ADD with a threshold value of + 10°C will be updated on a daily basis. The information to the orchards located in these zones will be transmitted by the sms-communication system and through the web GIS interface 1-2 days before the expected starting date of the codling moth's flight. Web GIS interface has already been worked out by Afonin et al. (2016) - http://app.o-gis.org/o-gis/web/app.php/editor/composition/88.

Conclusions

The study has shown that remote sensing data can be used as a prospective tool in plant protection by producing phenological maps for forecasting pest development stages. In particular, monitoring of ADD values calculated by Modis data makes possible to predict with sufficient accuracy the timing of the codling moth vulnerable developmental stages and to define optimal dates for farmers to implement pesticide treatment in apple orchards. Further precision

of forecasts may relate to the refinement of agro-ecological maps e.g. medium resolution satellite images (e.g. Landsat TIRS), forecasts can result in a spatial resolution of 30 m; a meteorological zoning technology and a technology for refined interpolation of under-cloudy data using the original cliche method and separate regression formulas for each zone would also significantly improve the methodological precision of maps (Afonin *et al*., unpublished).

The reported study was funded by RFBR according to the research project №19-05-00610 and Ministry of Education and Science of the Republic of Kazakhstan.

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Received: 27 December 2017; Accepted: 20 June 2019

Πρωτότυπο σύστημα χωρο-χρονικής πρόγνωσης της ανάπτυξης της καρπόκαψας της μηλιάς, *Cydia pomonella*, στο Καζακστάν

A. Afonin, B. Kopzhassarov, E. Milyutina, E. Kazakov, A. Sarbassova και A. Seisenova

Περίληψη Η εργασία αφορά στην ανάπτυξη ενός πρωτότυπου συστήματος πρόγνωσης εμφάνισης των βιολογικών σταδίων εντομολογικών εχθρών στο Καζακστάν με τη χρήση γεωχωρικών δεδομένων και οργανισμό – μοντέλο την καρπόκαψα της μηλιάς, *Cydia pomonella*. Η βασική μεθοδολογία περιελάμβανε την επιχειρησιακή ανάκτηση δεδομένων θερμοκρασίας στην επιφάνεια της γης από θερμικούς χάρτες του συστήματος MODIS και δεδομένων από μετεωρολογικούς σταθμούς, τον επανυπολογισμό και την απεικόνισή τους σε χάρτες ημεροβαθμών και τελικά σε χάρτες δυναμικής του πληθυσμού της καρπόκαψας. Η επαλήθευση των προβλεπόμενων ημερομηνιών εμφάνισης των βιολογικών σταδίων του εντόμου βάσει επιτόπιων δεδομένων από μηλεώνες έδειξε καλή πρόβλεψη. Η ακρίβεια πρόγνωσης είναι δυνατόν να βελτιωθεί περαιτέρω με τη χρήση ημερήσιων δεδομένων θερμοκρασίας από δορυφορικούς δέκτες και με τη βαθμονόμησή τους με δεδομένα από ένα δίκτυο μετεωρολογικών σταθμών εγκατεστημένων στους οπωρώνες.

Hellenic Plant Protection Journal **13:** 1-12, 2020

sciendo

Hellenic Plant Protection Journal **13:** 13-23, 2020
DOI 10.2478/hppj-2020-0002

Interactive effect of *Meloidogyne incognita* and *Macrophomina phaseolina* on the development of root–rot disease complex in relation to growth and physiological attributes of chickpea

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Summary The interactive relationship between the root–knot nematode *Meloidogyne incognita* and the root-rot fungus *Macrophomina phaseolina* in a root–rot disease complex of chickpea (*Cicer arietinum* var. *avrodhi*) was studied in a net house. The present study was carried out in such a manner so that the pathogenic potential of *M. incognita* and *M. phaseolina* individually, simultaneously and sequentially could be monitored. The pathogens singly as well as in combination led to significant reduction in growth, yield, nutrient and biochemical parameters. Gaseous exchange parameters like photosynthetic rate, transpiration rate and stomatal conductance were also reduced following infection of plants by the pathogens. However, maximum reduction was noticed in simultaneous inoculation with both pathogens. Sequential inoculation, where *M. incognita* preceded *M. phaseolina* by 15 days, was more damaging to the crop in comparison to that where *M. phaseolina* preceded *M. incognita* inoculation by 15 days. Infection by *M. phaseolina* caused a considerable reduction in the number of galls, egg–masses and nematode multiplication, with the highest reduction observed in plants simultaneously inoculated with the pathogens. Those plants also showed the highest disease severity in terms of percent root–rot. Thus, a manifold action plan to reduce the impact of the root-rot disease complex on chickpea crops has to be formulated.

Additional keywords: Cicer arietinum, gaseous exchange, interaction, nutrients, pathogenic potential

Introduction

Chickpea (*Cicer arietinum* L.) is the second most essential pulse crop after beans in the world both area wise (13.5 million ha) and production (13.1 million tons) (FAOSTAT, 2016). India is the largest producer of chickpea in the world contributing about 63% of the total production. Chickpea generally known as "Chana"/ "Gram" or "Bengal Gram" and widely appreciated as healthy food, is an essential legume having a broad variety of potential nutritional advantages due to its chemical composition (Aliu *et al*., 2016). In addition, it is important mainly for the developing countries, where people are mainly vegetarians and cannot afford the animal proteins for fulfilling their nutritional requirements. Despite India being the largest producer and processor of chickpea in the world, the country also imports large amounts of this pulse annually in order to meet its ever-increasing consumption requirements.

Chickpea production in India has suffered in the last few years due to various constraints that include both biotic and abiotic stresses. Among these constraints, fungal and nematode attacks are considered as the major biotic factors causing significant yield losses in the crop. *Meloidogyne incognita,* one of the most damaging root–knot nematodes, causes significant losses on chickpea. Parasitism by *M. incognita* is characterised by the formation of root galls and deformation of the vascular system of the plant due to formation of giant cells and transfer of nutrients to these cells for use by the nematodes (Palomares–Rius, 2011; Sumbul *et al*., 2015). *Macrophomina phaseolina*, the causal agent of charcoal rot of chickpea, is an important pathogen causing considerable yield losses (Ashraf *et al*., 2005). The fungus is regularly reported from temperate

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and tropical areas of the world, including India, where it is most commonly associated with chickpea (Srivastava *et al*., 2001; Kumar *et al*., 2007; Naseri *et al*., 2018). Infection by *M. phaseolina* results in the formation of red to brown lesions on the roots and stems due to the presence of dark coloured mycelia and black microsclerotia. Eventually the plants become defoliated and wilted (Iqbal and Mukhtar, 2014). Interactive association between *M. incognita* and *M. phaseolina* results in a root–rot disease complex on chickpea that causes more serious yield losses as compared to their individual action (Siddiqui and Husain, 1991; 1992). A plethora of studies has been performed on interaction of nematode–fungus complex, yet there are limited reports on the interactive effects of *M. incognita* and *M. phaseolina* on chickpea.

The present study was performed to monitor the interactive effect of *M. incognita* and *M. phaseolina* on chickpea taking into account different times of inoculation with the two pathogens (individual, concomitant and sequential inoculation) and the associated alterations in growth, yield, physiology, nutrients, and pathogen-related parameters.

Materials and Methods

Preparation and sterilization of soil mixture

Sandy loam soil obtained from a field of the Aligarh Muslim University (AMU), India, was sieved using a 10-mesh sieve. The soil was subsequently mixed with river sand and organic manure in the ratio of 3:1:1. Clay pots (15 cm in diameter) were filled with the soil mixture (1kg/pot). Small amount of water was added to each pot to wet the soil prior to the steam sterilization of the pots at 1.36 atm pressure for 30 minutes. The pot study was conducted in a net house of the Department of Botany, AMU, Aligarh (27°.52'N latitude, 78°.51'E longitude) during the winter season (October to January).

Growth and maintenance of test plants

The chickpea seeds (var. Avrodhi) were surface sterilized by dipping in 0.01% $HgCl_2$ for 2 min, followed by washing twice with distilled water. Prior to sowing, all seeds were treated with charcoal-based commercial culture of *Rhizobium*, chickpea strain. Five chickpea seeds were sown in each pot and the emerged seedlings were thinned to one seedling/pot. Watering of the pots was done as per requirement.

Inoculum preparation of root–knot nematode *Meloidogyne incognita*

Egg masses of *M. incognita* were hand-picked using sterilized forceps from heavily infested roots of eggplant (*Solanum melongena*) on which pure culture of the nematode was maintained. The egg masses were rinsed with distilled water and placed in a coarse sieve (16 mesh size, 10 cm in diameter) covered with crossed double layers of tissue paper and placed in Petri plates containing water just deep enough to contact the egg masses. The Petri plates were incubated at 25°C in the dark. After three days, most of the eggs hatched and the second stage juveniles (J_2) were collected by washing the Petri plates with distilled water.

The water containing hatched J_2 of *M. incognita* was thoroughly agitated for dispensing the nematodes homogenously in the suspension. The number of J_2 in the suspension was counted under the stereoscope. Five counts were made to calculate the average number of J_2/mL in the suspension of each sample and the final concentration was adjusted to 200±5 J_2/mL. Each plant was inoculated with 10 mL of the suspension containing 2000 freshly hatched J_2.

Mass culture of the root–rot fungus *Macrophomina phaseolina*

Macrophomina phaseolina inoculum was obtained from the roots of naturally infected chickpea plants collected from fields in Aligarh district. The fungal culture was purified and maintained on Potato Dextrose Agar medium. Koch's postulates were applied to assure the pathogenicity of *M. phaseolina* on chickpea plants. Large amount of fungal inoculum (mycelium and spores) was

obtained by mass culturing a *M. phaseolina* isolate in Richard's medium (Riker and Riker, 1936) for 15 days at 25°C. The mycelium and spores were subsequently placed on blotting sheets to remove excess water and nutrients. The final inoculum consisting of a mixture of 100 gr macerated wet mycelium and spores was added to 1 L of distilled water. Ten mL of the inoculum was used for the inoculation of each experimental plant.

Inoculation techniques

For the inoculation of plants with *M. incognita* and/or *M. phaseolina* the soil around the roots of one-week-old healthy chickpea seedlings was removed without causing any injury to the root system. Ten mL of inoculum suspension of *M. incognita* and/or *M. phaseolina* was poured around the roots, which were immediately covered with soil. An equal volume of distilled water was added to control plants.

Experimental Design

The experiment was carried out during the winter season in a completely randomized block design with the following variables:

(1) Uninoculated control
(2) *M. incognita* alone
(3) *M. phaseolina* alone
(4) *M. incognita* + *M. phaseolina* simultaneously
(5) *M. incognita* 15 days prior to *M. phaseolina*
(6) *M. phaseolina* 15 days prior to *M. incognita*

Five replicate pots were used for each treatment.

Measurement of plant growth parameters

The plants were harvested four months after emergence and washed gently under tap water to remove the adhering soil particles. Washed plants were labeled according to the treatments. Number of pods per plant and number of nodules per root system were counted visually. Plant height was measured with a measuring tape. Before estimating the plant fresh weight with a physical balance, the excess of water was removed from the plants with blotting paper. For the determination of dry weight, the plants were air-dried in an oven at 60°C for 24 - 48 h before weighing.

Leaf biochemical analysis

Nitrate Reductase Activity (NRA) in leaves was measured by the process of Jaworski (1971). The nitrogen (N) content of the shoot was determined by the method of Lindner (1944), whereas phosphorus (P) and potassium (K) contents were determined by the method of Fiske and Subbarow (1925) and flame photometer, respectively. Chlorophyll and carotenoid contents of leaves were determined by the method of Hiscox and Israelstam (1979) using dimethyl sulphoxide (DMSO).

Recording of gas exchange parameters of chickpea leaves

Gas exchange parameters, such as photosynthesis rate (Pn), transpiration rate (E) and stomatal conductance (Sc), were measured in fully expanded uppermost leaves of plants with an Infra-Red Gas Analyser (IRGA, CID–340, Photosynthesis System, Bio Science, USA). The measurements were carried out on a sunny day at 11 a.m–12 p.m.

Estimation of nematode reproduction in inoculated pots

Number of galls per root system was counted visually. For estimating the number of egg-masses per root system, the method of Daykin and Hussey (1985) was followed. In order to determine the nematode population in soil, 1 kg of soil from each sample was processed by Cobb's sieving and decanting method, followed by Baermann funnel extraction technique (Southey, 1986). The reproductive potential of *M. incognita* in terms of reproduction factor (Rf) was calculated by dividing the final nematode population in soil by the nematode population used for inoculating the plants (Windham and Williams, 1987).

Observations on percent root–rot in inoculated plants

To estimate the disease severity in terms of percent root–rot caused by *M. phaseolina* in chickpea, roots of each plant were initially cut into 5 cm pieces. The pieces were mixed together, and 15 pieces were randomly selected from the mixture. Each root piece was observed visually, and the length of the rotted portion was measured. The percentage of root–rot was estimated by using the following formula:

$$\text{Root-rot}(\%) = \frac{\text{length of rotted portion on root pieces}}{\text{total length of root pieces}} \times 100$$

Root rot index was determined according to four categories: 0 = none; 1 = less than 25%; 2 = 26–50%; 3 = 51–75%; 4 = 76 = 100% (Aoyagi *et al*., 1998). Disease severity was calculated according to the following formula (Aoyagi *et al.*, 1998):

$$\text{Disease severity}(\%) = \Sigma \frac{\text{Disease index x No of plants in each category of index}}{\text{Higher value of the index x No of all inoculated plants}} \times 100$$

Statistical Analysis

All the data were subjected to analysis of variance (ANOVA). Least significant differences (LSD) were calculated at $P \leq 0.05$ using R software, version 2.14.0. Duncan's Multiple Range Test (DMRT) was deployed to denote significant differences between treatments.

Results

Effect of interaction on growth and yield parameters of chickpea

The highest growth parameters were observed in control plants. Both pathogens applied individually or in combination caused significant reduction in plant growth parameters, such as plant height and fresh as well as dry weights, compared to the control (Table 1). However, the highest reduction in plant growth was observed in plants inoculated simultaneously with the pathogens followed by those where nematode preceded the fungal inoculation by 15 days and those where the fungal preceded the nematode inoculation by 15 days. *Meloidogyne incognita* caused a higher reduction in plant growth as compared to that by *M. phaseolina*. Also, the statistical analysis of data showed that the reduction in all the growth parameters of plants inoculated simultaneously with the pathogens did not differ significantly from that of plants inoculated with *M. incognita* 15 days prior to *M. phaseolina*. Likewise, the highest reduction in the number of pods/plant was observed on plants treated with *M. incognita* + *M. phaseolina* and the lowest on plants inoculated with *M. phaseolina* alone (Table 1). A similar trend of reduction was observed in the number of nodules/root system.

Effect of interaction on biochemicals and nutrients of chickpea leaves

All the treatments, either individual or combined, caused significant reduction in the physiological and biochemical parameters of chickpea plants when compared to control plants. Biochemical and nutrients parameters, such as NRA, chlorophyll, carotenoids, N, P and K contents of the chickpea plants showed higher reductions in case of *M. incognita* + *M. phaseolina* inoculated plants compared to control plants. These reductions were not significant statistically when compared to those on plants inoculated with *M. incognita* 15 days prior to *M. phaseolina* inoculation (Table 2).

Effect of interaction on gaseous exchange rate of chickpea

The highest photosynthetic rate (Pn) was recorded in control plants while inoculation of plants with the pathogens, individually and in any combination, reduced photosynthetic rate significantly (Table 3). Maximum reduction in Pn was observed in plants treated with *M. incognita* + *M. phaseolina* followed by those where the nematode preceded the fungal inoculation by 15 days, those where the fungal preceded the nematode inoculation by 15 days, and those inoculated with *M. incognita* alone and *M. phaseolina* alone. Likewise, E and Sc exhibited the same trend of reduction as compared to control plants (Table 3).

Table 1. Effects of *Meloidogyne incognita* and *Macrophomina phaseolina,* singly and combined, on growth, yield and nodulation of chickpea plants (*Cicer arietinum* var. *avrodhi*).

Treatments	Plant height (cm)	Plant weight (g)		Number of pods/plant	Number of nodules/root system
		Fresh	Dry		
Uninoculated control	57.04*±2.29a	39.50±1.46a	7.12±0.31a	21.00±0.76a	43.00±0.71a
M. phaseolina alone	48.40±1.62b	32.71±1.45b	5.68±0.32b	17.60±0.43b	36.60±0.71b
M. incognita alone	44.94±1.30b	30.29±1.27b	5.24±0.27b	16.40±0.52bc	34.40±0.77bc
M. incognita+M. phaseolina	30.68±0.99d	20.48±1.18d	3.49±0.26d	11.00±0.32d	23.20±0.71d
M. incognita→M. phaseolina	34.40±1.16d	23.02±1.37d	3.94±0.24d	12.60±0.69d	26.20±0.63d
*M. phaseolina→M. incogni*ta	39.56±0.89c	27.71±1.17c	4.78±0.31c	15.00±0.45c	31.40±0.45c

+ = simultaneous inoculation with both pathogens, → = nematode or fungal inoculation preceded by 15 days the fungal or the nematode inoculation, respectively. *Values are means of five replicates. Means in each column followed by the same letter(s) do not differ significantly at $P \leq 0.05$.

Table 2. Effects of *Meloidogyne incognita* and *Macrophomina phaseolina,* singly and combined, on nitrate reductase activity (NRA), nitrogen (N), phosphorus (P) and potassium (K) contents of chickpea plants (*Cicer arietinum* var. *avrodhi*).

Treatments	NRA (µmol NO2/g/h)	Fresh leaf content (mg/g)		
		N	P	K
Uninoculated control	0.397*±0.006a	3.220±0.065a	0.310±0.006a	1.570±0.036a
M. phaseolina alone	0.353±0.008b	2.896±0.055b	0.282±0.006b	1.458±0.031b
M. incognita alone	0.330±0.008bc	2.727±0.058bc	0.266±0.007bc	1.364±0.029bc
M. incognita+M. phaseolina	0.280±0.006d	2.297±0.048d	0.229±0.006e	1.189±0.029e
*M. incognita→M. phaseolin*a	0.296±0.006d	2.428±0.030d	0.241±0.006de	1.228±0.028de
M. phaseolina→M. incognita	0.322±0.008c	2.645±0.055c	0.259±0.007cd	1.317±0.030cd

+ = simultaneous inoculation with both pathogens, → = nematode or fungal inoculation preceded by15 days the fungal or the nematode inoculation, respectively. *Values are means of five replicates. Means in each column followed by the same letter(s) do not differ significantly at $P \leq 0.05$.

Table 3. Effects of *Meloidogyne incognita* and *Macrophomina phaseolina,* singly and combined, on chlorophyll, carotenoid, photosynthesis rate (Pn), transpiration rate (E) and stomatal conductance (Sc) of chickpea plants (*Cicer arietinum* var. *avrodhi*).

Treatments	Fresh leaf content (mg/g)		Pn (µmol/ m2/sec)	E (nmol/ m2/sec)	Sc (nmolH2O/ m2/sec)
	Chlorophyll	Carotenoids			
Uninoculated control	2.140*±0.036a	0.142±0.001a	9.284±0.009a	1.711±0.006a	280.236±0.018a
M. phaseolina alone	1.876±0.035b	0.126±0.002b	7.441±0.011b	1.483±0.005b	244.114±0.016b
M. incognita alone	1.756±0.017bc	0.119±0.001bc	7.127±0.008bc	1.376±0.005bc	233.773±0.025bc
M. incognita+M. phaseolina	1.458±0.023d	0.099±0.001d	5.083±0.008d	1.057±0.003d	191.457±0.025e
M. incognita→M. phaseolina	1.548±0.022cd	0.105±0.001d	5.343±0.007d	1.152±0.006d	202.190±0.021de
M. phaseolina→M. incognita	1.675±0.023c	0.115±0.002c	6.655±0.007c	1.331±0.003c	220.686±0.019cd

+ = simultaneous inoculation of both pathogens, → = nematode or fungal inoculation preceded by 15 days the fungal or the nematode inoculation, respectively. *Values are means of five replicates. Means in each column followed by same letter(s) do not differ significantly at $P \leq 0.05$.

Effect of interaction on nematode and fungal multiplication related parameters on chickpea

The greatest Rf, number of galls and egg–masses/root system were recorded in plants inoculated with *M. incognita* alone (Table 4). The multiplication of *M. incognita* and the number of galls/root system in chickpea plants were significantly hampered in the presence of *M. phaseolina* as compared to plants inoculated with *M. incognita* alone. The greatest reduction was observed in plants inoculated with *M. phaseolina* 15 days prior to *M. incognita* inoculation, followed by those inoculated simultaneously with *M. incognita* and *M. phaseolina* and those where *M. incognita* preceded *M. phaseolina* inoculation by 15 days. Similar trend of reduction was recorded in case of the final population of *M. incognita* recovered from the soil of the treated pots. On the other hand, the highest disease severity was observed in *M. incognita* + *M. phaseolina* inoculated plants followed by plants inoculated with the nematode 15 days prior to fungal inoculation, by plants where the fungal preceded the nematode inoculation by 15 days, and by plants inoculated only with *M. phaseolina*. Similarly, the highest root–rot index was recorded in *M. incognita* + *M. phaseolina* inoculated plants, followed by those inoculated with *M. incognita* 15 days prior to *M. phaseolina,* by plants where the fungal inoculation preceded the nematode inoculation by 15 days and by plants inoculated only with *M. phaseolina* (Table 4).

Discussion

It is evident from the present study that the highest and most significant decrease in growth and yield parameters was observed in chickpea plants inoculated simultaneously with *M. incognita* and *M. phaseolina,* which shows a synergistic effect between the fungus and the nematode (Singh *et al*. 2010; Ganaie and Khan, 2011; Ahmed *et al*., 2014). Simultaneous inoculation of plants with the pathogens significantly damaged the roots and root hairs leading to low capacity of the plants to absorb water and nutrients from the soil. The lack of water and nutrients in the plants resulted in poor growth in terms of reduced plant height, fresh and dry weights (Ansari and Mahmood, 2017). The reduction in growth and yield observed in plants inoculated with *M. phaseolina* 15 days prior to *M. incognita* was equal to that in nematode inoculated plants although the fungus had enough time to colonize the roots and make them less suitable for the penetration by the nematode (Meena *et al*., 2016). It is also possible that the toxic metabolites produced by *M. phaseolina* may have destroyed the giant cells which are necessary for the nematode

Table 4. Effects of *Meloidogyne incognita* and *Macrophomina phaseolina,* singly and combined, on disease development in chickpea plants (*Cicer arietinum* var. *avrodhi*).

Treatments	Number of galls/root system	Number of egg-masses/ root system	Number of nematode juveniles/kg soil	Reproduction factor (Rf)	Disease severity (percent root-rot)	Root-rot disease index (0-4)
Uninoculated control	0.00*±0.00e	0.00±0.00e	0.00±0.00e	0.00	0.00±0.00e	0
M. phaseolina alone	0.00±0.00e	0.00±0.00e	0.00±0.00e	0.00	21.23±1.45d	1
M. incognita alone	114.40±4.17a	107.20±2.17a	19659±321.89a	9.82	0.00±0.00e	0
M. incognita+*M. phaseolina*	79.20±3.40c	61.00±0.98c	12771±155.05c	6.38	64.21±2.55a	3
M. incognita→*M. phaseolina*	93.20±4.73b	83.60±1.50b	14416±222.99b	7.20	59.14±2.70b	3
M. phaseolina→*M. incognita*	69.40±2.84d	53.00±2.97d	11605±248.16d	5.80	46.41±1.73c	2

+ = simultaneous inoculation of both pathogens, → = nematode or fungal inoculation preceded by 15 days the fungal or the nematode inoculation, respectively. *Values are means of five replicates. Means in each column followed by same letter(s) do not differ significantly at $P \leq 0.05$.

feeding and reproduction (Ogaraku, 2008; Ahmed *et al*., 2014).

The significant damage to the root nodules observed in plants inoculated with the pathogens, either individually or simultaneously may be due to the heavy galling resulting from *M. incognita* infection, destruction of root tissue by the rotting caused by *M. phaseolina* and/or the inhibitory effects of *M. incognita* and *M. phaseolina* generated toxic metabolites on *Rhizobium* (Hussain and Siddiqui, 1991; 1992). Plants with lower number of nodules were able to fix lesser nitrogen into nitrate, depriving the plants with suitable substrate for the nitrate reductase enzyme. The decrease in NRA in inoculated plants indicates adverse effect of *M. incognita* and *M. phaseolina* on protein synthesis (Naik *et al*., 1982). This decrease also resulted in reduced growth and yield of chickpea plants. Chlorophyll and nutrient (N, P and K) contents of plants also decreased with the highest reduction observed in plants inoculated simultaneously with the pathogens. Plants inoculated simultaneously with both pathogens showed extremely damaged roots with hampered translocation of water and nutrients from roots to the upper parts (Ansari and Mahmood, 2017). Also, the root–knot nematode directs nutrient contents towards the infected giant cells for their own feeding and reproduction, thus depriving the upper parts of the plants from proper nutrient content levels (Sumbul and Mahmood, 2017).

Gaseous exchange parameters, Pn, E and Sc, were highly reduced in *M. incognita* + *M. phaseolina* inoculated plants which may be due to severe infection of the roots resulting in hampered water absorption and nutrient translocation acropetally (Lorenzini *et al*., 1997; Saeed *et al*., 1999; Strajnar, 2012). Ghazalbash and Abdollahi (2012) reported a decrease in gaseous exchange parameters in tomato plants infected simultaneously with *Meloidogyne javanica* and *Fusarium oxysporum* f. sp. *lycopersici*. The authors assumed that stomatal closure reduces the intercellular CO_2 concentration, which might be the cause behind the reduced net photosynthesis.

The reduced number of galls and egg–masses per root system in the presence of *M. phaseolina* indicates that this fungus is deleterious for the multiplication of *M. incognita*. The detrimental effect of *M. phaseolina* on *M. incognita* multiplication may be due to the destruction of root tissues which become unable to support a large number of galls thus affecting *M. incognita* reproduction (Back *et al*., 2002; Al–Hazmi and Al–Nadri, 2015; Meena *et al*., 2016). Decrease in feeding sites impaired nutrient supply to nematode (Hasan 1993; Fazal *et al*., 1998). Moreover, the toxic substances produced by the fungus resulted in the destruction of the giant cells induced by the nematode, as well as in reduction in hatching and immobilization of J_2 (France and Abawi, 1994; Mokbel *et al*., 2007). Plants inoculated with *M. incognita* 15 days prior to *M. phaseolina* produced higher number of egg–masses, galls and nematode population as compared to those inoculated simultaneously with both pathogens (Ogaraku, 2008).

The lowest disease severity was recorded in plants inoculated with *M. phaseolina* alone. Our results are in conformity with those of Senthamarai (2006), Ganaie and Khan (2011) and Ahmed *et al*. (2014). The low disease severity indicates that *M. phaseolina* could not infect the host in the absence of the predisposing factor, i.e. *M. incognita* in this case (Siddiqui and Hussain, 1991; Lobna *et al*., 2016). The highest rotting of chickpea roots was observed when plants were inoculated simultaneously with *M. incognita* and *M. phaseolina*. This may be because both pathogens had equal opportunities to infect the plants, but the presence of the nematode further enhanced the susceptibility of roots to fungal infection (Ganaie and Khan, 2011; Ahmed *et al*., 2014).

The root–rot fungus has an inherent mechanism to get entry into the root and cause root-rot disease. However, in the case of root–rot disease complex, nematode plays a crucial role in assisting the fungus in its pathogenesis and enhancing host susceptibility (Khan, 1984). Wounds caused by

the nematode on plant roots provide entry points for the fungus to infect the roots more rigorously (Inagaki and Powell, 1969). Apart from the wounds, nematodes also lead to different forms of damage to plant roots like split root galls, cracks and crevices due to emergence of swollen females etc. thus allowing the fungus to infect the host root (Evan and Haylock, 1993, Back *et al*., 2002). In addition to morphological disruptions, alterations in the physiological and nutrient status of the root cells infected by the nematode may also be responsible for the appearance of the root-rot disease complex. Giant cells produced by the root–knot nematode are the regions of high metabolic activity (Jones, 1981). These physiological alterations lead to better nutrients availability to the invading fungus and serve as the key factor in establishing the nematode–fungus disease complex (Khan and Muller, 1982; Khan, 1987; Abdel–Momen and Starr, 1998; Castillo *et al*., 1998). Plant root exudates play a key role in attracting both nematode and fungal pathogens (Grayston, 1997; Clarke and Henessy, 1987). Therefore, the root–knot nematode might have altered the root exudates either quantitatively or qualitatively, making them more favourable for the growth of the fungus (Bergeson, 1972; Golden and Van Gundy, 1975; Reddy 1980).

Conclusions

It can be inferred from the present study that the presence of *M. incognita* increased the severity of the root–rot disease caused by *M. phaseolina* in chickpea plants. The interaction between *M. incognita* and *M. phaseolina* even modified the biochemical composition in the plants to assist the growth and multiplication of the pathogens. Therefore, inoculation of plants with both pathogens (either simultaneous or sequential) caused higher damage compared to individual inoculations. However, the greatest damage was observed in plants inoculated simultaneously with the pathogens. When present together, the pathogens caused significant reduction in chickpea growth and yield and modified physiological and biochemical components of the plants to support their growth accordingly. Moreover, *M. incognita* proved to act as a predisposing factor for the infection of plants by *M. phaseolina*. Thus, the interaction between *M. incognita* and *M. phaseolina* should be taken into consideration for the development of strategies for the effective management of the root-rot disease complex in chickpea crops.

Both authors declare that they do not have any conflict of interest.

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Received: 3 August 2018; Accepted: 23 July 2019

Αλληλεπίδραση μεταξύ του κομβονηματώδη *Meloidogyne incognita* και του μύκητα *Macrophomina phaseolina* στην εμφάνιση του συμπλόκου της ασθένειας "σήψη των ριζών" σε σχέση με την ανάπτυξη και τα φυσιολογικά χαρακτηριστικά των φυτών ρεβιθιού

A. Sumbul και I. Mahmood

Περίληψη Η εργασία αφορά στη μελέτη της αλληλεπίδρασης μεταξύ του κομβονηματώδους *Meloidogyne incognita* και του φυτοπαθογόνου μύκητα *Macrophomina phaseolina* στο σύμπλοκο της ασθένειας "σήψη των ριζών" του ρεβιθιού (*Cicer arietinum* var. avrodhi). Η παθογόνος δύναμη των *M. incognita* και *M. phaseolina* μελετήθηκε μεμονωμένα, ταυτόχρονα και διαδοχικά. Τα παθογόνα τόσο ξεχωριστά όσο και σε συνδυασμό προκάλεσαν σημαντική μείωση της ανάπτυξης, της παραγωγής και των θρεπτικών και βιοχημικών παραμέτρων των φυτών ρεβιθιού. Οι παράμετροι ανταλλαγής αερίων, όπως ο ρυθμός φωτοσύνθεσης, ο ρυθμός διαπνοής και η αγωγιμότητα των στοματίων μειώθηκαν επίσης μετά τη μόλυνση των φυτών από τα παθογόνα. Εντούτοις, η μέγιστη μείωση των παραπάνω παραμέτρων διαπιστώθηκε μετά από ταυτόχρονη μόλυνση των φυτών με τα παθογόνα. Η διαδοχική μόλυνση των φυτών, όπου ο νηματώδης *M. incognita* προηγήθηκε του μύκητα *M. phaseolina* κατά 15 ημέρες, ήταν περισσότερο επιβλαβής για την καλλιέργεια σε σύγκριση με εκείνη όπου ο *M. phaseolina* προηγήθηκε του *M. incognita* κατά 15 ημέρες. Η μόλυνση των φυτών από το μύκητα *M. phaseolina* προκάλεσε σημαντική μείωση στον αριθμό των όγκων των μαζών ωών και στον πολλαπλασιασμό του νηματώδους, με τη μέγιστη μείωση να παρατηρείται στα φυτά που μολύνθηκαν ταυτόχρονα με τα παθογόνα. Αυτά τα φυτά εμφάνισαν επίσης τη μεγαλύτερη ένταση της ασθένειας. Τα αποτελέσματα της παρούσας μελέτης έδειξαν ότι για την μείωση των επιπτώσεων του συμπλόκου της ασθένειας "σήψη των ριζών" στην καλλιέργεια του ρεβιθιού είναι απαραίτητη η διαμόρφωση μιας στρατηγικής πολλαπλών μέτρων διαχείρισης.

Hellenic Plant Protection Journal **13:** 13-23, 2020

sciendo

Hellenic Plant Protection Journal **13:** 24-28, 2020
DOI 10.2478/hppj-2020-0003

SHORT COMMUNICATION

Plant-parasitic nematodes associated with cultivated and wild olive trees in Crete, Greece

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Summary The present study is part of a survey for the identification of plant-parasitic nematodes in the rhizosphere of cultivated and wild olive trees in Crete, Greece. Sixteen species corresponding to 13 genera are added to 20 species belonging to 8 genera, previously reported in the survey. Seven nematode species, *Filenchus ditissimus*, *Filenchus vulgaris*, *Ogma civellae*, *Pratylenchoides crenicauda*, *Psilenchus hilarulus*, *Tylenchus elegans*, and *Zygotylenchus guevarai*, are recorded for the first time in Greece.

Additional keywords: Ogma civellae, *Pratylenchoides crenicauda, Zygotylenchus guevarai*

Introduction

Olive (*Olea europaea* L.) is the most important tree crop for the island of Crete occupying 177,000 ha, which is c. 22% of the total olive cultivated area in Greece. Clusters of wild olives are also located in some areas of the island. Surveys on plant parasitic nematodes associated to cultivated and wild olive trees, which were carried out during the period 2013-2015, revealed the presence of ten species of the family *Longidoridae*, two species from each of the genus *Rotylenchus* and *Rotylenchulus*, three species of *Helicotylenchus*, *Bitylenchus hispaniensis*, *Pratylenchoides alkani* and *Merlinius brevidens* (Table 1), on the rhizosphere of both olive types (Tzortzakakis *et al*., 2014, 2015, 2016ab, 2018; Palomares-Rius *et al*., 2018ab). The main goal of this study was to identify the remaining nematode species which were found in these soil samples.

Material and methods

In this study, soil samples were collected from 146 cultivated olive orchards (73 irrigated and 73 non irrigated), distributed from 3 to 611 m above sea level and from 36 wild olive trees, individuals or clusters, located in non-agricultural areas from 17 to 343 m above sea level. Wild olive trees had no human activity related to cultivation practices, being usually foraged by goats living free in a semi-wild condition.

In each olive orchard, the soil samples were taken from the rhizosphere of 2-5 randomly chosen olive trees, using a sampler and a mattock from 20 up to 40 cm depth, depending on soil condition. Soil samples from wild olives were taken from individual plants or clusters with a mattock at a lower depth due to the stony soil condition.

Nematodes were extracted from two samples of 500 cm^3 soil per site by the wet sieving and decanting method (Cobb, 1918) and final separation of nematodes from soil debris with an extraction dish. Additional soil samples were collected, when required

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Table 1. Plant-parasitic nematode species reported in the rhizosphere of cultivated and wild olive areas in Crete, Greece, and their prevalence (%).

Nematode species	Cultivated olives			Wild olives (36 plants)
	Irrigated (73 fields)	Non irrigated (73 fields)	Total (146 fields)	
Bitylenchus hispaniensis[1]	-	2.7	1.4	13.9
Helicotylenchus microlobus[1]	4.1	-	2.1	-
Helicotylenchus oleae[2]	2.7	4.1	3.4	5.6
Helicotylenchus vulgaris[1]	4.1	2.7	3.4	2.8
Longidorus cretensis[3,4]	1.4	-	0.7	-
Longidorus closelongatus[3,4]	2.7	-	1.4	2.8
Longidorus iranicus[3,4]	-	1.4	0.7	-
Longidorus pseudoelongatus[3,4]	6.8	5.5	6.2	-
Merlinius brevidens[1]	15.1	27.4	21.3	27.8
Pratylenchoides alkani[1]	1.4	1.4	1.4	16.7
Rotylenchus cretensis[5]	-	1.4	0.7	-
Rotylenchus cypriensis[5]	-	1.4	0.7	-
Rotylenchulus macrodoratus[6]	11	5.5	8.3	-
Rotylenchulus macrosoma[6]	9.6	2.7	6.2	-
Xiphinema cretense[3,4]	1.4	2.7	2	-
Xiphinema herakliense[4,7]	-	1.4	0.7	-
Xiphinema index[3,4]	1.4	2.7	2.1	-
Xiphinema israeliae[3,4]	8.2	6.8	7.5	2.8
Xiphinema italiae[3,4]	5.5	9.6	7.6	2.8
Xiphinema pachtaicum[3,4]	41.1	37	39.1	5.6

[1]Tzortzakakis *et al*., 2018; [2]Palomares-Rius *et al*., 2018b; [3]Tzortzakakis *et al*., 2104; [4]Tzortzakakis *et al*., 2016a; [5]Tzortzakakis *et al*., 2016b; [6]Palomares-Rius *et al*., 2018a; [7]Tzortzakakis *et al*., 2015

to obtain sufficient specimens for morphological identification of the nematodes. Root fragments present in samples were separated from soil and were macerated to extract endoparasitic nematodes. Nematode specimens were killed by gentle heat, fixed in a solution of 4% formaldehyde and 2% glycerin and processed to pure glycerin using the De Grisse's method (1969). The specimens were observed and identified under a light microscope (LM) using the keys by Siddiqi (2000).

Results and discussion

In this study, 16 nematode species were identified associated with olive trees (Table 2). Nine of these species, *Coslenchus costatus, Criconemoides informis, Criconemoides xenoplax, Ditylenchus dipsaci, Helicotylenchus digonicus, Meloidogyne javanica, Pratylenchus thornei, Tylenchorhynchus clarus, Tylenchus davainei*, have already been reported in Greece in association with various crops, including olive (Hirschmann *et al*., 1966; Koliopanos and Vovlas, 1977; Koliopanos and Kalyviotis-Gazelas, 1969, 1973, 1979; Vlachopoulos, 1991; Karanastasi *et al*., 2008). Seven species, *Filenchus ditissimus, Filenchus vulgaris, Ogma civellae, Pratylenchoides crenicauda, Psilenchus hilarulus, Tylenchus elegans, Zygotylenchus guevarai*, are recorded in Greece for the first time (Table 2). All these nematode species are well known (Palomares-Rius *et al*., 2015), hence there was no need for additional molecular characterization with the exception of samples containing juveniles of *Meloidogyne* and cysts of *Heterodera*, which were identified molecularly by coxII and 28S large ribosomal subunit (LSU) D2-D3 expan-

Table 2. Plant-parasitic nematode species found in the rhizosphere of cultivated and wild olives in Crete, Greece, in this study and prevalence (%).

Nematode species	Cultivated olives (146 fields)			Wild olives (36 plants)
	Irrigated (73 fields)	Non irrigated (73 fields)	Total (146 fields)	
Coslenchus costatus	-	-	-	2.8
Criconemoides informis	1.4	1.4	1.4	-
Criconemoides xenoplax	1.4	-	0.7	-
Ditylenchus dipsaci	-	-	-	5.6
*Filenchus ditissimus**	8.2	9.6	8.9	2.8
*Filenchus vulgaris**	9.5	13.7	11.6	5.6
Helicotylenchus digonicus	64.3	56.2	60.3	75
Meloidogyne javanica	2.7	-	1.4	-
*Ogma civellae**	1.4	-	0.7	-
Pratylenchus thornei	1.4	4.1	2.8	2.8
*Pratylenchoides crenicauda**	-	1.4	0.7	-
*Psilenchus hilarulus**		1.4	0.7	-
Tylenchorhynchus clarus	30.1	15.1	22.6	16.7
*Tylenchus elegans**	4.1	2.7	3.4	5.6
Tylenchus davainei	-	4.1	2.1	2.8
*Zygotylenchus guevarai**	1.4	4.1	2.8	11.1

* Plant-parasitic nematode species reported for the first time in Greece.

sion segments.

Taking into account all findings of this survey (Tzortzakakis *et al*., 2018; Palomares-Rius *et al*., 2018a; Palomares-Rius *et al*., 2018b; Tzortzakakis *et al*., 2104; Tzortzakakis *et al*., 2016a; Tzortzakakis *et al*., 2016b; Tzortzakakis *et al*., 2015), a total of 36 species belonging to 19 genera were found in both cultivated and wild olives (Tables 1 and 2). The prevalence of each of them was calculated as the percentage of samples in which the nematode species was diagnosed with respect to total number of samples (Tables 1 and 2). A similar number of nematode species was found in irrigated (26 species) and non-irrigated (28 species) olive orchards. The diversity of nematodes was higher in cultivated olives as 17 more species were found compared to those occurring in wild olives.

Concerning the nine nematode species already reported in Greece, *Coslenchus costatus* and *Ditylenchus dipsaci* were found exclusively in the rhizosphere of wild olives, the two species of *Criconemoides* (*C. informis* and *C. xenoplax*) only in cultivated olives and *Helicotylenchus digonicus*, *Tylenchus davainei*, *Tylenchorhynchus clarus* and *Pratylenchus thornei* in both cultivated and wild olives (Table 2). The root-knot nematode *Meloidogyne javanica* has been reported parasitizing olive trees in Greece (Hirschmann *et al*., 1966). In our study, females and egg masses were not found in the olive roots, but second-stage juveniles (J_2s) were recorded in two soil samples from cultivated olives. Identification was conducted by DNA extraction and PCR assays (Castillo *et al*., 2003). The detection of root-knot nematode J_{2s} in the soil of these fields may be explained by the previous cultivation of vegetables between the tree rows.

Cysts of *Heterodera* were found in one sample from cultivated olive. Although species identification was not possible, *H. mediterranea* was excluded with the use of 28S large ribosomal subunit (LSU) D2-D3 expansion segments. *Heterodera* J_{2s} were also found in another sample from cultivated olive but identification to species level was not possible.

Filenchus ditissimus, *F. vulgaris*, *Tylenchus elegans* and *Zygotylenchus guevarai* were found in both cultivated and wild olives while *Ogma civellae*, *Pratylenchoides crenicauda* and *Psilenchus hilarulus* were found only in cultivated olive (Table 2). All these species, except *Pratylenchoides crenicauda*, have been detected in olive orchards in southern Spain (Palomares-Rius *et al*., 2015) and *P. crenicauda* has been reported in natural habitats in southeastern Spain (Castillo and Gomez Barcina, 1988) and in crops (*Citrus aurantium* L., *Gossypium barbadense* L., *Pyrus communis* L., *Vitis vinifera* L.) in Egypt (Ibrahim *et al*., 2010). Nematode species associated with the rhizosphere of olive trees in previous studies (Hirschmann *et al*., 1966; Koliopanos and Kalyviotis-Gazelas, 1969, 1973; Vlachopoulos, 1991) were *Filenchus filiformis*, *Pratylenchus neglectus*, *Tylenchorhynchus brevidens*, *T. dubius*, *T. striatus* and *Tylenchulus semipenetrans,* but none of them were found in our survey.

Examination of the roots did not reveal endoparasitic nematodes in any sample except for a few cases, in which *Helicotylenchus* were recovered from both roots and soil of the same sample. Therefore, it remains to be investigated whether olive or/and the weeds growing in the olive orchards are the hosts of the recorded nematode species and whether they can be of some concern for olive cultivation.

The present research was funded by grant KBBE 219262 ArimNET-ERANET FP7 2012-2015 Project PESTOLIVE 'Contribution of olive history for the management of soilborne parasites in the Mediterranean basin' conducted by the Hellenic Agricultural Organization-DEMETER and Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), grant AGR-136 from 'Consejería de Economía, Innvovación y Ciencia' from Junta de Andalucía, and Union Europea, Fondo Europeo de Desarrollo regional, 'Una manera de hacer Europa'. The authors thank J. Martín Barbarroja and G. León Ropero from IAS-CSIC for the excellent technical assistance.

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Received: 22 January 2019; Accepted: 3 September 2019

ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Φυτοπαρασιτικοί νηματώδεις στη ριζόσφαιρα καλλιεργούμενης ελιάς και αγριελιάς στην Κρήτη

A. Archidona-Yuste, C. Cantalapiedra-Navarrete, J.E. Palomares-Rius, P. Castillo και Ε.Α. Τζωρτζακάκης

Περίληψη Η παρούσα μελέτη αποτελεί μέρος επισκόπησης που πραγματοποιήθηκε με δειγματοληψίες εδάφους σε καλλιεργούμενες ελιές και αγριελιές στην Κρήτη, για την καταγραφή φυτοπαρασιτικών νηματωδών. Δεκαέξι είδη που ανήκουν σε 13 γένη προστίθενται στα 20 είδη ανήκοντα σε 8 γένη, που έχουν ήδη καταγραφεί κατά την επισκόπηση. Επτά είδη, *Filenchus ditissimus*, *Filenchus vulgaris*, *Ogma civellae*, *Pratylenchoides crenicauda*, *Psilenchus hilarulus, Tylenchus elegans* και *Zygotylenchus guevarai*, αναφέρονται για πρώτη φορά στην Ελλάδα.

Hellenic Plant Protection Journal **13:** 24-28, 2020

sciendo

Hellenic Plant Protection Journal **13:** 29-41, 2020
DOI 10.2478/hppj-2020-0004

A qualitative and quantitative comparison of mite fauna between bifenthrin-treated and non-pesticide treated alfalfa hay fields in Central Greece

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Summary The mite fauna in foliage and litter of a sprayed alfalfa hay field with the acaricide-insecticide bifenthrin, was studied based on monthly samplings from foliage and litter in Central Greece between 2008-2009. Potential differentiations between this field and two adjacent alfalfa hay fields, which were not subjected to pesticide applications and were managed with different number of cuttings, were also evaluated in terms of population fluctuation over time, population density, species richness, diversity and spatial distribution. The sprayed field hosted 50 and 68 species and morphospecies in foliage and litter respectively, depicting high relative abundance of oribatid and prostigmatic mites. *Neoseiulus aristotelisi* Papadoulis, Emmanouel and Kapaxidi, was a new record for alfalfa, previously found in rice in Macedonia, Greece. The seasonal fluctuation of mites, particularly in foliage, was similar in all fields. The spatial distribution of a *Zygoribatula* species, which was common and dominant in all fields, was also aggregated. Finally, the sprayed field shared similar mite diversity with the two non-sprayed fields, but not similar species richness.

Additional keywords: alfalfa, bifenthrin, mites, qualitative, quantitative study

Introduction

The ecological role of mites in terrestrial ecosystems is significant, since they can be plant pests, predators of other mites and insects (e.g. phytoseiid mites as thrips predators), decomposers, detritivores, scavengers, and parasites (Sabelis and Van Rijn, 1997; Schneider *et al.*, 2004; Krantz, 2009). Apart from faunistical surveys, a lot of work has been carried out within the framework of studying the impact of agricultural practices, such as the use of slurry in combination with tillage (Bosch-Serra *et al.*, 2014) or post-mining restoration treatments (Andrés and Mateos, 2006) on the population density, species richness and diversity of soil mites. Several studies have also emphasized population parameters of mites of the aerial part of crops. For instance, Wissuwa *et al.* (2012) studied how habitat age and plant species affected mesostigmatic mites in grassy arable fallows in Eastern Austria in terms of population density, species richness and diversity. The spatial distribution of mites has been limitedly studied worldwide, although new research has been added over the last few years. For instance, Alatawi *et al.* (2011) studied how the spatial patterns of *Phytoseiulus persimilis* Athias-Henriot (Phytoseiidae) and its prey, *Tetranychus urticae* Koch (Tetranychidae), affect the biological control of the latter in a greenhouse.

Alfalfa or lucerne is a major crop in Greece cultivated for hay production. According to the most recent published state statistical data (2016), the total cultivated area with alfalfa and other perennial clovers in Greece is about 119,723 ha or 46% of total cultivated area with hay plants in the country producing 1,369,377 t of hay (Hellenic Statistical Authority, 2019). Although alfalfa cuttings help remove arthropod pests hosted in foliage, pyrethroid insecticides, such as bifenthrin, may be used to control lucerne flower gall-midge (*Contarinia medicaginis* Kieffer) (Dip-

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tera: Cecidomyidae) in Greece.

Despite the economic importance of this crop in Greek agriculture, little is known about the mite fauna of alfalfa in Greece. In this respect, Badieritakis *et al.* (2014) contributed with results regarding qualitative and quantitative information over mite assemblages of foliage and litter mites of two considerably similar and non-pesticide treated alfalfa hay fields in Central Greece, which only differed in the number of cuttings. In that study we found that the population fluctuation of mites in the foliage of both fields was similar, unlike that of litter. The population density of mites also significantly differed between the fields except for Prostigmata. Moreover, litter was more species abundant in the less harvested hay field, although the opposite was observed in the foliage of that field when compared to the foliage of the more harvested field. The latter also exhibited higher mite diversity apart from Prostigmata. Finally, the spatial distribution of mites was aggregated in all habitats.

Based on these findings (Badieritakis *et al.*, 2014), the present work has as main objective to compare the mite fauna as well as the relative abundance of mites in the foliage and litter of a bifenthrin-treated alfalfa hay field located in the same area (approximately 100 meters away) with the non-pesticide treated fields as well as the species richness, diversity and spatial distribution of mites in the foliage and litter.

Materials and methods

Sampling sites

This study took place between 2008 and 2010 within the experimental farm of Agricultural University of Athens in Kopais Valley (Central Greece) (38°23′51.68″N, 23°5′23.87″E). The field used for this purpose was about 1,000 m^2 and succeeded a maize crop. This field, thereafter indexed as "C", was approximately 100 m away from those reported as field A and field B by Badieritakis *et al.* (2014), and was subject to the same agricultural practices and sown with the same alfalfa cultivar on the same day with the others. All fields were cut between May and October every year. Both fields, A and C, were harvested once a month on the same day, in comparison to field B which was cut almost bimonthly. In addition, field C was also sprayed with bifethrin 100 g/l EC in August and October 2008 and approximately bimonthly between March and September 2009. This pesticide was applied by local farmers at the application rate of 25 ml/50 l per 1,000 m^2 to control the population of *C. medicaginis*.

Sampling procedure and identification of mites

The sampling procedure and mite identification has been described by Badieritakis *et al.* (2014). In total, 240 foliage samples and 288 litter samples (10 foliage samples and 12 litter samples collected once a month) were randomly collected from each field during the two-year sampling period (2008-2010) with metallic quadrats of 25*40 cm for foliage sampling and 14*16 cm for litter sampling. Mites were extracted with a modified apparatus following the Berlese-Tullgren method in the laboratory and identified to species and morphospecies (e.g. sp1, sp2, etc. or species A, B, etc.) based only on adult mites (Minor and Cianciolo, 2007). The Orders and suborders of main interest under study were Mesostigmata, Sarcoptiformes: Oribatida and Trombidiformes: Prostigmata. Morphospecies were used in cases were identification was difficult due to lack of suitable dichotomous keys or mite species descriptions. Due to unsuitability of the Berlese-Tullgren method for extracting eriophyid and tetranychid mites (Badieritakis *et al.*, 2014), the latter were only recorded in Table 1 without being quantified. The dry weight of samples was also recorded for comparison reasons.

Data analysis

The classification of mite taxa as dominant, influent or recedent was carried out according to specific criteria of dominance (Palyvos

Table 1. Taxa, relative abundance (%) and total counts of adults and juveniles of mites recorded in foliage and litter of a bifenthrin-sprayed alfalfa field in Kopais Valley (Central Greece), between 2008-2010.

TAXA	Relative abundance (%)	
	Foliage	Litter
Order Mesostigmata Canestrini	0.91R	1.85R
Ameroseiidae Evans		
Ameroseius sp.	-	-
Kleemania sp.	0.01R	0.01R
Ascidae Voights & Oudemans		
Arctoseiodes sp.	0.01R	0.04R
Arctoseius sp.	-	-
Asca bicornis Canestrini & Fanzago[1]	-	-
Gamasellodes sp.	0.03R	0.21R
Protogamasellus sp.	0.01R	0.10R
Blattisociidae Garman		
Blattisocius sp.	0.01R	0.01R
Cheiroseius sp.	-	-
Lasioseius sp.	0.18R	0.15R
Digamasellidae Evans		
Dendrolaelaps sp.	-	-
Laelapidae Berlese		
Gymnolaelaps sp.	-	-
Hypoaspis sp.	0.03R	0.30R
Laelaps sp.	-	0.01R
Ololaelaps sp.	-	-
Macrochelidae Vitzthum		
Macrocheles sp.	-	-
Pachylaelapidae Berlese		
Pachylaelaps sp.	-	-
Parasitidae Oudemans		
Paragamasus sp.	-	-
Parasitus sp.	-	0.08R
Phytoseiidae Berlese		
Amblyseius andersoni (Chant)[1]	-	-
Neoseiulus aristotelisi Papadoulis, Emmanouel and Kapaxidi[1]	0.01R	0.01R
Neoseiulus barkeri Hughes	0.27R	0.28R
Neoseiulus bicaudus (Wainstein)[1]	-	-
Proprioseiopsis messor (Wainstein)	-	-
Typhlodromus kerkirae Swirski & Ragusa	-	-
Uropodoidea. not identified to Family		
Uropodoidea (one species)	-	-
Juveniles of Mesostigmata†	0.35	0.65
Order Sarcoptiformes Reuter	74.71D	85.41D
Suborder Endeostigmata Reuter		
Alycidae Canestrini & Fanzago		
Alycidae (one species)	0.01R	0.13R
Nanorchestidae Grandjean		
Nanorchestidae (one species)	0.02R	0.01R
Terpnacaridae Grandjean		
Terpnacaridae (one species)	0.01R	0.01R
Suborder Oribatida van der Hammen	74.67D	85.26D
Acaridae Latreille		
Rhizoglyphus sp.	-	0.05R
Thyreophagus sp.	0.01R	0.02R
Tyrophagus curvipenis Fain & Fauvel	0.01R	0.02R
Tyrophagus longior (Gervais)	0.43R	0.51R
Tyrophagus similis Volgin	0.05R	0.05R
Tyrophagus palmarum Oudemans sensu Robertson	0.05R	0.48R

TAXA	Relative abundance (%)	
	Foliage	Litter
Tyrophagus perniciosus Zakhvatkin	0.08R	0.24R
Tyrophagus putrescentiae (Schrank)	0.03R	0.03R
Brachychthoniidae Thor		
Brachychochthonius sp.	0.01R	-
Chortoglyphidae Berlese		
Chortoglyphus sp.	-	-
Epilohmanniidae Oudemans		
Epilohmannia sp.	-	0.10R
Euphthiracaridae Jacot		
Euphthiracarus sp.	-	0.08R
Oppiidae Grandjean		
Berniniella sp.	-	0.01R
Ramusella sp.	0.03R	0.19R
Oribatellidae Jacot		
Oribatella sp1	-	0.03R
Oribatella sp2	-	-
Oribatulidae Thor		
Zygoribatula sp1	0.84R	5.41I
Zygoribatula sp2	0.13R	1.62R
Zygoribatula sp3	15.75D	18.53D
Zygoribatula sp4	0.95R	1.25R
Scheloribatidae Grandjean		
Scheloribates sp1	-	-
Scheloribates sp2	0.16R	0.66R
Scheloribates sp3	-	-
Scheloribatidae (one species)	-	0.01R
Tectocepheidae Grandjean		
Tectocepheus sp.	0.64R	6.35I
Juveniles of Oribatida†	55.50	49.62
Juveniles of Sarcoptiformes†	55.50	49.62
Order Trombidiformes Reuter	24.38D	12.74D
Suborder Prostigmata Kramer	24.38D	12.74D
Anystidae Oudemans		
Anystidae (one species)	-	-
Bdellidae Dugès		
Bdella sp.	-	-
Bdellodes sp.	-	0.01R
Camerobiidae Southcott		
Neophyllobius sp.	-	-
Cheyletidae Leach		
Cheletogenes sp.	-	-
Hemicheyletia sp.	-	0.03R
Cunaxidae Thor		
Cunaxoides croceus (Koch)[1]	-	-
Cunaxoides paracroceus Sionti & Papadoulis[1]	0.01R	0.01R
Pulaeus subterraneus (Berlese)[1]	-	-
Ereynetidae Oudemans		
Ereynetidae (one species)	-	0.04R
Eriophyidae Nalepa		
Aceria medicaginis (Keifer)*	-	+
Erythraeidae Robineau-Desvoidy		
Abrolophus sp.	0.01R	-
Curteria sp.	-	-
Eupalopsellidae Willmann		
Eupalopsellus sp.	-	-
Eupodidae Koch		
Eupodidae (species A)	0.02R	0.07R
Eupodidae (species B)	-	0.01R
Eupodidae (species C)	-	0.05R

TAXA	Relative abundance (%)	
	Foliage	Litter
Iolinidae André		
Pronematus sp1	-	-
Pronematus sp2	-	-
Pyemotidae Oudemans		
Pyemotes sp.	-	-
Pygmephoridae Cross		
Acinogaster sp.	-	-
Pygmephorus sp1	-	0.05R
Pygmephorus sp2	-	0.02R
Pygmephorus sp3	-	0.02R
Pygmephorus sp4	-	-
Pygmephorus sp5	0.01R	-
Pygmephorus sp6	0.03R	0.14R
Pygmephorus sp7	-	0.02R
Siteroptes sp.	0.05R	0.03R
Rhagidiidae Oudemans		
Rhagidiidae (one species)	-	0.01R
Raphignathidae Kramer		
Raphignathus sp.	-	0.01R
Scutacaridae Oudemans		
Imparipes sp.	-	-
Scutacarus sp.	-	-
Stigmaeidae Oudemans		
Eustigmaeus jiangxiensis Hu. Chen & Huang[1]	-	0.03R
Eustigmaeus sp.	0.01R	-
Stigmaeus sp.	-	-
Tarsonemidae Kramer		
Neotarsonemoides sp.	0.18R	0.84R
Steneotarsonemus konoi Smiley & Emmanouel	0.15R	0.10R
Tarsonemus sp1	2.43R	3.72R
Tarsonemus sp2	1.21R	1.04R
Tarsonemus confusus Ewing	0.32R	0.05R
Tarsonemus fusarii Cooreman	0.03R	0.32R
Tarsonemus lacustris Schaarschmidt	9.65I	1.18R
Tarsonemus talpae Schaarschmidt	0.11R	0.36R
Tarsonemus waitei Banks	2.64R	0.56R
Xenotarsonemus belemnitoides (Weis-Fogh)	1.00R	0.77R
Tenuipalpidae Berlese		
Brevipalpus sp.	0.04R	0.01R
Tetranychidae Donnadieu		
Bryobia praetiosa Koch*	-	-
Bryobia sp.*	-	+
Petrobiini sp.*	-	+
Tetranychini sp.*	-	+
Triophtydeidae André		
Triophtydeus sp.	0.02R	0.01R
Trombiculidae Ewing		
Trombiculidae (one species)	-	-
Trombidiidae Leach		
Trombidiidae (one species)	0.01R	-
Tydeidae Kramer		
Lorryia ferula Baker	0.05R	-
Lorryia nesziyyonensis (Gerson)[1]	-	-
Lorryia sp.	0.13R	0.17R
Tydeus kochi Oudemans	4.86R	1.81R
Juveniles of Prostigmata†	1.41	1.25
Juveniles of Trombidiformes†	1.41	1.25
Total number of samples	240	288
Total number of individuals	10,938	12,325
Total number of species	50	68

TAXA	Relative abundance (%)	
	Foliage	Litter
Number of common species in foliage of fields A and C: 37		
Number of common species in foliage of fields B and C: 41		
Number of common species in litter of fields A and C: 61		
Number of common species in litter of fields B and C: 59		

D: Dominant (> 10%), I: Influent (5%-10%) and R: Recedent (< 5%)
[1]New records for the mite fauna of *Medicago sativa* L. ssp. *sativa* of Greece
† juveniles of mite Orders and suborders are not considered as separate taxa and are not consequently classified as dominant, influent or recedent
*not counted in the calculation of population fluctuation, population density, *Sobs*. Jackknife 1 estimator and Shannon-Weaver index (*H'*) of diversity. Instead of relative abundance (%), results are presented as "+" and "–" for these mites indicating their presence or absence respectively
Field A: Unsprayed alfalfa field managed with monthly cuttings
Field B: Unsprayed alfalfa field managed with bimonthly cuttings
Field C: Bifenthrin-sprayed alfalfa field managed with monthly cuttings

et al., 2008). The non-parametric estimator of species richness, Jackknife 1, was used (Krebs, 1999; Colwell, 2013) as well as Shannon index (*H'*) for the calculation of species diversity of mites (Hutcheson, 1970; Magurran, 2004). The spatial distribution of common and influent mite species was estimated with Taylor's power law and Iwao's regression of patchiness (Badieritakis *et al.*, 2014).

GLM of SAS JMP 7.0.1. statistical package at $\alpha = 0.05$ (after a log (x+1) transformation of the dataset) was used to compare the population density of mites between field C and fields A and B. The population density was expressed as the mean number of individuals per quadrat. The dry weight of foliage and litter samples collected was also compared between field C and the other two fields by using GLM after a log (x) transformation of the data.

Results

Dry weight comparison

The mean dry weight of foliage samples of field C was 7.70 g (± 1.37 g) without being significantly different from the respective dry weight calculated for field A (*d.f.=1*, $\chi^2 = 0.1168$, $p = 0.7325$) and field B (*d.f.=1*, $\chi^2 = 0.0286$, $p = 0.8657$). In addition, the mean dry weight of litter samples in field C was 4.09 g (± 0.40 g) revealing no significant difference with the respective dry weight calculated for field A (*d.f.=1*, $\chi^2 = 0.0369$, $p = 0.8475$) and field B (*d.f.=1*, $\chi^2 = 1.3887$, $p = 0.2490$). Therefore, no significant differences were found regarding the plant biomass of field C compared to that of the other two fields.

Mite fauna and relative abundance

In total, 23,263 individuals of mites (adults and juveniles) were collected from the foliage and litter samples collected from field C between 2008-2010. In particular, 50 and 68 species and morphospecies were respectively recorded from foliage and litter samples belonging to Mesostigmata, Sarcoptiformes and Trombidiformes, many of which were common with those found in fields A and B (Table 1). Many species were also common between field C and fields A and B. A new species record for the mite fauna of alfalfa of Greece was that of the phytoseiid, *Neoseiulus aristotelisi* Papadoulis, Emmanouel and Kapaxidi. Sarcoptiformes and Trombidiformes were the most abundant mite Orders in both foliage and litter samples. Among Sarcoptiformes the family Oribatulidae recorded high relative abundance.

Population fluctuation

Between 2008-2010 the population of total Acari hosted in the foliage of field C (Fig. 1a) presented high density in spring and summer. A similar seasonal pattern of population fluctuation was detected in the case of Oribatida (Fig. 1b). However, both Prostigmata and Mesostigmata had low population densities during the two-year study. In the

case of prostigmatic mites, their population density peaked in April 2009 (Fig. 1b).

In comparison to the findings in foliage samples, the population fluctuation of mites in litter revealed high population densities of mites in summer and autumn in both years (Fig. 1c). The same seasonal pattern was detected in the case of Oribatida, although Prostigmata and Mesostigmata showed lower population densities, almost zero during long time periods (months) (Fig. 1d).

Population density

Taking into account the results presented by Badieritakis *et al.* (2014) and Table 2 of the present study, significant higher population density of mites was detected in the foliage and litter samples of field B, when compared to field C, except for Prostigmata, which were more abundant in field C. On the other hand, the population density of mites was similar in the foliage of fields C and A, except for mesostigmatic mites, whose density was lower in field C. In the case of litter, oribatid and prostigmatic mites recorded higher population density in field C than in field A, although that of total Acari and mesostigmatic mites was similar between these two fields.

Species richness and diversity

Litter hosted more mite species than foliage. Prostigmata were generally more species abundant when compared to other taxa. Taking into account Jackknife 1 estimator and its confidence limits calculated for the mite fauna in all fields, it seems that field C hosted similar number of mite species with field B in foliage and litter, but lower number of species in foliage when compared to field A. However, both fields, A and C, hosted similar number of mite species in litter. On the other hand, field C shared also the same diversity of mites with fields A and B in foliage and litter (Table 3).

Spatial distribution

Only *Zygoribatula* sp3 was dominant in foliage and litter and *Zygoribatula* sp1 was influent in litter of all fields (Table 4). These morphospecies had an aggregated pattern of spatial distribution ($b > 1$). Since parameter a of Iwao's regression of patchiness was not significantly different from zero, we can also assume that the basic component of mite populations could have been one individual per quadrat. Moreover, Taylor's power law had a better fit to the data than Iwao's regression of patchiness (correlation coefficients r).

Discussion

The findings of the present study stressed that the bifethrin-sprayed field hosted many common mite species with the two unsprayed fields. The high relative abundance of oribatid mites in the bifenthrin-sprayed field could be attributed to the previous maize crop. Maize has been reported to host Oribatida in New York by Minor and Cianciolo (2007). *Neoseiulus aristotelisi*, a new phytoseiid species, was first reported in Greece by Papadoulis *et al.* (2009) on *Oryza sativa* (Poaceae) in Macedonia, Greece. However, no other information is available on the preferred habitats of this species.

The population fluctuation of total Acari in foliage of the bifenthrin-sprayed field was similar with that in the unsprayed fields (Badieritakis *et al.*, 2014). Due to their high relative abundance among total Acari in foliage, a similar population fluctuation of oribatid mites was also observed. The similar population fluctuation of Oribatida could be attributed to their high relative abundance among total Acari. Since *Zygoribatula* was dominant in foliage and litter of the bifenthrin-sprayed field, as it happened in the unsprayed, managed with different number of cuttings, fields (Badieritakis *et al.*, 2014), we speculate that the high densities of oribatid mites in summer represented this genus. The population fluctuation of prostigmatic and mesostigmatic mites in the foliage of the bifenthrin-sprayed field was more or less similar with that in the unsprayed fields. Hence, the findings demonstrate that the

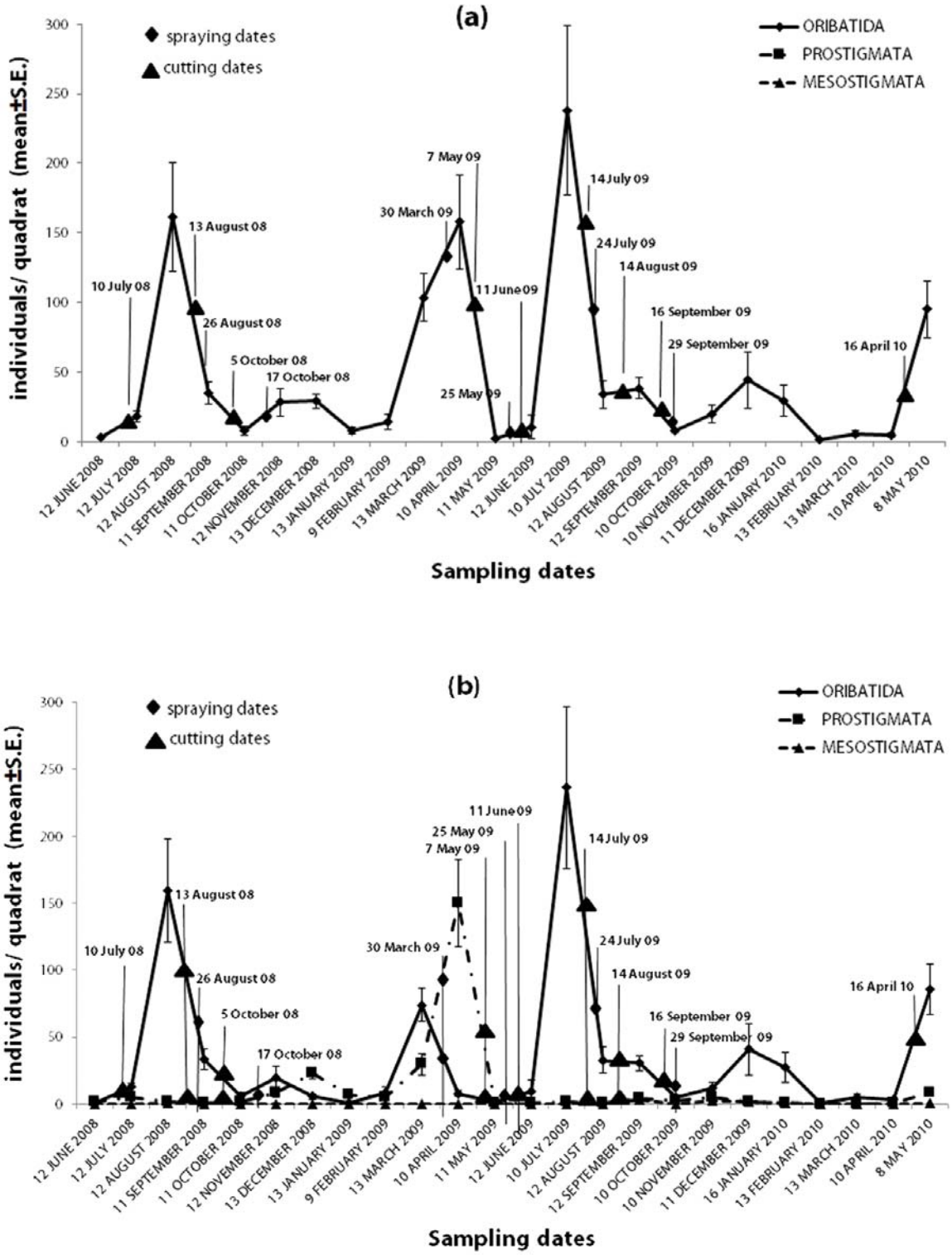

Figure 1. (a) Population fluctuation of total Acari and **(b)** main mite taxa, in foliage of a bifenthrin-sprayed alfalfa field in Kopais Valley (Central Greece) between 2008-2010.

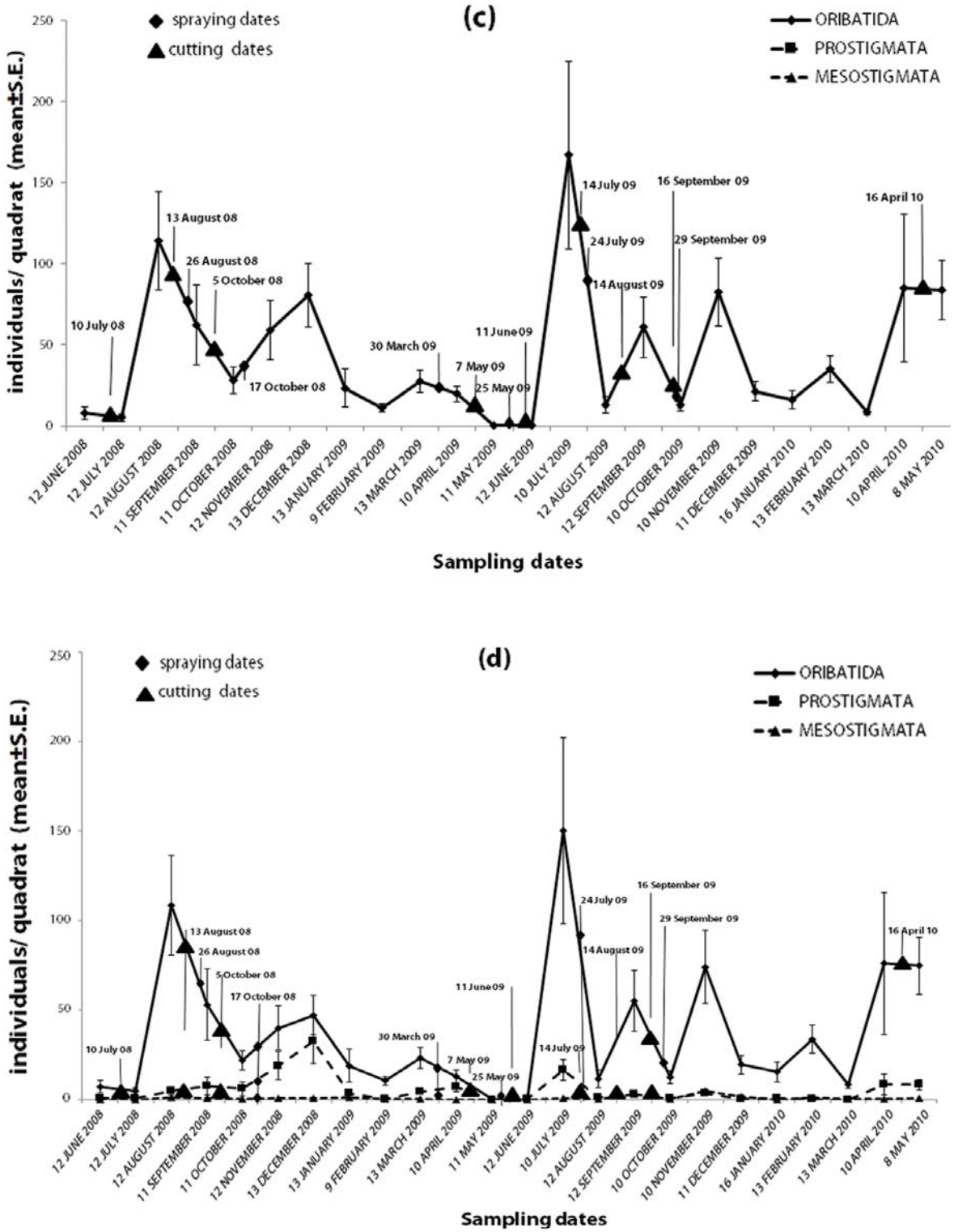

Figure 1. (c) Population fluctuation of total Acari (a) and **(d)** main mite taxa, in litter of a bifenthrin-sprayed alfalfa field in Kopais Valley (Central Greece) between 2008-2010.

Table 2. Population density (mean ± S.E.) of total Acari, order Mesostigmata, suborder Oribatida (Acari: Sarcoptiformes) and suborder Prostigmata (Acari: Trombidiformes) found in the foliage and litter of alfalfa fields receiving different management between 2008-2010 in Kopais Valley, Central Greece (GLM, $\alpha = 0.05$) for the hypothesis of similar population density .

Habitat	Taxa	Field C	Field A	Field B
Foliage	Total Acari	45.58 ± 5.28	χ^2= 0.1889, p= 0.6638	χ^2= 14.1997, p= 0.0002*
	Mesostigmata	0.4125 ± 0.0824	χ^2= 4.3212, p= 0.0376*	χ^2= 13.7020, p= 0.0002*
	Oribatida	34.18 ± 4.79	χ^2= 0.3834, p= 0.5358	χ^2= 31.1286, p<0.0001*
	Prostigmata	10.97 ± 2.36	χ^2= 0.2176, p= 0.6408	χ^2= 7.9220, p= 0.0049*
Litter	Total Acari	42.79 ± 4.55	χ^2= 3.2959, p= 0.0695	χ^2= 34.7409, p<0.0001*
	Mesostigmata	0.77 ± 0.12	χ^2= 1.5526, p= 0.2128	χ^2= 21.7506, p<0.0001*
	Oribatida	36.57 ± 3.99	χ^2= 8.6078, p= 0.0033*	χ^2= 40.0851, p<0.0001*
	Prostigmata	5.39 ± 0.88	χ^2= 4.0045, p= 0.0454*	χ^2= 0.5156, p= 0.4727

* significant difference
Field A: Unsprayed alfalfa field managed with monthly cuttings
Field B: Unsprayed alfalfa field managed with bimonthly cuttings
Field C: Bifenthrin-sprayed alfalfa field managed with monthly cuttings

Table 3. Number of species observed (*Sobs*), estimation of species richness (Jackknife 1) and diversity (Shannon-Weaver index, *H'*) of total Acari, order Mesostigmata, suborder Oribatida (Acari: Sarcoptiformes) and suborder Prostigmata (Acari: Trombidiformes) in foliage and litter of alfalfa fields receiving different management in Kopais Valley, Central Greece, between 2008-2010 for the hypothesis of similar species richness and diversity ($\alpha = 0.05$).

Habitat	Taxa	*Sobs*	Jackknife 1 estimator (95 % CL)[2]	Shannon - Wiener index (*H'*)		
				Field C	Field A[3]	Field B[3]
Foliage	Total Acari[1]	50	67.93 (60 – 76)	2.06	d.f.=4676, t=0.00286	d.f.=4676, t=0.3171
	Mesostigmata	9	13.98 (10 – 18)	0.79	d.f.=62, t=0.1907	d.f.=62, t=0.2515
	Oribatida	15	18.98 (15 – 23)	1.74	d.f.=2133, t=0.01735	d.f.=2132, t=0.0024
	Prostigmata[1]	23	28.98 (24 – 34)	1.35	d.f.=2481, t=0.0889	d.f.=2482, t=0.1497
Litter	Total Acari[1]	64	86.93 (77 – 97)	2.35	d.f.=5980, t=0.0927	d.f.=5980, t=0.2809
	Mesostigmata	11	20.98 (16 – 26)	1.50	d.f.=145, t=0.0977	d.f.=145, t=0.2041
	Oribatida	20	22.99 (20 – 26)	2.21	d.f.=4419, t=0.1332	d.f.=4419, t=0.1581
	Prostigmata[1]	30	39.97 (34 – 46)	2.00	d.f.=1401, t=0.2135	d.f.=1401, t=0.2566

[1] species belonging to Eriophyidae and Tetranychidae are not included
[2] confidence limits are rounded
[3] degrees of freedom (d.f.) and t calculated according to Hutcheson's method for the comparison of field C with fields A and B
Field A: Unsprayed alfalfa field managed with monthly cuttings
Field B: Unsprayed alfalfa field managed with bimonthly cuttings
Field C: Bifenthrin-sprayed alfalfa field managed with monthly cuttings

seasonal fluctuation of mites in foliage is not affected by agricultural practices, such as pesticide application and different number of cuttings. However, this was not the case for mites found in litter; it seems that no seasonal pattern of population fluctuation of mites in litter can be designated for alfalfa. The population densities of prostigmatic and mesostigmatic mites in litter and foliage were very low, almost zero, for many months. This could be possibly attributed to the intensive management in the sprayed field (cuttings and pesticide applications), which did not help the populations of these taxa to restore.

Mite population density in the bifen-

Table 4. Parameters of Taylor's power law and Iwao's patchiness regression of mite species in foliage and litter, which were common and concurrently dominant or influent in alfalfa fields receiving different management during 2008 - 2010 in Kopais Valley (Central Greece) for the hypothesis of aggregated pattern of spatial distribution.

Habitat	Mites	n[1]	Taylor's power law			Iwao's patchiness regression		
			log (a)[2]	b[3]	r[4]	a[5]	b[6]	r[7]
Foliage	*Zygoribatula* sp3 (dominant)	22	0.42 ± 0.07*	1.77 ± 0.08*	0.98*	5.32 ± 4.35	1.78 ± 0.26*	0.84*
Litter	*Zygoribatula* sp1 (influent)	21	0.52 ± 0.04*	1.59 ± 0.08*	0.98*	1.02 ± 0.77	2.22 ± 0.22*	0.91*
	Zygoribatula sp3 (dominant)	23	0.43 ± 0.08*	1.66 ± 0.09*	0.97*	0.59 ± 1.56	2.09 ± 0.14*	0.95*

[1] Number of mean - variance and mean - mean crowding pairs used in the regressions
[2,3,4] Parameters of Taylor's power law. Parameters log (a) and b (± S.E.) and correlation coefficient r
[5,6,7] Parameters of Iwao's patchiness regression. Parameters a and b (± S.E.) and correlation coefficient r
* significant difference of parameters log (a), a and r from 0 and parameter b from 1 in both models ($\alpha = 0.05$, t - test) at n - 2 degrees of freedom
Different management: bifenthrin-sprayed + monthly cuttings (current study); unsprayed + monthly cuttings; unsprayed + bimonthly cuttings

thrin-sprayed field was lower compared to the unsprayed field with half number of cuttings, except for the density of prostigmatic mites in litter and foliage, which were lower in the unsprayed field. By contrast, the mite population density did not differ in the case of the bifenthrin-sprayed and the unsprayed field managed with the same number of cuttings, except for mesostigmatic mites in foliage and oribatid and prostigmatic mites in litter. Agricultural practices, such as the application of pesticides can affect the mite communities of soil, particularly Mesostigmata whose density can be significantly reduced in conventional fields in comparison to uncultivated sites (Bedano and Ruf, 2007). In addition, many predatory arthropods tend to find refugia in soil or litter of grasslands after the application of pesticides (Roberts *et al*., 2011), which could explain the lower population density of mesostigmatic mites in the foliage of the bifenthrin-sprayed field. In the case of oribatid mites the use of agrochemicals and intensive agricultural practices may reduce the organic matter of soil leading to lower population densities which cannot easily recover in the short term (Bedano *et al*., 2006). On the other hand, Clapperton *et al.* (2002) concluded that prostigmatic mites are abundant in disturbed sites (heavily grazed prairies) in comparison to other mite taxa of soil.

In terms of species richness, the bifenthrin-sprayed field was poor in species in foliage when compared to the unsprayed field with the same number of cuttings (Badieritakis *et al*., 2014). This result is in accordance with the findings of Koehler (1999), that species richness of mites is negatively affected by agricultural practices.

The diversity of mites was similar between the bifenthrin-sprayed field and the unsprayed fields. However, the diversity of mites was generally higher in the field with monthly cuttings than that in field with bimonthly cuttings (Badieritakis *et al*., 2014). The diversity of mesostigmatic mites is reduced in conventional fields when compared to sites which are not disturbed (Bedano and Ruf, 2007). On the other hand, the diversity of oribatid mites is usually negatively affected by the intensity of agricultural practices (Minor and Cianciolo, 2007). In the case of the bifenthrin-sprayed field, the increased disturbance seems not to have affected the diversity of prostigmatic and oribatid mites, possibly due to an increase of relative abundance of mite species that balanced the decrease in species richness.

An aggregated pattern of spatial distribution of common and dominant or influent mites (*Zygoribatula* sp1 and *Zygoribatula*

sp3) was confirmed ($b > 1$ in Taylor's power law and Iwao's regression of patchiness). In addition, Taylor's power law fitted better to the data when compared to Iwao's regression of patchiness. We also assume that *Zygoribatula* sp3 did not form colonies (parameter *a* of Iwao's regression of patchiness not significantly different from zero) as it happened in many cases of the unsprayed fields managed with different number of cuttings (Badieritakis *et al.*, 2014).

Our results show that an occasionally sprayed with an acaricide-insecticide alfalfa hay field hosted a rich mite fauna similar to that of two adjacent unsprayed alfalfa hay fields. The seasonal fluctuation of mites was also similar in all fields, although similarity in species richness mainly occurred between the sprayed field and the unsprayed field with half number of cuttings. Some differentiations in population density of mites also occurred between the sprayed field and the unsprayed ones, possibly due to the extra disturbance in the sprayed field. The spatial distribution of mites was aggregated in all fields. Our results indicate that spraying alfalfa with a pesticide slightly affects the mite populations in foliage and litter. We have to stress, however, that more replicates of fields are needed to be sure about the impact of the spraying with pesticides on mite communities.

The authors would like to express their gratitude towards the Farm Unit of the Agricultural University of Athens for their help with the experiments in the Kopais region as well as towards the Laboratory of Plant Breeding and Biometry of the same University for providing SAS JMP 7.0.1. statistical package. The authors are also grateful to Professor George Th. Papadoulis for his help with the identification of Cunaxidae and Phytoseiidae and to Dr Eleftheria V. Kapaxidi for identifying E. jiangxiensis. This study was funded by the State Scholarships Foundation of Greece.

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Received: 18 March 2019; Accepted: 3 September 2019

Ποιοτική και ποσοτική σύγκριση της ακαρεοπανίδας σε μηδικεώνες με ή χωρίς επέμβαση με bifenthrin

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Περίληψη Στην παρούσα εργασία μελετήθηκε η ακαρεοπανίδα της βλάστησης και των φυτικών υπολειμμάτων ενός περιστασιακά ψεκαζόμενου μηδικεώνα με το ακαρεοκτόνο/εντομοκτόνο bifenthrin στην Κωπαΐδα Βοιωτίας, κατά τη διετία 2008-2010, μέσω μηνιαίων δειγματοληψιών. Ο συγκεκριμένος μηδικεώνας συγκρίθηκε με άλλους δύο γειτνιάζοντες, παρόμοιους ως προς τις καλλιεργητικές πρακτικές μηδικεώνες, στους οποίους δεν διενεργήθηκαν ψεκασμοί και πραγματοποιήθηκε διαφορετικός αριθμός κοπών, με σκοπό να διερευνηθούν τυχόν διαφοροποιήσεις μεταξύ τους ως προς την πληθυσμιακή διακύμανση μέσα στο χρόνο, την πληθυσμιακή πυκνότητα, την αφθονία των ειδών, τη βιοποικιλότητα, αλλά και τη χωροδιάταξη σε ό,τι αφορά τα ακάρεα. Στον ψεκαζόμενο μηδικεώνα καταγράφηκαν 50 και 68 είδη και μορφοείδη ακάρεων, στη βλάστηση και τα φυτικά υπολείμματα αντίστοιχα, με τις υποτάξεις Prostigmata και Oribatida να εμφανίζουν υψηλή πληθυσμιακή πυκνότητα. Το *Neoseiulus aristotelisi* Papadoulis, Emmanouel and Kapaxidi (Mesostigmata: Phytoseiidae), που είχε παλαιότερα αναφερθεί για πρώτη φορά σε ρύζι στην Πιερία, αποτελεί πρώτη καταγραφή για την ακαρεοπανίδα της μηδικής. Επίσης, η πληθυσμιακή διακύμανση των ακάρεων της βλάστησης σε όλους τους μηδικεώνες εμφανίστηκε να είναι παρόμοια, όπως ομαδοποιημένη βρέθηκε να είναι και η χωροδιάταξη ενός μη αναγνωρισμένου είδους *Zygoribatula* και στους τρεις μηδικεώνες, στους οποίους καταγράφηκε ως κυρίαρχο. Ο ψεκαζόμενος μηδικεώνας δεν φιλοξένησε παρόμοιο αριθμό ειδών ακάρεων σε σχέση με τους άλλους δύο μηδικεώνες, ενώ εμφάνισε με αυτούς παρόμοια βιοποικιλότητα.

Hellenic Plant Protection Journal **13:** 29-41, 2020

sciendo

Hellenic Plant Protection Journal **13:** 42-53, 2020
DOI 10.2478/hppj-2020-0005

Efficacy of communication disruption of *Thaumetopoea pityocampa* (Lepidoptera: Thaumetopoeidae) with low pheromone formulation

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Summary Mating disruption (MD) has been a successful approach for pest control of several lepidoptera. Field trials to evaluate the efficacy of communication disruption of low pheromone load formulation on *Thaumetopoea pityocampa* were carried out in 2010 and 2011 in an urban park. The efficacy of MD was assessed by comparing male *T. pityocampa* catches in pheromone traps, between MD and Control areas. In the 1st year of the application the percentage of male inhibition ranged from 85 to 100% during the 1st month of the flight period and 95-100% during the whole flight period in the 2nd year. The pheromone remained in the polymeric matrix was almost 30% after 7 weeks under laboratory aging conditions. Combining the pheromone release results with the male disorientation results we can assume that after 7 weeks the remaining pheromone concentration was still sufficient to achieve MD. This study indicates that air permeation with the major sex pheromone component (*Z*)-13-hexadecen-11-ynyl acetate, at a rate of 20 g/ha for one application per season, can affect the orientation of *T. pityocampa* males. Since mating disruption is an environmentally safe method for pest control, it could be a valuable tool to control *T. pityocampa* in urban areas and parks.

Additional Keywords: mating disruption, pheromone formulation, pine processionary moth, release rate, wax matrix.

Introduction

The pine processionary moth (PPM), *Thaumetopoea pityocampa* (Denis and Schiffermüller) (Lepidoptera: Thaumetopoeidae), is an endemic insect of pine and cedar trees, existing both in rural and urban areas causing economic damage throughout temperate regions of the Mediterranean, including southwestern Europe, the Balkan Peninsula and North Africa (Huchon and Démolin, 1971; Battisti 1988; Battisti *et al.*, 2005; Simonato *et al.*, 2007; Yilmaz *et al.*, 2013; Kerdelhué *et al.*, 2014; Battisti *et al.*, 2015; Castagneyrol *et al.*, 2016). This lepidopteran pest causes significant economic losses. It impairs the vitality of pine forests due to defoliation, and growth retardation of pine trees and also augments their susceptibility to secondary pests in temperate regions (Devkota and Schmidt, 1990; Masutti and Battisti, 1990; Hódar *et al.*, 2002; Carus 2004; Erkan 2011; Jacquet *et al.*, 2012).

PPM larvae defoliate pine trees by feeding gregariously, from autumn to spring (Semiz *et al.*, 2006; Yilmaz *et al.*, 2013). They complete their life cycle annually, but in unfavorable conditions they may remain in pupal stage in the soil for several years (Salman *et al.*, 2019). With their urticating hairs PPM larvae induce a variety of allergic reactions, such as skin irritation, conjunctivitis, respiratory congestions and asthma in humans and animals (Battisti *et al.*, 2011; Vega *et al.*, 2011). Outbreaks of the species in areas previously unaffected by the insect can be favored by the presence of *Pinus* hosts (Stastny *et al.*,

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2006) or as a result of climate change (Battisti *et al*., 2005, 2006, 2017).

In recent years there is an increasing interest in the prospect of exploiting environmentally safe strategies to control pine pests. This also applies for the PPM since the use of chemical pesticides for the control of this pest is not permitted in urban and suburban areas and parks. Although research has provided solutions for many agricultural problems, control of forest pest insects via integrated pest management tactics has developed at a slower pace (Corley and Jervis, 2012). Biological pest management practices have been used as alternatives to insecticides against *Thaumetopoea* sp. to reduce their damage on forestlands (Rausell *et al*., 1999; Kanat and Ozbolat, 2006; Semiz *et al*., 2006; Barbaro and Battisti, 2011; Yilmaz *et al*., 2013). Various methods such as destruction of winter nests and treatment with *Bacillus thuringiensis* have been tested in many studies for reducing the population of PPM larvae (Cebeci *et al*., 2010; Yilmaz *et al*., 2013).

Mating disruption (MD) has been a successful approach for pest control over the past few decades, and is now an accepted control option for a number of lepidopteran pests of fruits and vegetables (Cardé and Minks, 1995; Byers, 2007; Witzgall *et al*., 2010; Miller and Gut, 2015). To make MD successful as an insect management technique, synthetic pheromone needs to be present in the air in sufficient quantities over the course of the insects' mating period. As a general guide, application rates ranging between 10 g to 100 g per ha per season are required to achieve communication disruption, equivalent to, at least, 1 ng/m^3 aerial concentration (Bengtsson *et al*., 1994; Cork *et al*., 2008). Furhermore, the matrix that holds and releases the pheromones plays a significant role in the success of the mating disruption systems (Wilkins *et al*., 1984; Zdarek *et al*., 1988; Chamberlain *et al*., 2000; Zada *et al*., 2009).

There are several successful commercial formulations made of polyethylene tubes, cotton rolls, ropes, or bags baited with pheromones (Brown *et al*., 1992; Suckling 2000; Johansson *et al*., 2001; Hegazi *et al*., 2007; 2009). However, problems may arise when the matrix does not preserve the pheromone and allows either too high a release that exhausts the dispenser too soon, or too low a release that is less than optimal, primarily depending on the volatility of the components. Waxy blobs and similar materials are increasingly being used to dispense pheromone from many sources in mating disruption (Atterholt *et al*., 1998; Stelinski *et al*., 2005, 2007a,b; deLame *et al*., 2007).

The major sex pheromone component (*Z*)-13-hexadecen-11-ynyl acetate produced by the female *T. pityocampa* was first identified by Guerrero *et al.* (1981). Since its identification, research has mainly focused on the synthesis of the sex pheromone in sufficient quantities for the monitoring of PPM populations and the development of a mass-trapping method for direct control (Cuevas *et al*., 1983; Halperin, 1984, 1985, 1986; Tiberi and Niccoli, 1984; Roversi, 1985; Nicolini ,1987; Fabrias *et al*., 1989; Arsequell *et al*., 1990; Camps *et al*., 1990a,b; Devkota *et al*., 1992; Zhang and Paiva, 1998; Jactel *et al*., 2006; Battisti *et al*., 2015; Athanassiou *et al*., 2007, 2017).

Effective pheromone mating disruption formulations based on synthetic polymers have been developed for many species (*Grapholita molesta, Lymantria dispar, Paralobesia viteana* etc*)* and are commercially available. However to the best of our knowledge no such formulation with (*Z*)-13-hexadecen-11-ynyl acetate as active ingredient is commercially readily available.

Our study reports results of using a polymer formulation with the PPM sex pheromone as active ingredient applied consecutively for 2 years in "Attiko Alsos", a major hill-park in the Attica district. This park is a focal point within the city network with outdoors caffes, promenade walkways, sports courts etc. The treated area suffered severe PPM infestation raising concerns to visitors and the authorities. An effective treatment with minimal ecological footprint which would respect the non-target entomofauna could serve as a model for many parks

in the Mediterranean basin facing similar problems.

Materials and methods

Pheromone formulation

The sex pheromone formulations used for monitoring and MD of *T. pityocampa* were provided by Novagrica Hellas SA (Athens, Greece). The MD formulation was a wax matrix plus pheromone [(Z)13-hexadecen-11-ynyl acetate, 95% pure by GC], similar to that reported by Atterholt et al. (1999). It contained 2 % (w/w) of the PPM pheromone.

Experimental fields

MD trials were conducted for two consecutive seasons from 2010 to 2011 in "Attiko Alsos" (23 hectares with trees, mostly pines). It is an urban, green and recreational area of significant ecological value for Athens (E23°45'37.37"; N38° 0'16.08"). The pines in that area are mainly *Pinus brutia* Ten. and to a smaller extent *Pinus halepensis* Mill., representing a low elevation Mediterranean forest.

Three experimental plots (0.7 ha each) were selected, one plot for control (CO) and two plots for MD trials. The CO plot was separated by 250m from the MD plots and the distance between the MD plots were approx. 30m. Baker *et al.* (2009) report that males of the sibling species *Thaumatopoea processionea* L. fly long distances whereas gravid females fly much shorter ones. Gravid PPM females are also expected to be weaker fliers than males. In addition, time limitation of short-lived females to lay a single batch of eggs is a factor suggesting limited dispersion of females in an area where their natural host is abundant (Stansthy *et al.,* 2006). Plots were more or less elongate and on a declivity of the park to avoid trap disturbance by passing-byers. MD plots were clearly demarcated (secondary traffic road and barren land strips). Trees of the experimental plots were of medium size (reaching 4m in height). Tree density was approximately 345 trees/ha.

A data-logging unit **(**EBI 20-TH, ebro Electronic GmbH & Co. KG, Ingolstadt Germany) was placed in area of study to measure temperature and air relative humidity during the period of MD implementation. Data logger was interfaced with a computer to record air temperature and air humidity at 1 hour intervals.

Pheromone application

In 2010 on the first week of August and in 2011 on the fourth week of August MD treatments took place in the experimental plots to control the PPM. Waxy polymer formulation containing 2 % (w/w) of the PPM pheromone was applied manually on the trunks of the pines at head height using caulking guns. On each application point a small blob, approx. 2 g was placed. The point sources were placed at the base of branches for protection against the sun. In our effort to have as much as possible homogenous spread of the polymeric matrix effort was made to apply at least one blob of the waxy formulation on every tree depending on tree density and canopy volume. The amount of pheromone applied was 20 g/ha.

Assessment of MD efficacy

The efficacy of the MD was assessed by comparing: i) male *T. pityocampa* catches in pheromone-baited traps and ii) egg density and hatchability in control and pheromone-treated plots and iii) monitoring the pheromone release rate at different temperatures under laboratory conditions.

Trap catches

In the MD and CO plots four Delta traps per plot (12 traps in total) (Novagrica Hellas S.A, Athens, Greece) were hung on the external south part of tree canopy at head height and not in the immediate proximity of any of the pheromone releasing waxy blobs.

Each trap was baited with gray septa (Novagrica Hellas S.A, Athens, Greece) loaded with 1 mg of (*Z*)-13-hexadecen-11-ynyl-acetate. The traps were serviced once per week. Moth inhibition due to pheromone

treatment was assessed weekly by comparing the number of *T. pityocampa* captured in the Delta traps in MD and CO plots and was used to calculate the percentage of male inhibition according to the following formula: {[CO – MD)/CO]x100} representing the average male catches in untreated and treated plots respectively.

Egg density

The effect of mating disruption on oviposition of *T. pityocampa* was assessed by recording egg densities on pine needles. Egg masses were collected randomly in MD and CO plots five times during the experimental period (approx. every two weeks). Each time five sampling points were randomly selected in the MD or CO plots, but not at the border of the plot. On each sampling point, two neighboring trees were sampled and from these pines, 2 shoots (ca. 30 cm long) bearing egg masses were removed. In the laboratory, the number of unhatched eggs/sample was recorded. Collected eggs were kept in a climatic chamber (25 ±1 °C; 70% RH; 16:8 h L:D) until they either hatched, or were considered non-fertilized.

Pheromone release rate

Due to practical difficulties of collecting blobs samples (waxy polymer formulation) from the trees trunks, the determination of the release rate of the major sex component (*Z*)-13-hexadecen-11-ynyl acetate was carried out under laboratory conditions in two temperatures. Two (2) g of the wax polymer were weighed in small glass petri dishes and placed in constant temperature rooms at 20 and 28 °C. Three samples thereof were analyzed per sampling date and temperature as follows: prior to incubation, at 1, 2, 3, 4, and 7 days following incubation and then weekly for a total of 7 weeks. Samples were placed in a 20-mL vial (Machery-Nagel, Düren, Germany). Five mL of an internal standard solution of 1 mg/mL of methyl hexanoate in acetone (99.9% purity; Acros Organics, Geel, Belgium) was added to each vial. The samples were placed at -18 °C until analyses. Prior to analysis, samples were placed in a water bath at 65-68 °C for 3 min. Then they were vortexed for 1 min and placed again in the water bath for 2 more min. Samples were filtered through a polytetrafluoroethylene filter (PTFE) with a 0.45 μm pore size (Machery-Nagel, Düren, Germany).

A Thermo Scientific TRACE 1300 Series GC chromatograph (Milan, Italy) equipped with a flame ionization detector (FID) and a TG-1 ms capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness) with helium as carrier gas at 1 mL /min was used for the analysis of samples. Column temperature was initially kept for 2 min at 50 °C, then gradually increased to 240 °C with a rate of 2 °C/min, and held for 10 min. The injector and detector temperatures were set to 220 and 250 °C, respectively. One microlitre of each sample was injected manually in splitless mode. For quantification of pheromone content, the internal standard method was used and was normalized for the original weight of each sample.

Data analysis

The field data were subjected to analysis of variance (ANOVA) (SAS Institute, 2000). Data are presented as means of male catches per trap and mean number of eggs, and egg hatchability. Means were normalized using the log(x+ 0.5) transformation. The data for eggs density and status were tested by using the tailed t-test at n-2 and P=0.01 (Snedecor and Cochran, 1989).

Results

Trap catches

During the 1st year of the study (2010), the average air temperature of the area was 29.6, 23.5 and 17.6°C and the average rainfall was 0.0, 37.0, and 116.6 mm in August, September and October respectively. In 2011 the average air temperature of the area was 27.6, 25.4 and 16.4°C and the average rainfall was 0.0, 0.0, and 37.2 mm in August, September and October respectively.

Mean weekly catches per pheromone trap for treated (MD) and untreated (CO)

plots are shown in Fig. 1A for the 2010 trials. Capture data from the untreated plot indicated that the flight peak occurred on 17 September (40.25±5.8 males/trap/week). Of total male catches (20 August to 25 October), 65.6% were captured during September. In 2010, MD was applied on August 6th. Total trapped males were significantly lower (F=7.361, df=11, P=0.000) in MD plots (114.6±29.3 males/trap) than in CO (325 ± 49.7 males/trap) during the MD period. The percentage of male inhibition ranged from 85% to100% during the 1st month of the flight period (Fig. 1B).

In 2011 (Fig 2A), catches of males in the untreated plot indicated that flight peak occurred on 23 September (56±0.5 males/trap). Capture of moths in the MD plots was significantly low compared to CO traps (F=47.053, df=11, P=0.000). Trap catches on the CO plot started on 29 August. Of the total trapped males (29 August to 9 November), 77% were captured in September. Pheromone application was done on August 22nd. Trap captures in MD plots were significantly lower than in CO plot. Mean capture rates in MD and CO plots differed significantly each year. In 2011, the population density of *T. pityocampa* moths was generally, higher than in the previous year. The percentage of male inhibition ranged from 95-100% during the whole flight period (Fig. 2B).

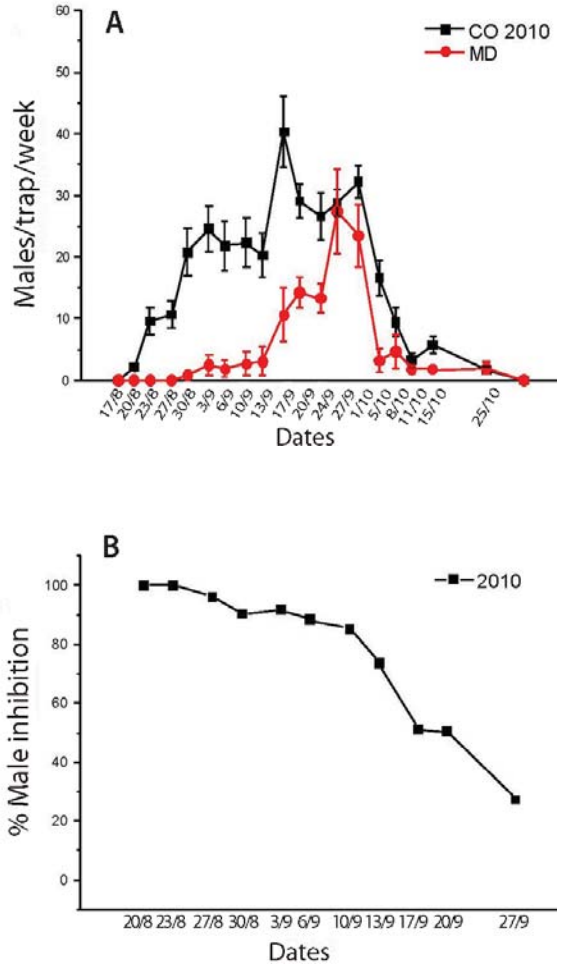

Figure 1. Captures of males (A) and male inhibition (B) of *Thaumetopoea pityocampa* in pheromone traps installed in plots treated with pheromone (MD) *vs* untreated-control (CO) plots at "Attiko Alsos", Attiki, Greece in 2010.

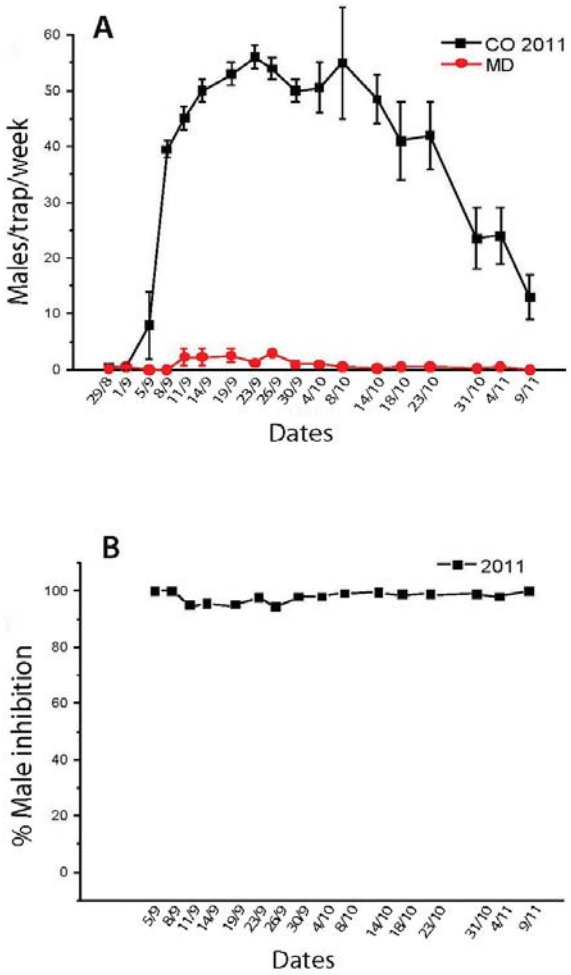

Figure 2. Captures of males (A) and male inhibition (B) of *Thaumetopoea pityocampa* males in pheromone traps installed in plots treated with pheromone (MD) *vs* untreated-control (CO) plots at "Attiko Alsos", Attiki, Greece, in 2011.

Egg density

In 2010 (Table 1) the number of *T. pityocampa* eggs/egg mass was significantly lower (t=14.370; df=49; P=0.000) in MD (91.24 ± 4.59 eggs/egg mass) compared to CO (172.24 ± 5.67 eggs). In 2011 (Table 1) number of *T. pityocampa* eggs was significantly lower (t = 12.610; df=49; P=0.000) in MD (76.44 ± 3.0 eggs) compared to CO (154.56 ± 3.62 eggs).

In 2010 hatchability of *T. pityocampa* eggs (Table 1, Fig. 3) was significantly lower (t= 9.023; df=49; P=0.000) in MD (35.28 ± 3.58 % eggs/egg mass) compared with CO (75.8 ± 1.49 %). In 2011 hatchability of *T. pityocampa* eggs (Table 1, Fig. 4) was also significantly lower (t = 16.851; df=49; P=0.000) in MD (20.34 ± 2.71 %) compared with CO (81.64 ± 1.56 %).

Pheromone release rate

The release rates of the sex pheromone component (*Z*)-13-hexadecen-11-ynyl acetate at the two examined temperatures are shown in Fig. 5 and fit a logarithmic decay curve. After 7 weeks of incubation almost 30% of the pheromone remained in the polymeric matrix (Fig. 5A). During the first week, the release rate was 1 mg per day at both temperatures. In the next week, the rate was slightly decreased at 0.63 and 0.7mg per day at 20 and 28°C, respectively. Over the next weeks the average rate was 95μg per day (Fig.5B). As indicated by male disorientation results, after 7 weeks the remaining pheromone concentration was still sufficient to achieve mating disruption.

Discussion

The results indicated that air permeation with the major sex pheromone component (*Z*)-13-hexadecen-11-ynyl acetate, with 20 g/ha for one application per season, can affect

Table 1. *Thaumetopoea pityocampa* egg density (No ± SE) and hatchability from untreated (CO) and treated plots (MD) at "Attiko Alsos", Attiki, Greece, in 2010 and 2011.

Year	No of eggs/egg mass		% of hatched eggs/egg mass	
	CO	MD	CO	MD
2010	172.24 ±5.67	91.24 ±4.59	75.8 ±1.49	35.28 ±3.58
2011	154.56± 3.62	76.44 ±3.0	81.64 ±1.56	20.34 ±2.71

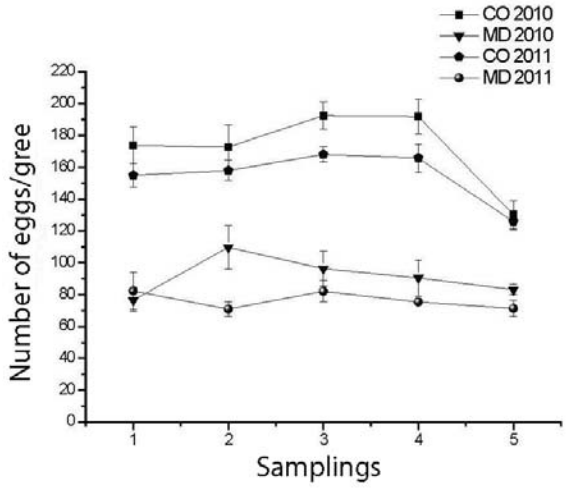

Figure 3. Egg density of *Thaumetopoea pityocampa* from samplings in untreated (CO) and treated plots (MD) at "Attiko Alsos", Attiki, Greece, in 2010 and 2011.

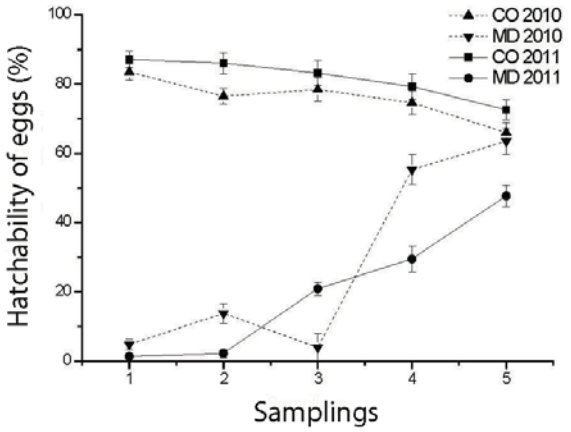

Figure 4. Hatchability of *Thaumetopoea pityocampa* from samplings in untreated (CO) and treated plots (MD) at "Attiko Alsos", Attiki, Greece, in 2010 and 2011.

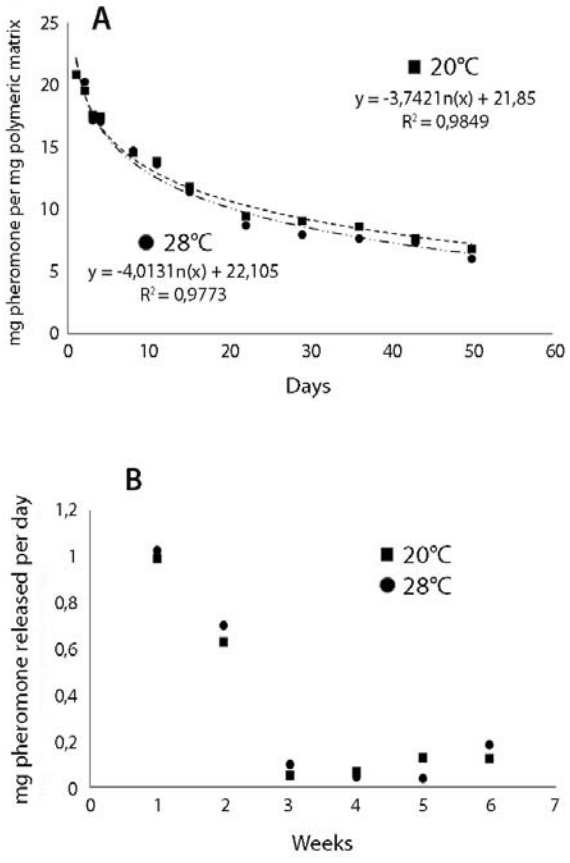

Figure 5. Release pattern of the major sex pheromone component (*Z*)-13-hexadecen-11-ynyl acetate at 20 and 28 °C (A) and the corresponding weekly loss (B).

the orientation of *T. pityocampa* males to female pheromone sources.

Successful mating disruption by a single pheromone component has also been reported for several Lepidoptera such as *Platyptilia carduidactyla* (Klun *et al.,* 1981), *Prays oleae* (Mazomenos *et al*., 1999; Hegazi *et al*., 2009) and *Phyllocnistis citrella* (Lapointe and Stelinski, 2011).

Captures of males in pheromone-baited traps were significantly lower in all pheromone-treated (MD) plots in comparison with the control (CO) during the study years. In 2010, polymer formulation of the pheromone was applied much earlier than the beginning of the flight. Thus, male disorientation was partially achieved ranging 85-100% during the first 4 flight weeks and then was reduced. For this reason, during the next trials in 2011 the MD was applied only a few days before the beginning of the flight, as has been verified by monitoring traps already set in place. Thus male disorientation was successfully achieved ranging 95-100% during the whole flight period. In 2011, the inhibition of successful orientation was almost complete even at the peak of the flight period of the moth. This observation is consistent with other studies on mating disruption, where the technique is most effective when applied in the correct time (Moffitt and Westigard, 1984; Borchert and Walgenbach, 2000; Hegazi *et al*., 2007; 2009; Witzgall *et al*., 2010).

Although during the second year of the application the population size of *T. pityocampa* was higher than in the first one, the number of *T. pityocampa* eggs collected from treated plots was significantly lower than those found in the control plot. The results are in agreement with the findings of Mazomenos *et al.* (1999), who reported that, in mating disruption trials, the reduction of moth populations is a gradual process and requires 2–3 years of continuous pheromone application to achieve control measures close to economically acceptable levels.

The shutdown of the trap captures was noticeable even from the first year and resulted to a further reduction in the oviposition patterns. During the two years, oviposition by female moths was reduced on the treated trees in comparison to untreated ones, but still indicated the presence of mated females. The fertile eggs in the treated plots may have been laid by females fertilized despite MD treatment or by gravid females immigrating from adjacent plots into the MD plots. Battisti et al. (2015) refer that *T. pityocampa* males may disperse over distances of 50–100 km. In general, immigration of gravid females into pheromone-treated areas from the surroundings is a common obstacle when using the MD technique (Knight, 1996; Mazomenos *et al*., 1999), but can be avoided by extending pheromone application to a larger spatial scale. Besides the risk of invasion by mated females from neighbouring areas, the existence of "hot spots" *i.e.* areas with high moth

densities, within the treated area, may allow significant mating. More knowledge on mating behaviour, dispersal and oviposition behaviour of this pest is still needed and studies in this respect should be encouraged.

The used pheromone formulation in our study with a load of 20 g/ha for one application per season, allowed the presence of sufficient pheromone concentration in the field for more than ten weeks of field exposure. This is a low pheromone load formulation compared to the pheromone concentration used in other species to achieve insect disorientation and communication disruption i.e. between 10 g and 100 g per ha (Bengtsson *et al*., 1994; Cork *et al*., 2008). In the case of *Prays oleae* the pheromone concentration used was 40 g a.i./ha per season (Hegazi et al. 2009), for *Palpita unionalis* was 80 g a.i./ha per season (Hegazi *et al*., 2007), for *Spodoptera littoralis* was 40-60 g of a.i/ha (De Souza *et al*., 1992), for *Grapholita molesta* was 44 g of a.i/ha (Arioli *et al*., 2014), for *Phylocnistis citrella* was 75 g of a.i/ha (Lapointe and Stelinski, 2011), for *Cydia pomonella* was up to 100 g of a.i/ha (Witzgall *et al.,* 2010).

As both the formulation and the matrix to deliver pheromone are of paramount importance in determining the mode of action and the success of MD programs (Cardé and Minks 1995, Leonardt *et al*., 1990, Weatherston 1990), the tested formulation-matrix combination seems promising for the effective MD of *T. pityocampa,* especially because it was attained with 20 g a.i./ha is. Since the cost of pheromone is still very high, achieving adequate levels of male sexual disorientation with less amounts of pheromone could make the use of this method more cost-effective (Gordon *et al*., 2005). Last but not least, MD is an environmentally safe method for pest control in urban and suburban areas and parks and a valuable tool in Integrated Pest Management programs.

This research has been co-financed by the Region of Attica, Greece (former Prefecture of Athens – Piraeus) through the project "Protection of woods in urban areas from Thaumetopoea pityocampa: Control of the moth by pheromone mating disruption method combined with Bacillus thuringiensis".The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Received: 9 April 2019; Accepted: 16 November 2019

Αποτελεσματικότητα της παρεμπόδισης επικοινωνίας της πιτυοκάμπης, *Thaumetopoea pityocampa* (Lepidoptera: Thaumetopoeidae), με χαμηλής συγκέντρωσης φερομόνη φύλου

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Περίληψη Η μέθοδος παρεμπόδισης των συζεύξεων (ΠΣ) έχει εφαρμοστεί με επιτυχία για τον έλεγχο πληθυσμών πολλών επιβλαβών λεπιδοπτέρων εντόμων. Τα έτη 2010 και 2011 πραγματοποιήθηκαν σε ένα αστικό πάρκο, πειράματα πεδίου για την αξιολόγηση της αποτελεσματικότητας της παρεμπόδισης της χημικής επικοινωνίας της πιτυοκάμπης, *Thaumetopoea pityocampa,* χρησιμοποιώντας μορφοτυποποιημένη φερομόνη φύλου σε χαμηλή συγκέντρωση. Η αποτελεσματικότητα της ΠΣ αξιολογήθηκε συγκρίνοντας τις συλλήψεις των αρσενικών *T. pityocampa* σε παγίδες φερομόνης, σε περιοχές που εφαρμόστηκε η ΠΣ και σε περιοχές που δεν έγινε καμία εφαρμογή (μάρτυρας). Τον 1ο χρόνο της εφαρμογής της μεθόδου το ποσοστό του αποπροσανατολισμού των αρσενικών κυμάνθηκε από 85 έως 100% κατά τη διάρκεια του 1ου μήνα της πτητικής περιόδου και 95-100% σε όλη τη διάρκεια ολόκληρης της πτήσης κατά το 2ο έτος. Το ποσοστό φερομόνης που παρέμεινε στη μήτρα του πολυμερούς μετά από 7 εβδομάδες σε εργαστηριακές συνθήκες γήρανσης ήταν σχεδόν 30%. Συνδυάζοντας τα αποτελέσματα απελευθέρωσης της φερομόνης με τα αποτελέσματα του αποπροσανατολισμού των αρσενικών μπορούμε να συνάγουμε ότι μετά από 7 εβδομάδες η συγκέντρωση της φερομόνης που απομένει εξακολουθεί να είναι επαρκής για την επίτευξη της ΠΣ. Η μελέτη αυτή υποδεικνύει ότι η διάχυση του κύριου συστατικού της φερομόνης φύλου (Z) -13-δεκαεξανο-11-υνυλ οξικού εστέρα σε συγκέντρωση 20 g/ha στον αέρα σε μία εφαρμογή ανά πτητική περίοδο είναι επαρκής ώστε να επηρεάσει τον προσανατολισμό των αρσενικών του *T. pityocampa*. Δεδομένου ότι η παρεμπόδιση συζεύξεων είναι μια περιβαλλοντικά ασφαλής μέθοδος για τον έλεγχο των επιβλαβών εντόμων, θα μπορούσε να αποτελέσει ένα πολύτιμο εργαλείο για τον έλεγχο του *T. pityocampa* σε αστικές περιοχές και σε πάρκα.

Hellenic Plant Protection Journal **13:** 42-53, 2020

Τόμος 13, Τεύχος 1, Ιανουάριος 2020
ISSN 1791-3691

Περιεχόμενα

Volume 13, Issue 1, January 2020
ISSN 1791-3691

Hellenic Plant Protection Journal

Contents

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