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REVIEW ARTICLE

Evaluating the risks of occupational pesticide exposure

C.R. Glass[1] and K. Machera[2]

Summary In the European Union (EU), the estimation and/or measurement of the operator exposure levels to a plant protection product (PPP) during its mixing/loading and application to the crops (outdoors or indoors) is a key issue in the registration process in accordance with Directive 91/414/EEC. The predictive models currently applied for regulatory purposes within the EU (UK POEM, German and Dutch models) have been based on data generated in Northern Europe, not reflecting the Southern European conditions. Several data, including outdoor and indoor trials in Greece, where hand-held application techniques were used, have been generated recently in order to address this issue. The most important route of exposure to PPPs is dermal, while the contribution of inhalation exposure is lower. The selection of the method to measure the operator exposure in each study is a decisive step and many factors should be taken into account. On the other hand, there are a great number of factors to consider when using predictive operator exposure models in the risk assessment of a PPP. In any case, the operator is considered to be safe only if the specific application scenario examined each time leads to a systemic exposure level lower than the systemic Acceptable Operator Exposure Levels (AOEL) as defined from the toxicological evaluation of each active substance.

Additional keywords: operator exposure, pesticide safety, dermal, inhalation

Introduction

Plant Protection Products (PPPs) have become a key part of many crop protection programmes, allowing intensive production techniques for a wide range of crops. However, as a consequence, there are tasks involved with PPP use that can result in exposure of the user (operator) to the active ingredient(s) of the PPPs and potential risk for human health. The legislative basis for the regulation of PPPs in the European Union (EU) is the Directive 91/414/EEC, concerning the placing of PPPs on the market. This involves a harmonised approach to the official evaluation of PPPs and data requirements for applicants seeking to supply the market with PPPs. The authorization of PPPs (i.e. pesticides) in the EU Member States (MS) is essentially a two stage process. At present, one MS acts as a Rapporteur on behalf of the Commission (EC) and prepares the Draft Assessment Report (DAR) for each active substance of PPPs. In the DAR, the risk assessment for the substance is provided which includes hazard identification, setting of reference values, exposure assessment, and risk characterisation. The DAR is then considered by the Pesticide Risk Assessment Peer Review (PRAPeR) Unit of the European Food Safety Authority (EFSA). A comprehensive summary of the Risk Assessment is produced by EFSA and sent to the Commission, where the final decision is made. Active substances that are demonstrated not to present an unacceptable level of risk for human health and the environment are then included in Annex I to Directive 91/414/EEC.

The second stage of the process involves and it is carried out at MS (granting the au-

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thorization of the product) which has the responsibility of carrying out risk assessments for the examined PPPs which contain Annex I listed active substances. With the information from the hazard assessment for the active substance(s), as concluded during the Annex I inclusion, the regulatory authority for each individual MS decides whether the PPP can be used safely under national conditions. With decisions now made on a European basis for the selection of active substances for inclusion in the Annex I, it is important that data and information used in the initial risk assessment (such as exposure models) are valid for all MS, and not just for those that have generated the most information.

There are various tasks involved which result in occupational exposure to pesticides, but the greatest exposures are often associated with the operator, during both the handling of the concentrated pesticide when mixing and loading, and the application of the diluted pesticide in the field. Studies to measure operator exposure have been carried out since the 1960's using a range of methodologies to determine potential dermal and inhalation exposure. More recent studies have measured dermal exposure and the absorbed dose, and data is now available from a wide range of studies using different application techniques. The estimation or measurement of operator exposure is a key element of occupational health and a requirement of the risk assessment in pesticide registration (van Hemmen and Brouwer, 1997), which is carried out according to the directive 91/414/EEC.

Three predictive models are used for regulatory purposes within the EU: the UK model (Martin, 1990); the German model (Lundehn *et al*., 1992) and the Dutch model (van Hemmen, 1992). These models contain experimental data obtained from particular use scenarios and were incorporated into the European Predictive Operator Exposure Model (EUROPOEM) Expert Group under concerted Action AIR3-CT93-1370. The EUROPOEM is a database for reference rather than an actual tool for regulators, and tends to be used in conjunction with existing models developed in the UK and Germany. Data have been added since EUROPOEM was set up, with field assessments carried out, especially in southern Europe (Machera *et al*., 2001; Glass *et al*., 2002) as part of the project SMT4-CT96-2048. In North America, a Pesticides Handlers Exposure Database (PHED) provides generic mixer/loader/applicator exposure data (Krieger, 1995). Work is being done to combine PHED and EUROPOEM datasets in a new North American model, the Applicator and Handlers Exposure Database (AHED).

The modelling of operator exposure still relies on a number of assumptions related to the personal protective equipment (PPE) worn by operators, the protection factor offered by coveralls for example. The degree of dermal absorption of the compound is a substantial information for reliable human risk assessment. This information is usually derived from *in vivo* animal data and *in vitro* human and animal skin data. When there are no available data for a substance, default values of dermal absorption are used.

The methods used to measure operator exposure, and the subsequent use of these data together with toxicology data in risk assessments are discussed.

Routes of operator exposure

The most important route for exposure to pesticides is dermal for the majority of application techniques. The other routes are inhalation, particularly with fogging and misting application techniques, and by accidental ingestion (oral), for example by eating or smoking while working, or not washing adequately after work.

Potential dermal exposure is the total amount of pesticide landing on the body, including amounts landing on clothing. The mass of pesticide available on the skin for absorption into the body is the actual dermal exposure, which is the amount deposited directly on the skin plus any that penetrates clothing.

Inhalation exposure, generally contributes much less to the absorbed dose than

the dermal exposure. The concentration of the pesticide in the air inhaled by the operator is used as the basis for estimating operator exposure by the inhalation route. Particulates of up to 100 µm within the breathing zone of the operator may enter the nose or mouth. However, only particles of diameter <10 µm range are likely to reach the lungs, known as the inhalable fraction. The larger particles will be deposited on the surface and hairs of the nasal cavity, and subsequently swallowed in many cases.

Methodology to determine levels of operator exposure

Dermal Exposure

Early methods of measuring potential dermal exposure involved the use of absorbent cotton pads attached to different parts of the body (Durham and Wolfe, 1962). The amount of pesticide collected on each pad was used to extrapolate to various parts of the body. The method can also be used to estimate dermal exposure, by placing the patches inside the workers PPE. Although this method is relatively easy to use in practice, attaching 100 cm^2 pads to the outside of workers normal PPE, it has been criticised for providing inaccurate values for potential dermal exposure (Machera *et al.* 1998). Therefore in modern studies it has been superseded by the whole body dosimetry method, which uses a coverall as the dermal sampler, so avoiding the need for extrapolation. Care needs to be taken with this method, as pesticide deposit can either penetrate or be shed by the dosimeter, leading to underestimates of exposure. Therefore a useful variation of the whole body dosimeter method uses typical work clothing, such as cotton coveralls, as the sampling media (Chester, 1993). This technique allows potential dermal exposure to be estimated by dermal dosimetry in addition to allowing biological monitoring.

Dosimeters can be worn to measure potential dermal exposure, or as internal garments to measure dermal exposure. In both cases care needs to be taken in the use of the data, as pesticide can penetrate through to the skin, so not retained by the internal dosimeter, or included in the dermal exposure measurement.

Measuring the pesticide deposits on hands or the potential dermal exposure of the hands is complicated. The hands are often the part of the body most exposed to pesticides. The use of absorbent gloves (cotton) worn outside any other protection can give information about the potential hand exposure. However absorbent gloves will retain more liquid than the hand itself. Absorbent gloves worn inside protective gloves give an indication of dermal exposure, but this is only relevant for that particular scenario and the type of glove worn. Outer protective gloves worn more than once often contain internal pesticide deposit, carried into the inner glove during removal and donning by the operator, or by penetration through the material. To overcome this, hand rinse sampling has been used for monitoring dermal hand exposure. Prior to the study the hands need to be washed in the solvent to remove any background contaminants present. Data for the recovery of the pesticide for the handwash technique is not really available for such studies, and for this reason the technique has been criticised as underestimating dermal exposure of the hands.

Inhalation exposure

The inhalation exposure is carried out using personal air samplers, which sample the air in the breathing zone of the operator using a pump and appropriate filter to allow the airborne concentration to be determined. The breathing rate of the operator will vary depending on the type of task being done, generally considered to be 1.7-3.5 m^3/h.

Biomonitoring

The amount of pesticide which has been absorbed by the body is the ultimate measure of operator exposure, however lack of pharmacokinetic data makes interpretation difficult, together with human variability

and other confounding factors on rates of metabolism. Urine samples, taken over a period of at least 24 hours, tend to be used for biomonitoring studies.

Examples of data for operator exposure

The hand held application technique is generally considered to represent the worst-case scenario for applicator exposure, due to the proximity of the nozzle to the operator. In the EU-funded project SMT4-CT96-2048, data for hand held application techniques were generated in a number of southern European countries (see Figure 1). These studies provided data for the developing EUROPOEM database, which till then had few datasets for hand held applications, and those available concerned outdoor application in northern Europe.

In considering operator exposure to pesticides, studies should be done to allow exposure during the tasks of mixing/loading to be determined separately from the exposure during the application. The handling of the concentrated pesticide during mixing and loading generally results in greater levels of exposure than the application process. However this depends on the type of containers or transfer mechanism used for mixing and loading, as the size of the container and the number of containers to be handled is critical and varies greatly. In case large greenhouses are to be treated, the mixing and loading procedure of pesticides is often done by workers not involved with the application itself.

The data presented in Figure 1, concerning a hand held, upward application technique, show potential dermal exposure as ml/hour separately for the hands and the body. The potential dermal exposure of the hands was measured by placing cotton gloves on the volunteer operators, so it is a measure of the pesticide landing on the hands, and not necessarily what would be retained by hands or impermeable gloves.

These data are consistent with published data for potential dermal exposure for the hand held, upward application technique. The majority of the data for potential dermal

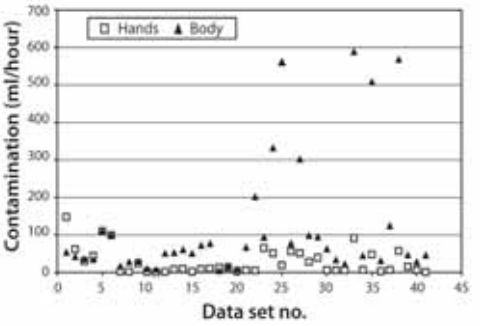

Figure 1. Data for potential dermal exposure of hands and body (ml/hour).

exposure excluding hands are between 60 and 120 ml/hour. The data are variable, with coefficient of variation of 122% for the hands and 139% for the rest of the body. Data in the German model (Lundehn *et al*., 1992) for an equivalent application technique have a coefficient of variation of 149%.

Several studies have been carried out in Greece concerning indoor and outdoor hand held application of pesticides yielding data for potential dermal exposure expressed in ml spray solution/hour (Machera *et al*., 2001; Machera *et al*., 2002; Machera *et al.,* 2003). Recent studies have provided the exposure levels in mg/kg a.i as expressed in the German Model. Two of them involved outdoor application in olives and vines indicating a potential dermal exposure between 61-317 mg/kg a.i. with the respective value calculated by the German model being 189 mg/kg a.i. The other two of them involved greenhouse trials, hand held application techniques with either spray guns or 4 nozzle lances fed by hoses at 25 bar pumping pressure. In the first greenhouse study with 11 operators using spray guns and two types of protective coveralls the potential dermal exposure ranged from 8.4 to 664.1 mg/kg a.i. applied, with a mean of 179.3 and a coefficient of variation of 112%. The lowest value was obtained with an application where the operator walked backwards away from the spray cloud. In the same study the data indicate that the potential dermal exposure measured (mg/kg a.i., 50% percentile) was 5-6 times the value estimated by

the German Model (Machera *et al.,* 2009). Data from the second greenhouse study conducted with a handheld lance indicate that potential dermal exposure measured (75% percentile) was 12-13 times the value estimated using the German Model (Machera & Tsakirakis, pers. comm.).

Interpretation of data

Use of predictive models

Within the EU most regulators use the UK POEM or the German model. The UK model is based mainly on local unpublished studies conducted mainly by industry and the Government laboratories such as the Central Science Laboratory. The predicted exposure is expressed in mass or volume of the formulation or the spray liquid per unit time (mg/h or ml/h). Surrogate exposure levels are chosen to be the 75th percentile values. An estimated value for potential dermal and inhalation exposure is given based on the input parameters, from which actual dermal exposure is estimated, to give a final figure for the systemically absorbed dose based on default values for clothing penetration/permeation and dermal absorption. This value is compared to the systemic Acceptable Operator Exposure Level (AOEL) value for a particular compound.

In the UK model exposure during mixing and loading is assumed to be confined to the hands with respiratory exposure not taken into account. The dermal exposure estimation is based on the number of pesticide containers or packs (operations) which the worker has to deal with during one working day. The estimation of operator exposure is therefore based on the amount of active ingredient handled during mixing and loading together with the exposure during the application itself which is time rather than mass dependant. The concentration of the spray liquid but not the treated area is included for the calculation of the exposure during application.

The German model is based on the amount of the pesticide handled during one working day, and exposure level is expressed as units of mass per amount of a.i. handled (mg/kg a.i.). The potential exposure is calculated, including potential respiratory exposure, for both mixing and loading and application. Again the actual exposure is calculated as the mass of pesticide on the workers skin area after penetration through clothing. The actual dermal and inhalation exposure is then compared with the AOEL value.

There is also a Dutch model which is a literature-based model using internationally published studies. The units for exposure values are similar to the UK model (ml/h or mg/h), but the dermal exposure during mixing and loading is not limited to the hands. The potential exposure is calculated as for other models; however the estimation of the actual exposure is left to expert judgement, and is often close to the potential exposure (Kangas and Sihvonen 1996).

In the Dutch model, the estimation of the operator exposure for outdoor applications is based on the working time, the concentration of the formulation and the concentration of the spray liquid. This is supported by an additional model for mixing and loading based on field studies carried out in the Netherlands. The exposure is dependent on the amount of pesticide handled and is expressed in mass units per amount of active ingredient handled (mg/kg a.i.) as in the German model.

A number of assumptions are made, which often differ, in the different models, such as the wearing of clothing, both the workers own clothing and PPE, and the penetration and permeation of PPE which is worn. The rates of uptake from the skin also vary as do the statistical parameters on surrogate values used such as the geometric mean used in the German model, the 75th percentile in the UK POEM and the 90th percentile in the Dutch model.

The rate of coverall contamination is one of the factors that determine the protective factor of the PPE worn during pesticide application, representing the challenge to the PPE. In an ideal scenario, the rate of

PPE contamination would be a factor to be taken into account when selecting appropriate PPE to be worn for a particular pesticide application task. Therefore, impermeable coveralls (e.g. CE marked Type 3 or Type 4 garments) should be worn for certain tasks involving hand held application techniques. However, in reality the climatic conditions in Southern Europe make the wearing of such PPE difficult. Therefore the types of coveralls worn by pesticide applicators tends to be constructed of permeable material such as cotton or cotton/polyester mixtures.

Working patterns in different regions of EU

In using models or evaluating data from operator exposure studies the working patterns typical for the region where the pesticide is to be used need to be considered. For mechanised applications such as tractor mounted or trailed boom sprayers the operator can be expected to work longer hours than a manual application, and treat much larger areas. Table 1 shows the default values of the three models.

As the common acceptance directive is developed, there is likely to be greater tendency for pesticides to be approved for use over more than one country, which provides another uncertainty factor into the risk assessment. For example, in the studies published by Glass *et al.* (2002), the working patterns in southern Spain were very different from those in Greece or Portugal, in terms of the length of the working day, types of application equipment used and the protective clothing worn.

Derivation of the AOEL and Risk Assess ment

The AOEL is "the maximum amount of the active substance to which the operator may be exposed without any adverse health effects", as defined in Annex VI to Directive 91/414/EEC. In this definition, "operators" are represented by mixer/loaders, applicators and re-entry workers, but the term may be extended to include non-occupational exposed groups (bystanders). The AOEL is based on the highest level at which No Adverse Effect (NOAEL) is observed in tests of the most sensitive relevant animal species. To translate the NOAEL values into an AOEL, assessment factors accounting for uncertainties in extrapolation from toxicity data to the exposed human population are applied. Often, the AOEL values relate to the internal (absorbed) dose available for systemic distribution from any route of absorption and expressed as internal levels (mg/kg body weight/day) (AOEL systemic). Thus, depending on the route specific NOAEL (oral, dermal, inhalation), the degree of oral/dermal/inhalation absorption should be considered in the correction of the AOEL and the estimation of AOEL systemic.

Following the setting of the systemic AOEL, a comparison to the estimated dose of exposure is performed. The systemic dose of exposure is the sum of the exposure from the dermal route, corrected for the degree of dermal absorption and the exposure from the inhalation route considering 100% absorption of the inhaled amount. The examined PPP is considered to be safe for the operator for the specific application scenario, when the systemic dose of exposure is lower than the systemic AOEL.

Discussion

There is a number of factors to consider when using predictive operator exposure

Table 1. Standard daily work rates for agriculture used for the models (Kangas & Sihvonen 1996).

Application method	UK	Dutch	German
Tractor, downward application	50 ha	10 ha	20 ha
Tractor, upward application	30 ha	6 ha	8 ha
Hand-held equipment	1 ha (or 400 L spray dilution)	1 ha	1 ha

models. Extrapolations often have to be made using data from the most suitable or similar scenario for crop or application technique. In selecting an indicative value for potential dermal exposure the variability of the data in the database supporting the model needs to be considered. The UK model uses the 75th percentile values, whereas the German model uses the geometric mean values. Having determined the potential operator exposure, a number of assumptions are then made with respect to transfer factors of the pesticide from the outside of PPE to the skin of the operator and the rate of subsequent dermal absorption of the active substance estimated to have reached the skin of the operator.

Performance of protective clothing

The protection factor offered by various types of PPE tends to be related to the performance of new garments in standard laboratory tests such as EN 463 and EN 468. These two tests are for whole garments of chemical protective clothing and those passing the test are CE marked as Type 3 (EN 463) or Type 4 (EN 468) garments. Type 4 garments offer more protection than Type 3 garments. Recently Type 6 garments (prEN 13034) became available, which offer limited protection against penetration by liquid contamination. However in many cases, such as with the orchards or greenhouses of southern Europe, working conditions for pesticide operators are such that specific chemical protective clothing is rarely used. Where protective clothing is worn it tends to be workwear such as polyester cotton coveralls, for which there are no test methods to determine penetration by aqueous liquids. Field and laboratory tests carried out within the framework of the project SMT4-CT96-2048 have shown that the rate of coverall contamination is a key factor in determining the protection factor offered by various types of coveralls (Moreira *et al.*, 1999).

Another factor which should be further evaluated is the age or condition of PPE. Disposable coveralls have a limited life, and the coating on the material begins to be damaged and removed by contact with the crop for example, or simply through the movement of the applicator creasing the material. Washable coveralls such as the polyester coveralls become more absorbent and less repellent after repeated washings, as the coating of the material is removed.

Rates of dermal absorption

Most models assume that 10% of the active substance which reaches the skin is absorbed into the body. In practice the proportion of the active substance which is absorbed by the body is influenced by many factors. These range from factors such as the physicochemical properties of the active substance (partition coefficient in octanol/water and molecular weight), concentration of the active substance on the skin and the area of skin exposed, to the relative humidity and temperature of the air. In order to improve model estimates of exposure, compound specific data are required for dermal absorption for likely ambient conditions in which the product would be used. The type of formulation can affect the rate of dermal absorption, such as the presence of lipophilic organic solvents such as xylene in emulsifiable concentrate formulations. The size of the molecule of the active substance also affects the rate of dermal absorption.

Biomarkers of exposure and effect

Biomonitoring studies to measure pesticides and metabolites in urine samples give an indication of the exposure levels and the absorbed dose, assuming pharmacokinetic data are available. Biomarkers of exposure can also measure the interactions between a pesticide and target molecules or cells, including detection of biologically effective doses (Lowry *et al.*, 1995; Decaprio *et al.*, 1997; Lopez *et al.*, 2007).

Biomarkers of effect can identify alterations of an organism that could indicate a potential for health impairment or disease. Therefore biomarkers can be used to detect the early effects of pesticides before adverse clinical health effects occur. Tech-

niques that measure DNA damage with e.g. the COMET assay provide a powerful tool for measuring effects of exposure, although not specific, being a response to oxidative stress (Bhalli *et al*., 2006; Muniz *et al*., 2008). Studies on the cytogenetic effects of pesticide exposure report increases in the frequency of chromosomal aberrations and/or sister chromatid exchanges (Ergene *et al*., 2007). Biomarkers of effect have been developed for detecting early stage effects of neurotoxic pesticides picking up delayed neuropathy and neurobehavioural effects of chronic pesticide exposure (Salvi *et al*., 2003; Battershill *et al*., 2004).

Genetic variation with the human population makes it difficult to be certain about the dose-response relationship. There has been a great deal of interest in the role of P450 enzyme gene polymorphisms, and the role played by metabolic pathways of toxic compounds such as pesticides (Buratti *et al*., 2007; Wang *et al*., 2008). Ultimately biomarkers of effect could be used to predict and therefore possibly prevent detrimental health effects and disease associated with pesticide exposure.

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ΑΡΘΡΟ ΕΠΙΣΚΟΠΗΣΗΣ

Αξιολόγηση της επικινδυνότητας κατά την επαγγελματική έκθεση σε φυτοπροστατευτικά προϊόντα

C.R. Glass και Κ. Μαχαίρα

Περίληψη Στην Ευρωπαϊκή Ένωση (Ε.Ε.), ο υπολογισμός ή/και η πειραματική μέτρηση των επιπέδων έκθεσης των ψεκαστών σε φυτοπροστατευτικά προϊόντα (φ.π.) κατά την ανάμιξη - φόρτωση του ψεκαστικού διαλύματος και κατά την εφαρμογή τους στις καλλιέργειες (θερμοκηπίου ή υπαίθριες) είναι ένα από τα κύρια σημεία της αξιολόγησής τους σύμφωνα με την Οδηγία 91/414/ΕΟΚ. Τα υπολογιστικά μοντέλα που εφαρμόζονται κατά τον έλεγχο των φ.π. στην Ε.Ε. (UK-POEM, Γερμανικό και Ολλανδικό μοντέλο) έχουν βασιστεί σε στοιχεία που έχουν παραχθεί σε χώρες της Βόρειας Ευρώπης και δεν είναι πάντοτε αντιπροσωπευτικά των συνθηκών των νοτίων Ευρωπαϊκών χωρών. Προκειμένου να αντιμετωπιστεί το θέμα, ένας μεγάλος αριθμός πειραμάτων έχει πρόσφατα πραγματοποιηθεί στην Ελλάδα με τεχνικές που περιλαμβάνουν χειροκίνητα μέσα εφαρμογών φ.π. σε υπαίθριες και σε υπό κάλυψη καλλιέργειες. Η σημαντικότερη οδός έκθεσης σε φ.π. είναι από δέρματος, ενώ η συνεισφορά της έκθεσης από αναπνοής είναι χαμηλότερη. Η επιλογή της μεθοδολογίας που θα εφαρμοστεί για τον προσδιορισμό των επιπέδων έκθεσης των ψεκαστών είναι ένα καίριο βήμα και πολλά στοιχεία της εφαρμογής θα πρέπει να ληφθούν υπόψη. Ακόμη, κατά την εφαρμογή των υπολογιστικών μοντέλων για την εκτίμηση επικινδυνότητας κατά τη χρήση ενός φ.π. ένας μεγάλος αριθμός παραμέτρων θα πρέπει να ληφθεί υπόψη. Σε όλες τις περιπτώσεις και για όλους τους τρόπους εκτίμησης των επιπέδων έκθεσης, ο ψεκαστής των φ.π. θεωρείται ότι είναι ασφαλής κατά την εφαρμογή ενός φ.π. μόνον όταν το συγκεκριμένο σενάριο εφαρμογής που εξετάζεται κάθε φορά οδηγεί σε επίπεδα συστηματικής έκθεσης χαμηλότερα από τα Αποδεκτά Επίπεδα Συστηματικής Έκθεσης των Ψεκαστών, όπως αυτά έχουν καθοριστεί από τον τοξικολογικό έλεγχο της κάθε δραστικής ουσίας.

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SHORT COMMUNICATION

Comparison of two methods for the determination of soil nitrate nitrogen in the field

Y.E. Troyanos[1], E. Roukounaki[1] and G. Gomoli[2]

Summary A "test strip" method (Merck quant®) was compared against the standard (hydrazine nitrate reduction) method for measuring soil nitrate nitrogen (NO_3^- - N) concentrations in soil samples from processing tomato fields in the area of Ilia. The agreement between the "test strip" and standard method was tested by using regression analysis and a simple "graphical" method. The regression analysis showed that the "test strip" method overestimated the mean soil NO_3^- - N concentrations ([NO_3^- - N]) by approximately 12% compared to the standard method. However, analysis of the results according to a more precise "graphical" method revealed that the maximum differences that could be expected to occur when the "test strip" method is used in the field are 10 ppm above or 6 ppm [NO_3^- - N] below the standard method. This discrepancy is acceptable for "on-farm" measurements of soil N and the "test strip" method could be used with adequate confidence to evaluate the soil [NO_3^- - N] in fields.

Efficient nitrogen fertilization management is essential to achieve optimum yields. A technique available to manage in-season N inputs efficiently, in terms of economic and environmental concerns, can be accomplished by monitoring the in-season NO_3^- - N status of the soil. The "quick test" Merck quant® method described by Hartz *et al.* (4) is currently used in California as an "on farm" procedure to manage the nitrogen fertilization of vegetables. Soil samples from processing tomato fields in the area of Ilia (Peloponnissos) were collected in order to compare the "quick test" method with an established method (e.g. hydrazine sulfate reduction method) for [NO_3^- - N] determination.

It is well known that comparisons of different analytical methods could be carried out using correlation or regression analysis. However, the correlation coefficient (r) measures the strength of the relation, not the agreement between the methods whereas, regression analysis has drawbacks since both the dependent ("test strip" method) and the independent (standard method) variables are measured with error. To overcome these problems, a simple "graphical" method has been suggested by Altman and Bland (1). In this study both the regression analysis and the simple "graphical" method were used to compare the two methods.

Soil cores were taken randomly between the drippers from 0-20 and 20–40 cm depth from processing tomatoes fields. The cores were bulked in a composite sample from each soil depth that contained approximately 10 soil cores per hectare. The composite samples were placed in a refrigerator until NO_3^- - N analysis. A total of 67 composite soil samples were sent to the laboratory for analyses. According to the standard method (hydrazine reduction method) (2), 2 sub-samples (25 g each) were taken from each composite sample, whereas one subsample was taken for the "test strip" meth-

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Abbreviations: NO_3^- - N; nitrate nitrogen, [NO_3^- - N]; nitrate nitrogen concentration

od (4).

[NO_3^- - N] measurements were carried out with the standard method after extracting each sub-sample with 250 ml de-ionized water for 0.5 hour. The extracts were filtered using a 125 mm filter paper which had been rinsed with 25 ml of de-ionized water. After filtration, 0.75 ml of a solution containing 1 M NaOH and 0.12 M $Na_3PO_4.12H_2O$ was added per 25 ml of extract to remove the Ca and Mg ions (5). Afterwards, a volume of 2 ml extract was taken from the clear supernatant and used for colorimetric determination of [NO_3^--N] (5). If the results of the analyses of the two sub-samples differed more than 10%, a new sub-sample was taken and analyzed for a third time and the means of the three measurements were used. Measurements with the "test strip" method were carried out according to Hartz *et al.* after extracting the soil with 0.01 M $CaCl_2$ (4).

Regression analysis was performed between the [NO_3^--N] determined by the "test strip" and the standard method (Figure 1). The analysis showed that a linear curve was significant (P<0.001) with a high coefficient of determination (R^2=0.85, n=67). The slope of the line was estimated to be 0.953, it was different from zero (P<0.001) and had a standard error (SE) of 0.053. The estimated constant was 2.408, it was different from zero (P<0.001) and had a SE 0.919 (P=0.011). Based on this analysis, the mean [NO_3^--N] measured by the standard method was 14.22 ppm and the mean [NO_3^--N] measured by the "test strip" method was 15.96 ppm showing an overestimation of approximately 12.23 % by the "test strip" method. From Figure 1 it is evident that the constant of the regression equation causes the regression line to be different from the line (y=x) which describes the agreement between the two methods.

To evaluate the differences between the two methods the procedure of Bland and Altman (3) was used. According to this method the plot of the differences between the [NO_3^--N] determined with the two methods against their means are indicative of their discrepancies. The mean of the differences ($\bar{x}$) ("test strip" – standard method) was 1.74 ppm and the standard deviation (SD) of the differences was 3.99 (Figure 2). Therefore, 95% of the differences between the methods are expected to lie between ±1.96 SD. From Figure 2 it is evident that the upper limit of the differences between the methods could be 9.56 ppm and the low limit 6.08 ppm. Therefore, the maximum differences that could be expected to occur when the "test-strip" method is used in the field are 10 ppm above or 6 ppm [NO_3^--N] below the standrd method. A 10 ppm overestimation corresponds to approximately 30 kg of N per ha (0-30 cm soil depth) which is acceptable and therefore, the "quick test" method could be used reliably to monitor the "in-season" soil N status of drip irrigated processing tomatoes.

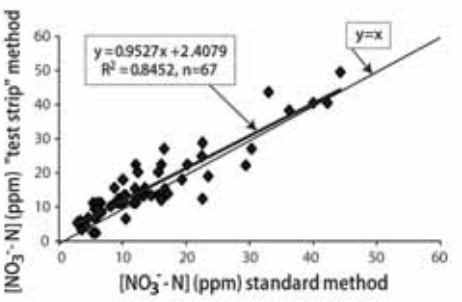

Figure 1. Regression analysis between the "test strip" and the standard method and the line of agreement (y=x) between the methods.

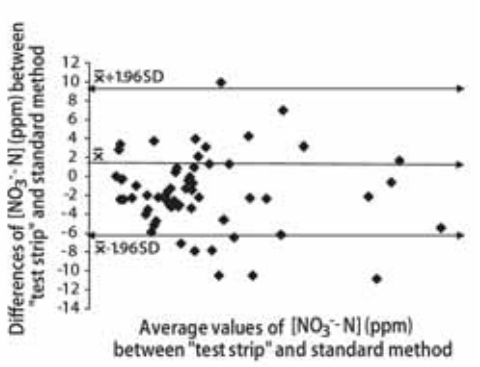

Figure 2. [NO_3^- - N] (ppm) differences between the methods vs. average values of the methods for each composite soil sample. ($\bar{x}$ = mean of the differences and SD = standard deviation).

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ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Σύγκριση δύο μεθόδων προσδιορισμού του εδαφικού νιτρικού αζώτου

Γ.Ε. Τρωγιάνος, Ε. Ρουκουνάκη και Γ. Γκομόλη

Περίληψη Στα πλαίσια μιας μελέτης προσδιορισμού των συγκεντρώσεων του νιτρικού αζώτου σε εδάφη του νομού Ηλείας μελετήθηκε η ακρίβεια μίας ημι-ποσοτικής μεθόδου προσδιορισμού τους με χρωματομετρικές ταινίες τύπου Merck quant® σε σύγκριση με μία πρότυπη μέθοδο (αναγωγή νιτρικών με θειική υδραζίνη). Η σύγκριση των δύο μεθόδων πραγματοποιήθηκε με την χρήση γραμμικής παλινδρόμησης και με μία απλή γραφική μέθοδο. Τα αποτελέσματα της ανάλυσης γραμμικής παλινδρόμησης έδειξαν ότι η μέθοδος των χρωματομετρικών ταινιών υπερεκτιμάει κατά μέσο όρο 12% τη συγκέντρωση του νιτρικού αζώτου σε σύγκριση με την πρότυπη μέθοδο. Τα αποτελέσματα μιας πιο ακριβούς "γραφικής" μεθόδου έδειξαν ότι η μέθοδος των ταινιών είναι πιθανό να υπερεκτιμήσει έως 10 ppm ή να υποεκτιμήσει έως 6 ppm τη συγκέντρωση του νιτρικού αζώτου σε σύγκριση με την πρότυπη μέθοδο. Τα αποτελέσματα της σύγκρισης των δύο μεθόδων έδειξαν ότι η μέθοδος των ταινιών Merck quant® μπορεί να χρησιμοποιηθεί αρκετά αξιόπιστα για τον προσδιορισμό των νιτρικών σε αγρούς.

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Method validation for the determination of pesticide residues in wheat flour by gas chromatography

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Summary A rapid multi-residue method for the simultaneous determination of residues of 28 multi-class representative pesticides is presented. The 28 pesticides were included in the 2007 Proficiency Test for cereals, organized by the European Commission Reference Laboratories on Cereals and Feedingstuff and Single Residue Methods. The extraction was based on acetone – dichloromethane – petroleum ether and the analysis was performed by gas chromatography (GC) with ECD and NPD detectors. The procedure was applied to the screening, confirmation and quantification of the 28 pesticides. The recoveries obtained from the validation data were from 66 to 120% with relative standard deviation (RSD) <10% and the attained limits of quantification were between 0.01 and 0.75 mg/kg. The method is characterized by good accuracy, precision and sensitivity.

Additional keywords: cereals, GC-ECD, GC-NPD, multi-residue, proficiency test

Introduction

The requirements related to sampling and analysis are set out in Article 11 and Annex III of Regulation 882/2004. Sampling and methods of analysis used for official control purposes should, wherever possible, be recognised by international organisations and be validated in accordance with Community legislation or with internationally accepted protocols. Article 32 of Regulation 882/2004 establishes the Community Reference Laboratories (CRLs) for food and feed. According to this article the CRLs are responsible for organization of Proficiency Tests. The objective of a proficiency test is to obtain information about the quality, accuracy and comparability of the pesticide residue data sent to the European Commission within the framework of the EU and national pesticide monitoring programs.

Many analytical methods have been studied for the simultaneous determination of multi-pesticide residues. In 1975 Luke (9, 11) proposed the extraction of pesticides from agricultural products with acetone, and liquid-liquid partitioning cleanup for determination of several pesticides by gas (GC) and liquid (LC) chromatography.

The objective of this study was to develop and validate a simple and rapid method for the determination of the pesticides used in the 2007 Proficiency Test C1-SRM2 organized by the CRL laboratory for cereals (National Food Institute, Department of Food Chemistry, Danish Technical University) on behalf of the European Commission. The sample of the test was wheat flour and the extraction was based on the Dutch Ministry of Public Health, Welfare and Sport (12) acetone – dichloromethane – petroleum ether multiresidue method extraction procedure. Determination was performed by GC-NPD/ECD and validation levels encompassing the minimum required performance levels (MRPLs) were achieved.

Materials and Methods

1. Chemicals and solvents

The following pesticide analytical standards (obtained from Dr Ehrenstorfer Labo-

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ratories GmbH Germany) were used in this study: bifenthrin, carbaryl, chlorpyriphos, chlorpyriphos methyl, cyhalothrin-λ, deltamethrin, diazinon, endosulfan-a, endosulfan-b, fenpropimorph, imazalil, iprodione, kresoxyl-methyl, lindane, malathion, methacrifos, parathion, penconazole, pirimicarb, pirimiphos methyl, procloraz, procymidone, propiconazole, thiabendazole, triadimefon, triadimenol, triazophos and vinclozolin.

Acetone, 2,2,4-trimethyl pentane and toluene were used for the preparation of stock and working standard solutions. Acetone, dichloromethane and petroleum ether were used in the extraction procedure. All solvents were pesticide residues grade, obtained from Lab Scan (Ireland).

2. Stock and working solutions

Stock standard solutions at 1000 mg L^{-1} were prepared in acetone for each pesticide and stored at -20° C. Standard mixture solutions of the compounds were prepared in 2,2,4-trimethyl pentane/toluene (90/10) at intermediate concentrations (1-10 mgL^{-1}) and stored at -20° C. In order to acquire the retention time of each analyte, working solutions containing only one analyte at 0.5 mgL^{-1} were prepared and injected in the chromatographic system.

Working standard mixture solutions for measurement were prepared in an extract of wheat flour, previously analyzed for the absence of compounds interfering with the analytes. In the quantification of an unknown sample one of the most serious problems is the presence of unexpected interferences in the matrix (6). The effect can be due to different reasons e.g. the presence of a blank due to solvent and/or reagents, or the presence of a compound in the sample that contributes to the analytical signal (8). The detection and correction of errors caused by matrix interferences have been extensively studied for a long time (2, 13). Matrix-induced enhancement is a phenomenon commonly found in the chromatographic analysis of pesticides in food (3) that has been noticed in the analysis of these contaminants by GC-ECD (7) and GC-NPD (4). For this purpose matrix matched standards (including matrix blanks) were used.

The concentrations of the working solutions were at 70 and 100% of the fortification concentrations and quantification was performed by bracketing. According to this technique the peak area of the analyte in the sample solution was bracketed between the peak areas of two standard solutions, not differing between them more than 20% (5).

3. Sample preparation

The sample processing according to the applied method was the following (5, 12): An aliquot of 10 ± 0.1 g of sample was weighted into a 250 mL PTFE centrifuge bottle (Nalgene, Rochester, NY), 10 mL of water and 30 mL of acetone were added and stirred for 1 min in an ultra-turrax homogenizer at 15.000 rpm, 30 mL of dichloromethane and 30 mL of petroleum ether were added and the mixture was stirred for 1 min and then centrifuged at 4.000 rpm for 2 min. Then, 25 mL of the supernatant liquid were evaporated to dryness on a water bath at 65–70° C and 1 mL of 2,2,4-trimethyl pentane/toluene (90/10) was added. Another 15 mL of the supernatant liquid were evaporated to dryness on a water bath at 65–70° C and 3 mL of 2,2,4-trimethyl pentane/toluene (90/10) were added. The two extracts were placed in ultrasonic bath for 30 sec and each was transferred into a separate vial with a Teflon stopper, ready respectively for NPD and ECD chromatographic analysis. Simultaneous injections were performed in the injectors with the aid of 2 separate autosamplers.

4. Criteria for validation of the method

The accuracy was estimated by calculating the attained recovery. For validating a method, mean recoveries of 70–120% are considered acceptable, while in the case of routine analysis, the acceptable recoveries are in the range of the mean recovery ± 2×RSD (5).

The precision of the method was evaluated by assessing the relative standard deviation (RSD) values under repeatability conditions (same analyst, same instrument,

same day). Repeatability with RSD≤ 20% is considered acceptable (5).

The sensitivity of the method was assessed by the **limit of quantification of the method (LOQ_m)**. The LOQ_m was established as the lowest concentration tested for which recovery and precision were satisfactory (70–120% and <20% RSD, respectively) in accordance with the criteria established for analysis of pesticide residues in foods (5).

The **limit of quantification of the analytical instrument (LOQ_i)** was calculated based on the requirement that the signal-to-noise ratio should be higher than 10.

5. Preparation of fortified samples

Control samples were prepared from organically produced wheat flour. Aliquots of 10 g of wheat flour were fortified at two levels, the LOQ_m and the $10\times LOQ_m$ which are shown in Table 2. Working standard mixture solutions for fortification were prepared in 2,2,4-trimethyl pentane/toluene (90/10) at $100\times LOQ_m$. The blank samples were spiked with 0.1 mL of the $100\times LOQ_m$ working standard mixture for the LOQ_m and 1 mL of the $100\times LOQ_m$ working standard mixture for the $10\times LOQ_m$ fortification level.

For validating the method a minimum of 5 replicates is required according to SANCO 2007/3131 (5). In this study 6 replicates in each level were performed.

6. Gas-chromatographic analysis

The studied analytes were separated and determined in an Agilent 6890 gas chromatograph, with two splitless injectors, a DB-5-MS column (30 m, 0.32 mm i.d. and 0.25 μm film thickness) connected to the ECD and a DB-17 MS column (30 m, 0.32 mm i.d. and 0.25 μm film thickness) connected to the NPD and equipped with a Chemstation chromatography manager data acquisition and processing software. The oven temperature program started from 60° C for 1.5 min increased to 220° C at a rate 14° C/min, held for 4 min, then increased to 280° C at 20° C /min and held for 20 min. The helium carrier gas flow rate was 1.5 mL/min for both columns. The temperature of both injectors was set at 230° C and splitless injection was carried out with the purge valve closed for 1 min. Hydrogen (3 mL/min) and air (60 mL/min) were used as fuel gases for the NPD, while nitrogen (60 mL/min) and helium (6 mL/min) were used as auxiliary gases for the ECD. The temperature of both ECD and NPD detectors was set at 310° C.

7. Confirmation

The confirmation of the analytes was conducted, as mentioned earlier, from the retention time of the analyte by using two different columns and two different detectors. The retention times acquired for each analyte by using a combination of two different columns and two different detectors are shown in Table 1. Most pesticides are sensitive to both detectors. For pesticides which are determined by only one detector, such as bifenthrin, endosulfan etc. confirmation is achieved using two different separation systems (2 different columns).

Results and Discussion

Acetone, dichloromethane and petroleum ether showed good performance for extraction of the tested analytes. The method was evaluated by assessing the basic parameters, accuracy, precision and sensitivity. The chromatograms of the compounds are shown in Figures 1 and 2. Furthermore the absence of any interference from matrix compounds was confirmed by the analysis of matrix matched blank samples which gave (recovery) values lower than 30% of the residue level corresponding to the LOQ_m (5).

Mean recoveries of the samples fortified at the LOQ_m were between 66.5 – 120% and at the $10\times LOQ_m$ between 86.4 – 120%. These results indicate satisfactory accuracy of the method.

The attained LOQ_i values are shown in Table 1 along with the MRLs. The lowest calculated LOQ_i value was 0.004 mg /kg for the analytes lindane and chlorpyriphos and the highest 0.37 mg/kg for the analyte thiabendazole.

Table 1. Retention times (R.T.), E.U. maximum residue level (MRL), minimum required performance level (MRPL) of the organizer and limit of quantification of the instrument (LOQ_i) of the 28 pesticides.

Pesticides	R.T. (min)		E.U. MRL (mg/kg)	MRPL (mg/kg)	LOQ_i (mg/kg)
	DB-5MS	DB-17MS			
Bifenthrin	26.7		0.5	0.05	0.03
Carbaryl		16.6	0.5	0.05	0.1
Chlorpyriphos	12.4	15.7	0.05	0.05	0.002
Chlorpyriphos methyl	10.5	13.9	0.05	0.05	0.003
Cyhalothrin-λ	30.4-31.4	33.8-34.1	0.02	0.02	0.004
Deltamethrin	37.1-37.4		1	0.05	0.003
Diazinon	9	10.5	0.02	0.02	0.006
Endosulfan-α	15.5		0.05	0.05	0.004
Endosulfan-β	18.7		0.05	0.05	0.004
Fenpropimorph		12.26	0.5	0.05	0.003
Imazalil	16.6	22.5	0.02	0.02	0.10
Iprodione	19.4-25.4	33.2	0.5	0.05	0.02
Kresoxim-methyl	18.2	26.2	0.05	0.05	0.025
Lindane	8.6		0.01	0.01	0.002
Malathion	11.9	16.2	8	0.05	0.03
Methacrifos	6.045	6.8	0.05	0.05	0.02
Parathion	12.4	16.4	0.05	0.05	0.006
Penconazole	13.9	18.3	0.05	0.05	0.006
Pirimicarb	11.6	13.6	0.05	0.05	0.05
Pirimiphos methyl	11.6	14.9	5	0.05	0.03
Procloraz	36.8	33.8	0.5	0.05	0.12
Procymidone	14.7	19.4	0.02	0.02	0.02
Propiconazole	22-22.4	29.2-29.5	0.05	0.05	0.07
Thiabendazole		23.6	0.05	0.05	0.37
Triadimefon	12.5	15.6	0.2	0.02	0.005
Triadimenol	14.4-14.7	18.4-19	0.2	0.05	0.03
Triazophos		32.4	0.2	0.1	0.02
Vinclozolin	10.4	12.7	0.05	0.1	0.006

The attained LOQ_m values are shown in Table 2. The lowest calculated LOQ_m value was 0.01 mg/kg for the analyte endosulfan and the highest 0.75 mg/kg for the analyte carbaryl.

As shown in Table 2, relative standard deviation values (RSD) at the LOQ_m level were 1.5 - 8.9% and at the $10\times LOQ_m$ level 0.7 – 8.1%. These results indicate satisfactory precision of the method.

In the 2007 Proficiency Test for cereals, organized by the European Commission Reference Laboratories on Cereals and Feedingstuff and Single Residue Methods, the above described method was applied in our laboratory and the following results were obtained: From the 28 pesticides that were validated deltamethrin, diazinon, pirimiphos methyl and propiconazole were detected in the sample of the proficiency test. All 4 compounds were determined with acceptable z score values, ranging between –0.2 and 1.2, verifying the acceptable accuracy of the method.

In conclusion in this study, 28 active ingredients of plant protection products of various physicochemical characteristics and chemical classes used for the control of pests

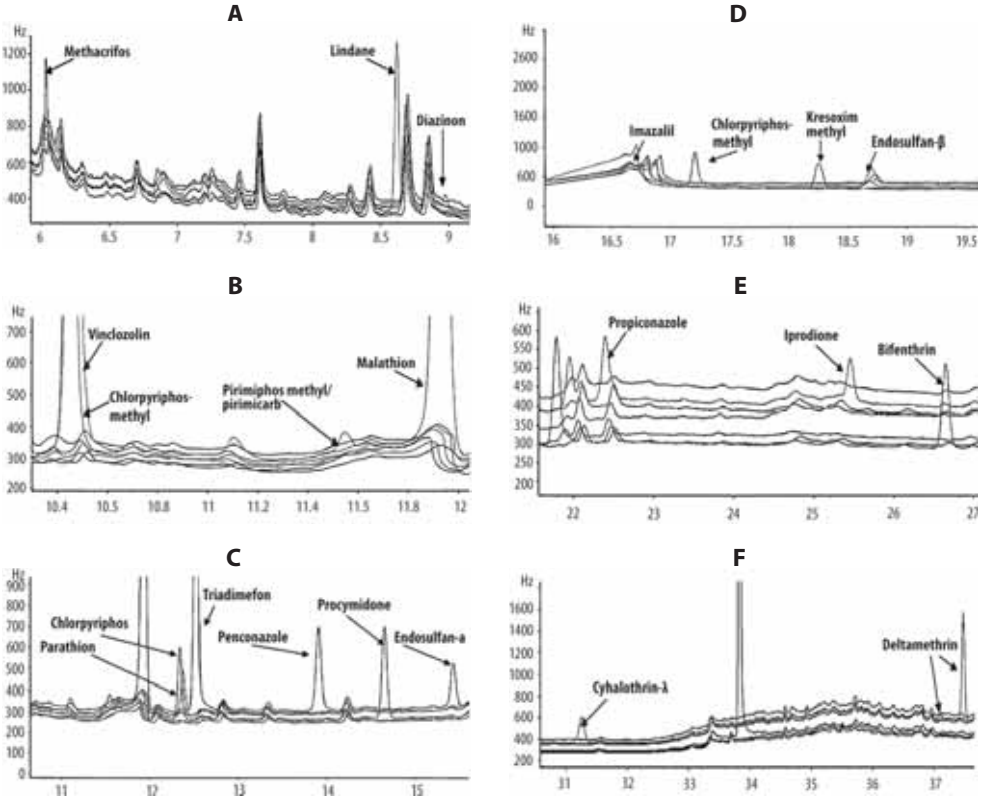

Figure 1. Chromatogram in 6 time segments (A to F) of 22 of the 28 analytes in wheat flour at fortification level equal to the limit of quantification (LOQ_m). Injection splitless column DB-5MS, detector ECD.

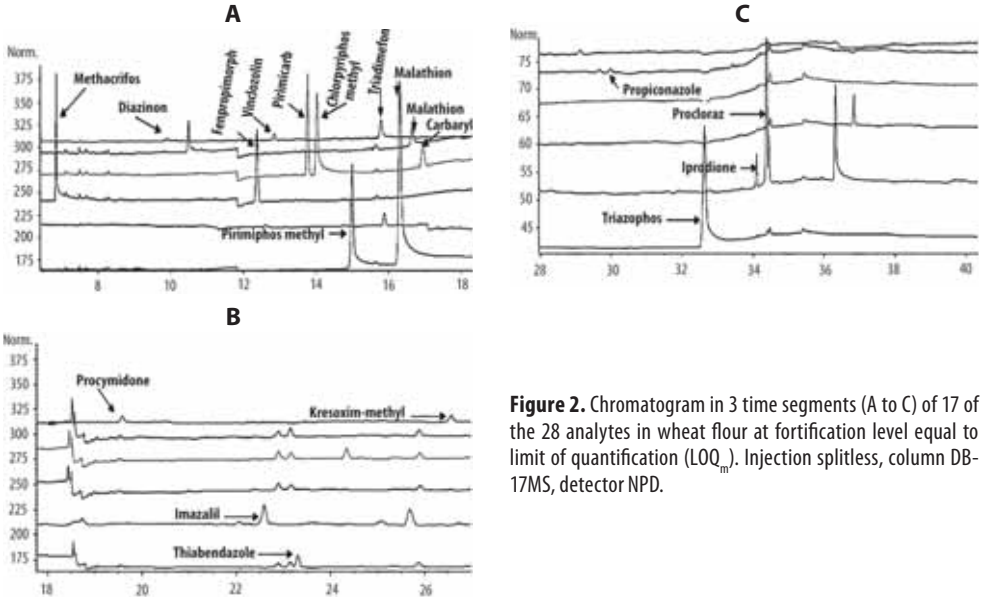

Figure 2. Chromatogram in 3 time segments (A to C) of 17 of the 28 analytes in wheat flour at fortification level equal to limit of quantification (LOQ_m). Injection splitless, column DB-17MS, detector NPD.

Table 2. Average recovery values and relative standard deviation for the 28 pesticides as derived from the fortification experiments (LOQ_m = Limit of Quantification of the method).

Analyte	Detector	LOQ_m (n=6)			$10\times LOQ_m$ (n=6)		
		C (mg/Kg)	Recovery (%)	RSD (%)	C (mg/Kg)	Recovery (%)	RSD (%)
Bifenthrin	ECD	0.045	100.1	6.4	0.45	101.9	3.8
Carbaryl	NPD	0.75	99.2	2.5	7.5	91.4	2.4
Chlorpyriphos	ECD	0.015	104.9	3.7	0.15	102.4	2.1
Chlorpyriphos methyl	NPD	0.075	103.7	3.4	0.75	86.4	2.6
Cyhalothrin-λ	ECD	0.03	90.4	4.9	0.3	116	2.6
Deltamethrin	ECD	0.075	96.9	4.4	0.75	119.9	2.5
Diazinon	ECD	0.03	94.7	2.6	0.3	118.3	0.7
Endosulfan-a	ECD	0.0075	92.9	8.9	0.075	106.8	2.0
Endosulfan-b	ECD	0.0075	66.5	6.8	0.075	107.3	1.9
Fenpropimorph	NPD	0.5	83.2	2.8	5	104.4	1.8
Imazalil	NPD	0.15	119.9	1.5	1.5	112.8	8.1
Iprodione	ECD	0.03	91.0	6.0	0.3	107.9	2.5
Kresoxim-methyl	ECD	0.075	100.5	4.7	0.75	102.0	2.8
Lindane	ECD	0.015	99.5	5.8	0.15	100.9	7.5
Malathion	NPD	0.375	103.9	5.8	3.75	106.1	7.5
Methacrifos	NPD	0.05	89.3	5.2	0.5	96.5	6.2
Parathion	ECD	0.03	95.4	4.6	0.3	119.1	1.7
Penconazole	ECD	0.03	98.2	5.1	0.3	100.1	1.5
Pirimicarb	NPD	0.15	100.8	2.8	1.5	88.5	2.1
Pirimiphos methyl	NPD	0.15	106.3	4.0	1.5	106.9	7.5
Procloraz	NPD	0.5	101.1	4.5	5	119.5	2.6
Procymidone	ECD	0.075	110.3	2.2	0.75	101.0	1.6
Propiconazole	NPD	0.075	100.8	4.1	0.75	114.1	0.8
Thiabendazole	NPD	0.75	112.4	3.7	7.5	88.2	2.7
Triadimefon	ECD	0.075	83.6	8.5	0.75	106.5	1.3
Triadimenol	ECD	0.075	100.9	5.0	0.75	119.8	1.9
Triazophos	NPD	0.15	113.3	4.3	1.5	114.3	4.4
Vinclozolin	ECD	0.075	104.3	3	0.75	100	1.7

and diseases in cereals and included in the European Commission Proficiency test 2007 for cereals were studied. The extraction procedure was based on liquid extraction with acetone followed by dichloromethane and petroleum ether. Water was added in the sample before the extraction for better performance. The method is simple, fast and suitable for routine analysis and with the same extraction method fruits, vegetables and cereals are analyzed. The validation of the method resulted in good accuracy with recoveries of 66.5 – 120% and precision with RSD of 0.7 – 8.9% and sensitivity meeting in most cases the EU legislation requirements for the detection limits. Prompted by the satisfactory performance of the method, we aim at further testing it for the determination of more active compounds of the same chemical classes.

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Επικύρωση μεθόδου προσδιορισμού υπολειμμάτων φυτοπροστατευτικών προϊόντων σε αλεύρι σίτου με την τεχνική της αέριας χρωματογραφίας

Χ.Ι. Αναγνωστόπουλος και Γ.Ε. Μηλιάδης

Περίληψη Μια γρήγορη πολύ-υπολειμματική μέθοδος αναπτύχθηκε και επικυρώθηκε σε αλεύρι σίτου, για τον προσδιορισμό υπολειμμάτων 28 αντιπροσωπευτικών φυτοπροστατευτικών ουσιών οι οποίες περιλήφθηκαν στην διεργαστηριακή δοκιμή που διοργανώθηκε από την Ευρωπαϊκή επιτροπή για υπολείμματα σε δημητριακά το 2007. Η εκχύλιση των ουσιών πραγματοποιήθηκε με ακετόνη και διάλυμα πετρελαϊκού αιθέρα/διχλωρομεθάνιου (50/50). Ο ποιοτικός και ποσοτικός προσδιορισμός των ουσιών πραγματοποιήθηκε με την τεχνική της αέριας χρωματογραφίας σε συνδυασμό με ανιχνευτές σύλληψης ηλεκτρονίων και αζώτου/φωσφόρου. Από τα στοιχεία επικύρωσης προκύπτει ότι η μέθοδος παρουσιάζει αποδεκτή ορθότητα με ποσοστά ανάκτησης 65-120%, καθώς και πιστότητα με σχε-

τικές τυπικές αποκλίσεις μικρότερες από 10%. Το όριο ποσοτικοποίησης της μεθόδου κυμαίνεται από 0.002 ως 0.37 mg/kg ανάλογα με την φυτοπροστατευτική ουσία. Η μέθοδος χαρακτηρίζεται από αξιοπιστία και ευαισθησία και κρίνεται κατάλληλη για αναλύσεις ρουτίνας υπολειμμάτων φυτοπροστατευτικών προϊόντων σε αλεύρι.

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Effect of superphosphate fertilizer on glyphosate adsorption by four Greek agricultural soils

C.N. Giannopolitis[1] and V. Kati[2]

Summary Single superphosphate fertilizer (0-20-0) applied to four distinct surface soils from Greek agricultural fields, at a rate that provided an elevated phosphorus supply (220-260 ppm P), increased glyphosate and aminomethylphosphonic acid (AMPA) adsorption by the two soils and did not have any effect on their adsorption by the other two soils. These effects of superphosphate are contrary to the expected reduced adsorption if phosphorus had competed glyphosate for the same adsorption sites in the soils. The superphosphate-induced increase of adsorption was associated with a parallel decrease of the soil pH which was caused by the fertilizer in the neutral or slightly acidic soils but not to the alkaline and calcareous soils. Further evidence that the effect of superphosphate on glyphosate soil adsorption is brought about by its effect on soil pH was obtained by measuring adsorption after liming of an acidic soil and after strong acidification (using sulfuric acid) of an alkaline soil. The increased glyphosate adsorption in one of the soils amended with superphosphate resulted in an apparent retardation of glyphosate decomposition and AMPA accumulation, indicating that it was sufficient to reduce availability of glyphosate to soil microorganisms. These results provide good evidence that superphosphate fertilizer applied to Greek agricultural soils can affect glyphosate adsorption more positively (by reducing the soil pH) than negatively (by a possible competition for adsorption sites between phosphorus and glyphosate) and thus cannot contribute to an increased risk of glyphosate leaching.

Additional keywords: AMPA, calcareous soils, herbicide leaching, herbicide persistence, liming, soil pH

Introduction

Glyphosate has been one of the world's most applied herbicides since it came into the market in 1974 and its current use is further expanded with the incorporation of resistance genes into genetically modified crops grown in large acreage. It is a non selective foliar-applied herbicide which is readily absorbed through foliage and shoots and translocated throughtout the entire plant. Root absorption does not normally seem to contribute to herbicide uptake by plants, as glyphosate is quickly adsorbed to soil becoming unavailable to roots.

Glyphosate is adsorbed mainly by the mineral phase of the soil, with aluminium and iron oxides to play an important role (9, 12). At present, it is generally accepted that the phosphonic moiety of the glyphosate molecule controls the adsorption by complexation through hydrogen bonding. Soil organic matter seems to have only an indirect effect by a blockage of adsorption sites while the pH of the soil solution is the most important factor for adsorption because it affects the electrical charge of both glyphosate and the soil hydrous oxides (2).

Since glyphosate is adsorbed to soil in a manner similar to phosphorus, phosphate fertilization has been suspected as able to negatively affect the adsorption of glyphosate through competition for similar adsorption sites (7). There has been concern that applying glyphosate on soils rich in inorganic phosphate or in soils with a low unoccupied P-adsorption capacity may result in free glyphosate in the soil solution which can be available for plant root uptake and

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injury to transplanted sensitive crops (3) as well as for leaching into the soil and contamination of underground waters (2, 13). Studies conducted so far have indeed established that a reduced adsorption may be observed when glyphosate is applied to soils that have prior been heavily fertilized with phosphates (4, 8, 11) but the practical implications of this have not been determined. Furthermore, recent studies show that the extent to which glyphosate adsorption is reduced by phosphates can vary dramatically in different soils making prediction even more difficult (2).

However, besides the variability of effects depending on soil type, variable effects might also be expected depending on the type of the phosphate fertilizer that is used. In addition, besides the competition between phosphorus and glyphosate, phosphate fertilizers might also affect glyphosate adsorption by other means, eg. by altering the soil pH and this may be true with a variety of other fertilizers as well. We examined this possibility by using superphosphate fertilizer, which is widely used for the basal fertilization of many crops at planting, with typical agricultural surface soils from Greece. The fertilizer was used at a high rate to assure maximum possible competition between phosphorus and glyphosate for the adsorption sites in all four soils and the results are presented here.

Materials and Methods

Origin of soil samples

The samples of soil used in these studies were collected in mid June from the top 10-cm layer of fields planted to peach orchards (region of Himathia in Northern Greece, designated as H1, H2, H3) or vineyards (region of Korinthia in Southern Greece, designated as K1, K2) and of uncultivated highland fields used as pastures (region of Kalavryta in Southern Greece, designated as KA1). The fields from which the soil samples were taken had been used as described for many years and received regular cultivation and fertilization according to the established practices in the respective area. Pasture fields (KA1 soil) were grazed by sheep and had received no fertilization or other treatment for years. Some basic characteristics of the soil types used are presented in Table 1.

Soil treatments

Soil samples were air-dried and sieved through a 2-mm sieve before use. Soil pH was determined by preparing 1:1 soil suspensions in deionized water and measuring with a pH/mV meter equipped with a combined pH electrode and automatic temperature compensation.

Superphosphate amendment of the soils was made using granular single superphosphate fertilizer (0-20-0) from the Phosphoric Fertilizers Industry SA (Greece). To ensure uniform distribution of the fertilizer the granules were first ground to a fine powder. The appropriate amount of the powder was thoroughly mixed with 100 g of soil and placed in a plastic cup (7-cm height, 6-cm upper diameter) with 4 holes at the bottom (for watering) covered with a filter paper. The soil was watered (from below) to the field capacity and kept for 1-4 weeks in a growth chamber with a light period of 16 hours, day temperature of 25° C and night temperature of 20° C.

Liming of the KA1 soil was made by using

Table 1. Basic characteristics of the soil types used in the experiments.

Soil type	Origin	pH	Texture	Other
H1	Peach orchard	7.2	Heavy clay	Dark fertile soil, high Al and Ca
H3	Peach orchard	8.2	Sandy clay	Dark poor soil
K1	Vineyard	7.8	Heavy calcareous	Whitish color, very high $CaCO_3$
K2	Vineyard	7.6	Heavy calcareous	Whitish color, very high $CaCO_3$
KA1	Pasture	5.9	Loamy	Red, washed soil, high Fe, low Ca

analytical grade $CaCO_3$ from Acros Organics (Belgium) and acidification of the H3 soil by using a 2 N solution of sulfuric acid (Merck, pro-analysis, 95-97%). After thorough mixing, 100 g samples of the treated soils were placed in the above plastic cups, watered to field capacity and left to equilibrate for 4-7 days in the growth chamber.

All the treated soil samples along with the respective controls were dried in the oven (40° C) for 4 hours and sieved through a 2-mm sieve before used for batch equilibration tests to determine glyphosate and AMPA adsorption.

Measurement of glyphosate and AMPA adsorption

The capacity of the various soil samples to adsorb glyphosate and AMPA was determined by conducting batch equilibration tests using aqueous glyphosate and AMPA solutions of various concentrations. The tests were performed by placing 1 or 1.5 g of the soil sample and 10 ml of the glyphosate + AMPA solution in 25-ml glass test tubes. The tubes were kept in an orbital shaker for 2 hours to equilibrate (12), then centrifuged at 5000 rpm for 10 min and the supernatants collected and analysed. The amount of glyphosate and AMPA adsorbed by the soil was calculated by substracting the amount found in the supernatant from that in the initial solution.

The glyphosate and AMPA solutions used in these studies were prepared using analytical reference standards (Monsanto, certified as 99.8 and 99.5% respectively). An aqueous stock solution containing 500 μg/ml of each, of the two compounds, was prepared in HPLC-grade water and working solutions of various concentrations were prepared by diluting with de-ionized water.

Glyphosate and AMPA were quantitatively determined using cation exchange HPLC and fluorescence detection following post-column derivatization with hypochloride and o-phthalaldehyde (OPA), which is an improved version of the US EPA method 547 (10, 14.). The instrumentation was as described before (5). Each sample solution was first diluted with de-ionized water as needed and filtered through a 0.22 μm disposable syringe filter with a PTFE membrane, into a 2 ml amber borosilicate glass vial and then directly injected into the HPLC system at 20-50 μl.

All tests were set in a completely randomized design with the treatments replicated three times. The combined data from each test were subjected to ANOVA and in most cases to an LSD comparison of the treatment means. Most of the conducted tests were repeated three times and the results obtained from a typical run of each test are presented here.

Results and Discussion

Addition of superphosphate fertilizer to the soil increased the amount of glyphosate adsorbed by the soils KA1 and H1 but did not affect glyphosate adsorption by the soils K2 and H3 (Table 2). The increased glyphosate adsorption by the soils KA1 and H1 was evident one week after the addition of superphosphate and lasted for at least one month. In further experiments with shorter time intervals after the addition of superphosphate, it was realized that the glyphosate adsorption started to increase as soon as two days after its addition (data not presented).

The addition of superphosphate also caused a decrease in the pH of all four soils (Table 2), although to a varying extent depending on the soil. This pH-decreasing effect of the superphosphate seems to be accompanied by an increase of glyphosate adsorption only in the two soils (KA1 and H1) in which the pH was lowered to a value below about 7.0.

The increased glyphosate adsorption, after the addition of superphosphate, by the soils KA1 and H1 but not by the soils K2 and H3, was further confirmed by comparing the adsorption isotherms obtained with three glyphosate concentrations and soils amended or not with superphosphate (Figure 1).

The applied rate of superphosphate fer-

Table 2. Glyphosate adsorption and pH of four soils, at weekly intervals after the addition of 0.3 g of superphosphate per 100 g of soil (260 ppm P). Sorption was determined with batch equilibration experiments using 1 g of soil and 10 ml of a 20 ng/ml solution of glyphosate.

Soil	KA1		H1		K2		H3	
	Control	+P	Control	+P	Control	+P	Control	+P
Days	Glyphosate adsorbed ng/g							
8	102.5	131.8	138.2	152.1	94.7	90.1	41.6	37.9
15	97.8	125.2	108.1	135.6	97.1	92.5	41.6	36.6
21	98.0	129.3	116.6	157.3	96.9	96.8	38.1	39.5
30	101.5	129.1	109.2	172.0	99.6	100.5	45.5	39.3
Mean	*100.0*	*128.9***	*118.0*	*154.3**	*97.1*	*95.0 NS*	*41.7*	*38.3 NS*
Days	pH (H_2O)							
8	5.7	5.2	7.1	6.7	7.6	7.4	8.2	7.5
15	5.7	5.1	7.2	6.6	7.6	7.5	8.3	7.3
21	5.8	4.9	7.3	6.4	7.6	7.5	8.2	7.3
30	5.8	5.0	7.2	6.4	7.6	7.5	8.2	7.4
Mean	*5.8*	*5.1***	*7.2*	*6.5***	*7.6*	*7.5**	*8.2*	*7.4***

Means for +P are statistically different at the 0.05 (*) and 0.01 (**) levels, or non-statistically different (NS), according to a t-test comparison with the respective control means.

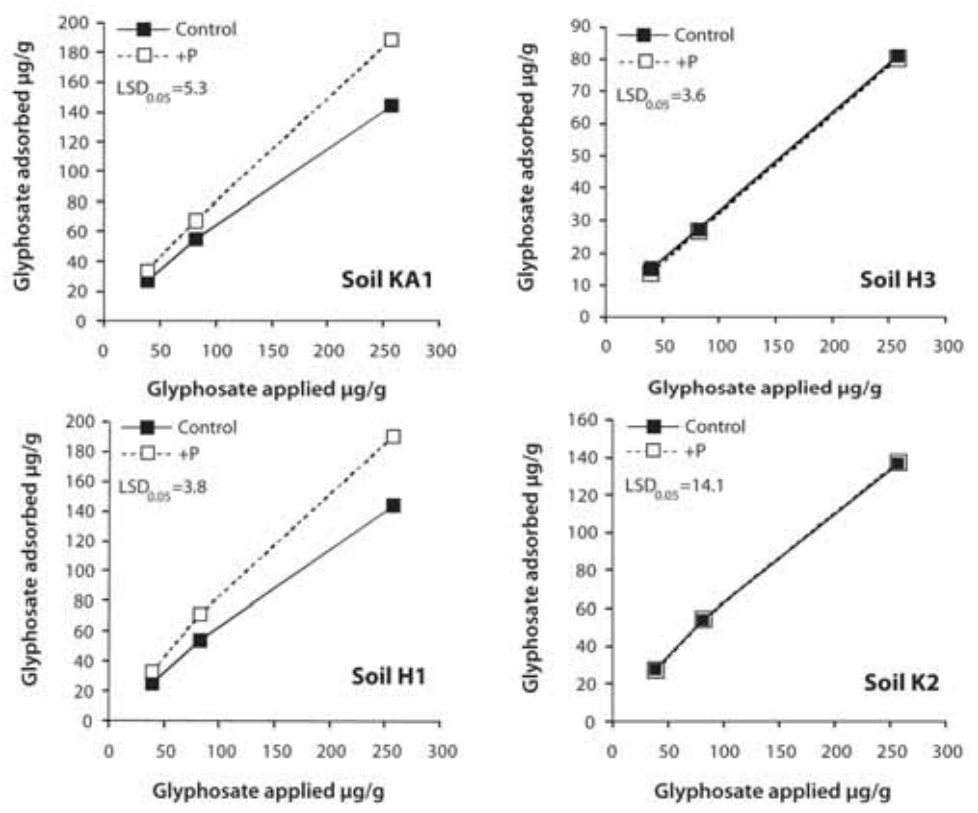

Figure 1. Glyphosate adsorption by the four soils, 3 weeks after the addition of superphosphate at 0.3 g/100 g (260 ppm P). Adsorption was determined with batch equilibration experiments using 1 g of soil and 10 ml of one of three glyphosate solutions (4.0, 8.3 and 25.7 µg/ml).

tilizer in the above experiments was 0.3 g of the 0-20-0 granular formulation per 100 g of soil. This corresponds to a supply of about 260 ppm of P which is well in excess of the recommended P fertility level of up to 50 ppm (1). It is of interest that at this superphosphate rate, at which P is assumed to maximally compete glyphosate for the soil adsorption sites, adsorption of glyphosate was not actually reduced.

In another series of experiments in which various levels of superphosphate fertilization were utilized, the increased adsorption by the soils KA1 and H1 was observed at all superphosphate levels tested (0.25, 0.5 and 1.0%) and with both glyphosate and AMPA (Figure 2). This effect of superphosphate in soils KA1 and H1 was most pronounced at high glyphosate and AMPA concentrations and this is consistent with the high adsorption capacity of these two soils. It is of interest, therefore, that in these two soils in

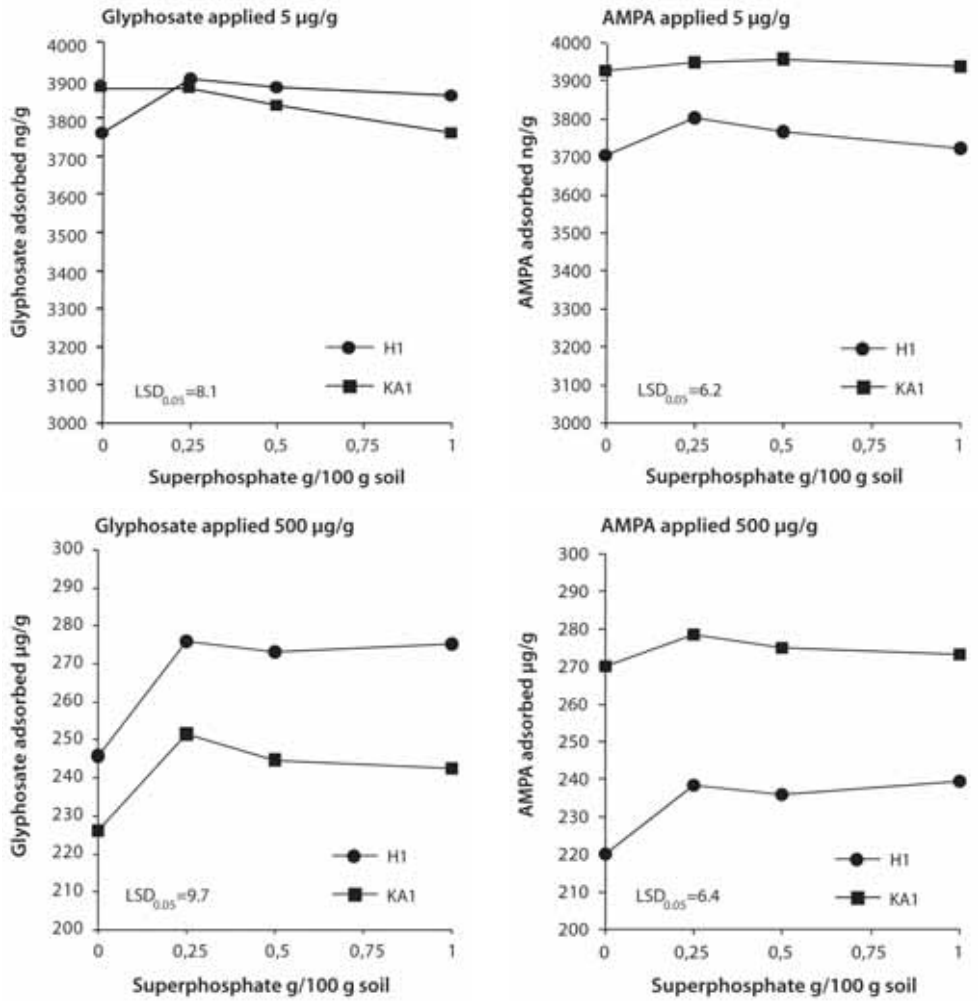

Figure 2. Glyphosate and AMPA adsorption on KA1 and H1 soil samples that had been previously amended with the indicated amounts of superphosphate and let to equilibrate for 5 days. Glyphosate and AMPA adsorption was measured by batch equilibration experiments utilizing 10 ml/g of solutions at the proper concentrations to supply the indicated amounts of each chemical.

which adsorption is increased with the addition of supephosphate, the rate of superphosphate does not seem to be as critical as the rate of glyphosate and AMPA. It is also worthy to note that soil H1 was found to adsorb more glyphosate than AMPA while the reverse was observed with soil KA1.

The same experiments with the soils H3 and K1, which are of a low adsorption capacity (a sandy and a calcareous soil, respectively), indicated that superphosphate at all tested levels had only a slight decreasing or increasing effect on glyphosate and AMPA adsorption which could be seen only at low concentrations of the two chemicals (Figure 3). Again, the rate of superphosphate is not so critical as that of glyphosate and AMPA.

The addition of increasing concentrations (0.25, 0.5 and 1.0%) of superphosphate had also a parallel decreasing effect on the pH of the soils (Figure 4). In soils KA1 and H1 in which adsorption is increased with the addition of superphosphate, the pH was actually lowered to values which are more fa-

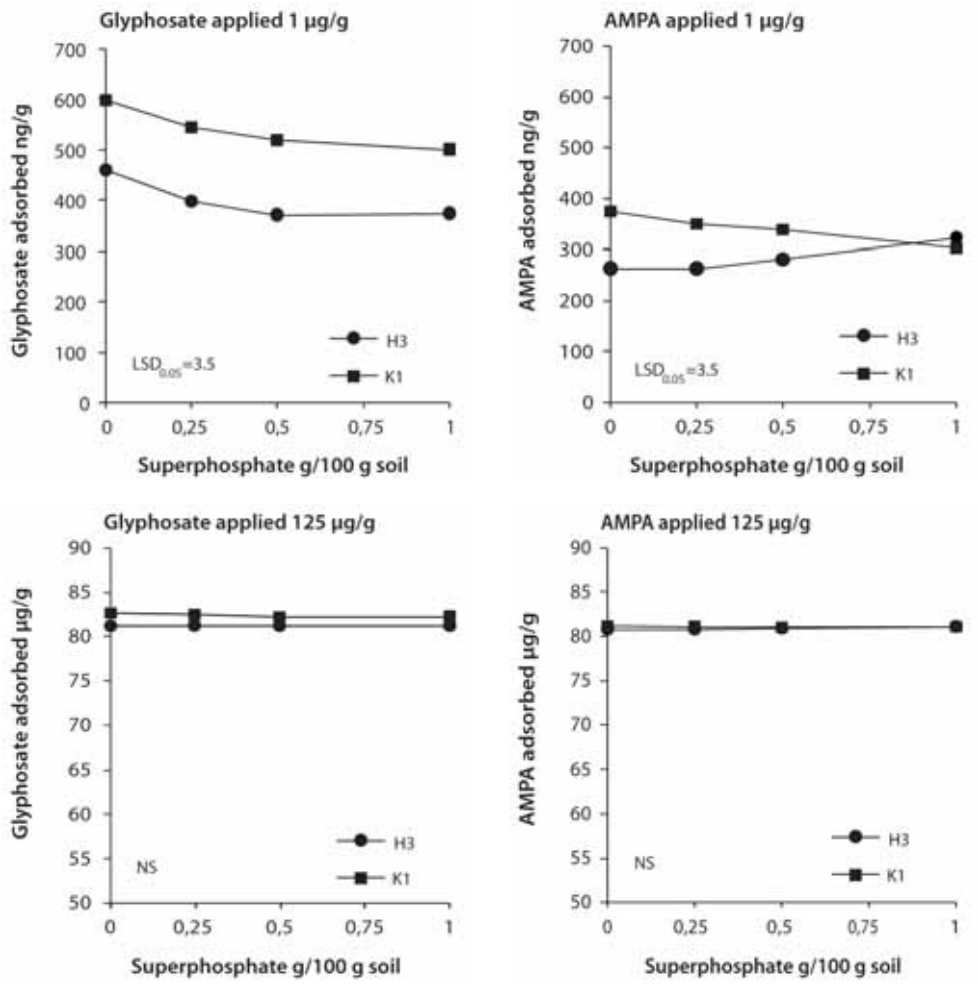

Figure 3. Glyphosate and AMPA adsorption on K1 and H3 soil samples that had been previously amended with the indicated amounts of superphosphate and let to equilibrate for 5 days. Glyphosate and AMPA adsorption was measured by batch equilibration experiments utilizing 10 ml/g of solutions at the proper concentrations to supply the indicated amounts of each chemical.

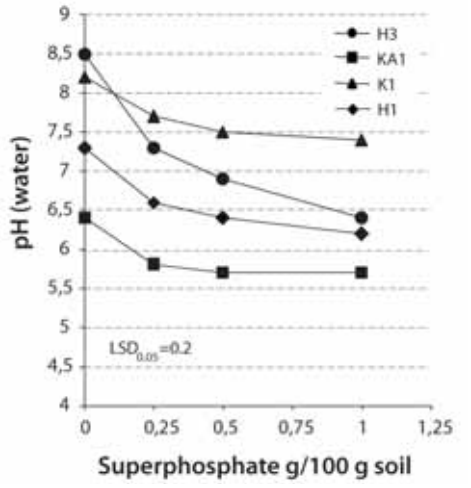

Figure 4. Effect of the superphosphate amendment (at three levels equivalent to 220-880 ppm P) on the pH of the four soils.

vourable for adsorption. Many researchers have already shown that glyphosate adsorption is stronger in acidic soils, with a pH well below 7.0, where electrical charge of both glyphosate and the soil aluminium and iron oxides are most favorable for complex formation (4, 6, 9).

To obtain a better insight of the possible correlation of the two superphosphate effects (a decrease of pH against an increase of glyphosate adsorption), the most acidic soil (KA1) was limed and the most alkaline and least adsorptive soil (H3) was acidified. Glyphosate adsorption was then compared with soil samples that had been limed or acidified at various levels and subsequently amended or not with superphosphate.

Liming of the KA1 soil with 0,5% $CaCO_3$ caused a sharp increase of pH and liming with 1 or 2% of $CaCO_3$ caused a slight further increase to the saturation pH of about 7.7 (Figure 5A). Glyphosate adsorption on these soil samples followed a pattern that mirrored that of the pH (Figure 5B). Samples amended with superphosphate had the same pH and glyphosate adsorption patterns but shifted to lower pH values and higher adsorption values, as it would be expected from a $CaCO_3$ neutralization by the

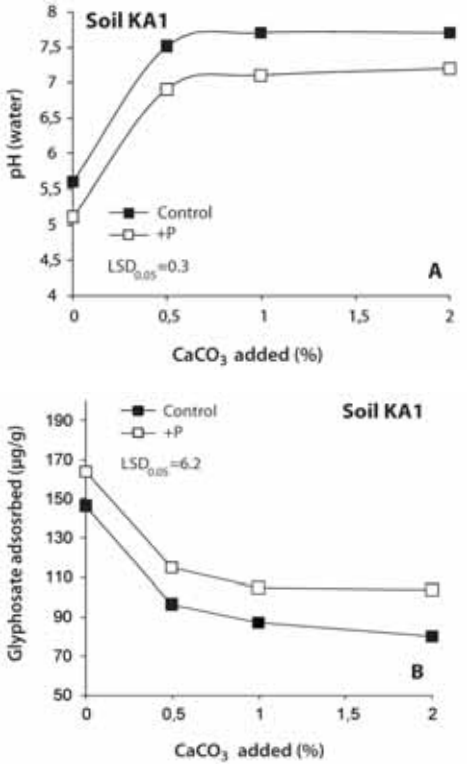

Figure 5. Effect of 0,3 g/100 g of superphosphate, added to KA1 soil which had previously been limed with increasing amounts of CaCO3 (0, 0.5, 1 and 2), on soil pH (A) and glyphosate adsorption (B).

superphosphate, which is a good evidence that the superphosphate-induced decrease of the soil pH leads to the increased glyphosate adsorption.

To acidify soil H3, samples were equilibrated with various sulphuric acid concentations which at saturation decreased the pH from 8.2 to 7.6. The pH of the same samples, after a subsequent superphosphate amendment, was even lower, ranging between 7.3 and 6.8 (Figure 6A). Acidification of the H3 soil increased glyphosate adsorption as expected (Figure 6B). The increase of adsorption brought about by increasing the acidification level was parabolic in the control soil samples (not amended with superphosphate) but almost linear in the superphosphate amended samples. As it can be noted in Figure 6B, regarding soil H3, at a certain

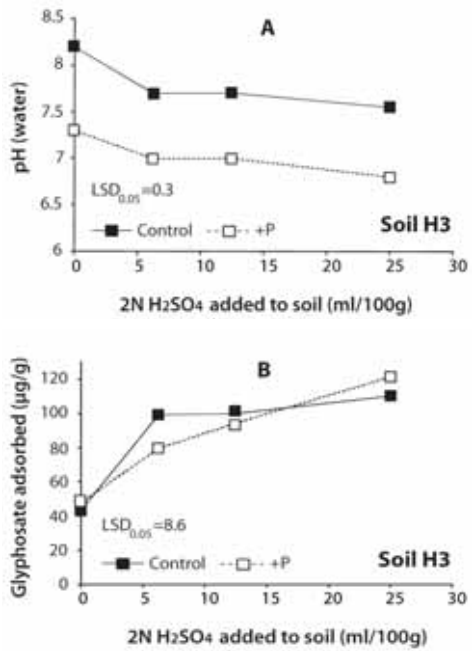

Figure 6. Effect of 0.3 g/100 g of superphosphate, added to H3 soil samples which had previously been acidified with increasing volumes of 2N H2SO4 (0, 6.25, 12.5 and 25 ml/100g), on soil pH (A) and glyphosate adsorption (B).

level of acidification (the resulting with the addition of 12.5 ml 2 N H_2SO_4/100 g of soil) superphosphate amendment seems not to differentiate glyphosate adsorption from that in the unamended control. At lower acidification levels superphosphate seems to have a slight negative effect and at higher levels a positive effect on glyphosate adsorption. It appears therefore that even in this alkaline soil, glyphosate adsorption can be increased by the addition of superphosphate if combined with an acidifying agent of sufficient strength to reduce the soil pH below 7.0.

The increased glyphosate adsorption induced by superphosphate in soil KA1 was further reflected in a slower decomposition of glyphosate to AMPA when this soil was amended with superphosphate. As indicated in Figure 7, glyphosate dissipated quickly in this soil and within two weeks most of the applied herbicide (3.1 µg/g) was decom-

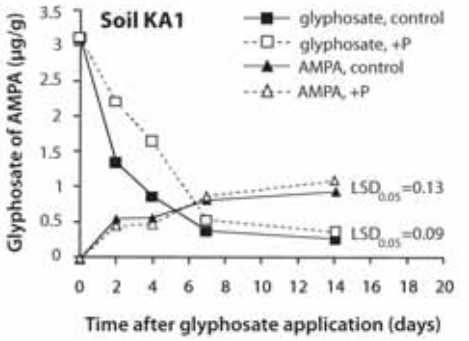

Figure 7. Dissipation of glyphosate and accumulation of AMPA in soil KA1 amended or not with 0,3 g/100 g of superphosphate fertilizer.

posed with a parallel accumulation of AMPA. Addition of superphosphate to the soil (one week before glyphosate application), which has been shown to increase glyphosate adsorption, caused an apparent decrease of the initial rate of glyphosate decomposition and AMPA accumulation, i.e. a slight retardation of both processes. This is a reasonable effect to expect since more adsorption means less herbicide available to soil microorganisms for decomposition. Not examined in theses studies but already well documented by others, increasing glyphosate adsorption in the soil may also mean reducing the risk of leaching and of underground water contamination. Increasing adsorption may also mean reducing the risk of root uptake and toxicity to crop plants transplanted to the soil soon after glyphosate application.

The results presented above clearly demonstrate that superphosphate fertilizer, even when applied at high rates, can not lead to any significant reduction of glyphosate adsorption to soil as it would be expected from a competition between glyphosate and phosphorus for the available adsorption sites. Contrary to that, excessive superphosphate fertilization of certain agricultural soils from Greece significantly increases glyphosate adsorption and this increase seems to depend more on the rate of glyphosate than on the rate of superphosphate. A study by Gimsing *et al.* (2004), us-

ing contrasting Danish surface soils, have revealed that adsorption of glyphosate and phosphate can be both competitive and additive, with the competition not always been as pronounced. Adsorption of the two anions seems actually to be only partially competitive.

Increased glyphosate and AMPA adsorption following the addition of superphosphate fertilizer was observed with the two most acidic soils (KA1 and H1) and the fact that this superphosphate amendment decreased the pH of these two soils to even lower values in the acidic range seems to contribute to that. It is well established that glyphosate and phosphate adsorption is mostly contributed to aluminium and iron in acid soils and to calcium in alkaline soils. Changing the pH of the soil from the alkaline to the acidic range would increase adsorption since higher charged cations (Al^{3+}, Fe^{3+}) are capable of complexing more glyphosate than lower charged cations (Ca^{2+}). Furthermore, with decreasing pH both the clay and glyphosate become less negatively charged and thus more interactive (more adsorption). The pH and glyphosate adsorption patterns of KA1 limed soil samples (Figure 5) and of the H3 acidified soil samples (Figure 6) in this study further document the validity of theses statements.

The strong acidifying action of the superphosphate fertilizers in the soil is already known (1). When granules of the fertilizer are incorporated into the soil, the sparingly soluble calcium dihydrogen phosphate [$Ca(H_2PO_4)_2$], which they contain, absorbs water and is hydrolyzed to calcium hydrogen phosphate [$CaHPO_4$] and ortho-phosphoric acid (H_3PO_4). The three phosphate compounds coexist in an equilibrium, forming the so called "triple point solution", with a pH of 1.0-1.5, which diffuses around the granules in the soil. It seems, therefore, that in acidic and neutral soils the ortho-phosphoric acid, during diffusion of the triple point solution, lowers the soil pH and solubilizes aluminium and iron oxides thus increasing the adsorption capacity of the soil. In alkaline calcareous soils, however, the ortho-phosphoric acid is more quickly neutralized by calcium carbonate and precipitates as insoluble tricalcium phosphate, thus being unable to affect glyphosate adsorption.

Many other fertilizers are known to alter soil pH and it would be of interest to examine how they affect glyphosate and AMPA adsorption.

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Επίδραση του υπερφωσφορικού λιπάσματος στην προσρόφηση του ζιζανιοκτόνου glyphosate από τέσσερα ελληνικά αγροτικά εδάφη

Κ.Ν. Γιαννοπολίτης και Β. Κατή

Περίληψη Εφαρμογή απλού υπερφωσφορικού λιπάσματος (0-20-0) σε τέσσερα χαρακτηριστικά επιφανειακά εδάφη από καλλιεργούμενους αγρούς στην Ελλάδα, σε δόσεις που εξασφαλίζουν υψηλή συγκέντρωση φωσφόρου στο έδαφος (220-260 ppm P), προκάλεσε σημαντική αύξηση της προσρόφησης του glyphosate και του AMPA (ο κύριος μεταβολίτης του) στα δύο εδάφη και δεν επηρέασε την προσρόφησή τους από τα άλλα δύο εδάφη. Η επίδραση αυτή του υπερφωσφορικού είναι αντίθετη από την αναμενόμενη μείωση της προσρόφησης εάν ο φωσφόρος ασκούσε έντονη ανταγωνιστική δράση στο glyphosate για τις ίδιες θέσεις προσρόφησης στα εδάφη. Η αύξηση της προσρόφησης συνδεόταν με μια παράλληλη μείωση του pH την οποία προκαλούσε το λίπασμα στα δύο ουδέτερα ή ελαφρά όξινα εδάφη και όχι στα δύο αλκαλικά ασβεστούχα εδάφη. Με ασβέστωση (χρησιμοποιώντας $CaCO_3$) ενός όξινου εδάφους και με οξύνιση (χρησιμοποιώντας H_2SO_4) ενός αλκαλικού εδάφους αποκτήθηκαν πρόσθετες ενδείξεις ότι το pH παίζει καθοριστικό ρόλο στην προσρόφηση του glyphosate και ότι η αυξημένη προσρόφηση μετά τη χρήση υπερφωσφορικού οφείλεται στη μείωση του pH που αυτό προκαλεί. Η αυξημένη προσρόφηση του glyphosate, μετά την εφαρμογή υπερφωσφορικού, σ' ένα από τα εδάφη, κατέληξε επιπλέον σε εμφανή επιβράδυνση της διάσπασης του glyphosate και της συσσώρευσης AMPA, γεγονός που αποδεικνύει ότι η αύξηση της προσρόφησης ήταν αρκετή για να μειώσει τη διαθεσιμότητα του glyphosate στους μικροοργανισμούς του εδάφους. Τα αποτελέσματα αυτά δείχνουν ότι εφαρμογή υπερφωσφορικού λιπάσματος σε ελληνικά αγροτικά εδάφη είναι δυνατόν να επηρεάσει την προσρόφηση του glyphosate περισσότερο θετικά (μειώνοντας το pH του εδάφους) παρά αρνητικά (λόγω πιθανού ανταγωνισμού μεταξύ φωσφόρου και glyphosate για τις θέσεις προσρόφησης) και επομένως δεν είναι δυνατόν να συμβάλει σε αυξημένη έκπλυση του ζιζανιοκτόνου.

Hellenic Plant Protection Journal **2:** 23-32, 2009

Hellenic Plant Protection Journal **2:** 33-35, 2009

SHORT COMMUNICATION

First records of armoured scale insects (Hemiptera: Coccoidea: Diaspididae) from the oil-rose, *Rosa damascena,* in Turkey

O. Demirözer[1], M.B. Kaydan[2], I. Karaca[1] and Y. Ben-Dov[3]

Summary The olive Parlatoria scale, *Parlatoria oleae* (Colvée) and the apple oyster-shell scale *Lepidosaphes ulmi* (L.) (Hemiptera: Coccoidea: Diaspididae) are recorded for the first time infesting the oil-rose, *Rosa damascena,* at Isparta, Turkey. These armoured scale insects are considered as potential pests to the cultivation of the oil-rose.

The oil-bearing rose, *Rosa damascena* Mill. (Rosaceae) is an agricultural crop cultivated in various countries of the northern hemisphere, such as Turkey, Bulgaria, Morocco, Iran, Egypt, France, China and India. Turkey and Bulgaria are the major producers of this crop in the world. The annual production in Turkey is estimated at 1.5 – 2 tons of rose oil, and Bulgaria produces approximately 1 – 1.5 tons (3, 7, 11).

The cultivation of *R. damascena* is damaged by several diseases and insect pests (e.g. 9). However, only one species of scale insects (Hemiptera: Coccoidea), the soft scale *Rhodococcus perornatus* (Cockerell & Parrott), has been recorded as a pest of oil-rose (4).

Here we report on infestations of twigs of *R. damascena* with two species of armoured scale insects (Hemiptera: Diaspididae), namely, *Lepidosaphes ulmi* (L.) (Figures 1, 2), and *Parlatoria oleae* (Colvée) (Figures 3, 4) at Isparta, Turkey. *L. ulmi* was found in 14 plots, among 40 orchards inspected, at population density of 75-100 scales per 10 cm length of a twig. *P. oleae* was remarkably less common, being found at only one plot of this crop at Isparta.

The two species, mentioned above, are widely distributed mainly in the Palaearctic, Nearctic and Oriental zoogeographical regions, and are highly polyphagous. *P. oleae* has been recorded from about 150 host plant species that belong to 51 families, while *L. ulmi* is known from about 270 species of host plants belonging to 63 families (5, 6). Therefore, we assume that their infestation on the oil-rose in Turkey was previously overlooked.

Both species are considered serious pests of fruit and ornamental trees. However, insect natural enemies play a significant role in their biological control (1, 2, 5, 6, 8, 10).

Figures 2 and 3 show the scale covers of both species with exit holes of hymenopterous parasitoids. Therefore, we draw the attention of growers, that if chemical control measures are applied within the frame of pest management in the cultivation of oil-rose, these should be cautiously recommended and applied, in order to prevent the upset or resurgence of these potential pests from becoming destructive pests in the oil-rose cultivation.

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Figure 1. Twig of *Rosa damascena,* Turkey, Isparta, infested with *Lepidosaphes ulmi* (L.).

Figure 2. Female scale covers of *Lepidosaphes ulmi* (L.).

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Figure 3. Twig of *Rosa damascena*, Turkey, Isparta, infested with *Parlatoria oleae* (Colvée).

Figure 4. Female scale covers of *Parlatoria oleae* (Colvée).

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ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Πρώτη αναφορά δύο κοκκοειδών Diaspididae από την τριανταφυλλιά ροδελαίου, *Rosa damascena*, στην Τουρκία

O. Demirözer, M.B. Kaydan, I. Karaca and Y. Ben-Dov

Περίληψη Τα κοκκοειδή *Parlatoria oleae* (Colvée) και *Lepidosaphes ulmi* (L.) (Hemiptera: Coccoidea: Diaspididae) αναφέρονται για πρώτη φορά ότι προσβάλλουν την τριανταφυλλιά ροδελαίου, *Rosa damascena*, στην Isparta της Τουρκίας. Τα κοκκοειδή αυτά θεωρούνται ικανά να προκαλέσουν ζημιά σε καλλιέργειες της τριανταφυλλιάς ροδελαίου.

Hellenic Plant Protection Journal **2**: 33-35, 2009

Hellenic Plant Protection Journal **2:** 37-40, 2009

SHORT COMMUNICATION

Galinsoga ciliata (Raf.) S.F. Blake and *Sida spinosa* L., two new weed records from Greece

S. Lymperopoulou and C.N. Giannopolitis

Summary Two weed species are reported for the first time to occur in Greece. *Galinsoga ciliata* (Raf.) S.F. Blake (Asteraceae) was found at high densities in vegetable crops in the area of Marathon, near Athens. *Sida spinosa* L. (Malvaceae) was found to be present as few scattered plants in cotton fields in the valley of Louros, near Preveza (Southwestern Greece) and in the area of Palamas, near Carditsa (Central Greece). Both species are considered as invasive alien plants, not previously included in the flora of mainland Greece (the former) and of Greece (the latter). Distinguishing characteristics of the two species are presented.

Additional keywords: alien plants, Asteraceae, *Galinsoga parviflora*, Greek flora, Malvaceae, *Sida rhombifolia*

Galinsoga ciliata (Raf.) S.F. Blake [synonym *G. quadriradiata* auct., non Ruiz et Pav.], Asteraceae, was found for the first time in October 2003 in cabbage crops in the area of Marathon, a vegetable producing area near Athens. Observations during the years 2004-08 indicated that the species is established in this area primarily in fields grown to vegetables, where it occurs at high densities during summer and autumn. Occasionally it is also found in greenhouses grown to vegetables or ornamentals and in uncultivated land. The plant seems to produce many seeds which germinate soon after their ripening and falling to the soil, as one can find plants of all stages to be present in a field at the same time. The system of continuous intensive vegetable growing, which is applied in the area, with frequent fertilization and irrigation, apparently favors the plant to attain a proliferous growth.

G. ciliata, a native to South and Central America, has now become one of the most common weeds in the United States, Canada and many European countries (6). It is regarded as an invasive alien plant species already established in most European countries, but not known to be present in Greece yet (2). Although there has been a report of its presence in the Greek island of Samos since 1993 (10) and in the Izmir province (West Anatolia) of Turkey since 2003 (7), its presence in mainland Greece is reported here for the first time.

The only other species of the genus, *G. parviflora* Cav., which is also an important weed, morphologically very similar to *G. ciliata*, has been reported to occur in Greece since 1983 (5) and is now thought to have spread throughout the country. It is very likely, therefore, that *G. ciliata* has been present in Greece for long but remained unrecognized from *G. parviflora*.

Both species are annual plants reproducing by seed. They have upright stems with many branches and reach a height of 10-80 cm at maturity. They are recognized from the opposite simple ovate leaves and the small (<1 cm) flower heads consisting of 4-5 white, 3-toothed ray florets and many yellow disk florets (Figure 1). Distinction of the *G. ciliata* plants was mainly based on the following specific characters (11):

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Figure 1. Part of a *G. ciliata* plant with the hairy stem, the opposite leaves and the small flower heads. More details of the flower head, particularly the white 3-toothed ray florets are shown in 1a.

- Peduncles with numerous long (more than 0.5 mm) patent glandular hairs (Figure 2),
- Receptacular scales entire (Figure 3),
- Pappus scales aristate

as opposed to few short (less than 0.5 mm) hairs on the peduncles, 3-fid receptacular scales and not aristate papus scales in *G. parviflora*. Furthermore, *G. ciliata* plants are larger (reach a height of 80 cm) with more branched stems covered with long glandular hairs and rather triangular leaf blades with a broader base and dentate (not serrate) at the margins.

Sida spinosa L. (synonyms *S. alba* L., *S. angustifolia* Lam., *S. angustifolia* Mill.), Malvaceae, was first found in a cotton field in the valley of Louros river, near Preveza (South Western Greece), in September 2003. During a weed survey in this area, at that time, only few scattered plants were present in a small acreage of cotton crops in the specified location. Furthermore, in summer 2004,

Figure 2. The long glandular hairs on the peduncles of *G. ciliata* (2a) as opposed to the short ones on the peduncles of *G. parviflora* (2b).

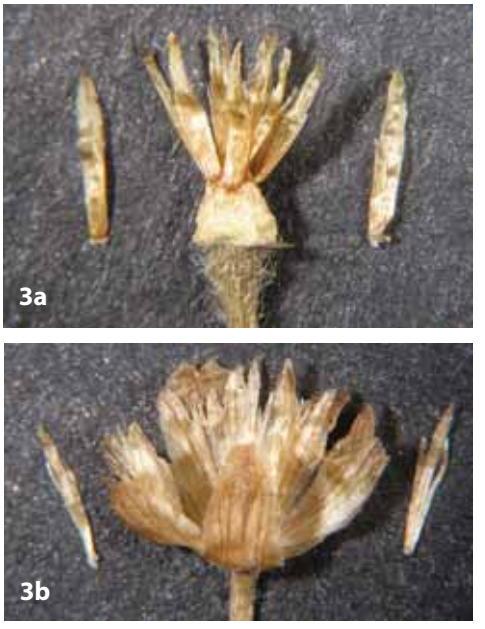

Figure 3. Receptacular scales, entire in *G. ciliata* (3a) and trifid in *G. parviflora* (3b).

a specimen of the same species arrived at the laboratory for identification from cotton crops in the area of Palamas, near Karditsa (Central Greece). A visit to Palamas in September 2005 verified the presence of the

Figure 4. A shoot of *S. spinosa* bearing leaves, the short stipules (arrow) at the base of the petioles and flower buds. Details of the flower are shown in 4a.

Figure 5. Fruit of *S. spinosa* at various stages of maturity (top) and the 2-spined mericarps (bottom). Each fruit is breaking up into 5 mericarps.

species at low densities in cotton crops. The plant seemed to grow normally in the area, reaching maturity and producing seeds. Observations and information received in subsequent years from the above two areas indicated the continuing occurrence of the species at low densities with no evidence for a fast spreading up to present.

S. spinosa, a native to tropical countries of South America, has become a common weed particularly in cotton and soybean fields in the USA, Mexico, Argentina, Chile, Peru and Uruguay, as well as in Australia (4). In Europe it has been reported only from Romania (8). It is regarded as an invasive weed presenting a risk mainly for the Mediterranean region (4).

It is an annual species reproducing by seeds (9). The plant reaches a height of about 1 m, with an upright stem, woody at the base, much branched and covered with hairs. The leaves are alternate, elongated, 2-4 cm long, with toothed margins. At the base of the petiole there are two filiform stipules shorter than the petiole (Figure 4). Flowers are axillary, single or in small clusters at the end of short pedicels, with 5 white to light yellow petals. The fruit is a capsule consisting of a ring of 5 mericarps, each with two sharp spines at the tip (Figure 5). The ring breaks up at maturity releasing one seed per mericarp. The number and the shape of the mericarps are the safest characteristics that distinguish *C. spinosa* (3) from *C. rombifolia* (12) and possibly other species.

Based on the above information it seems most likely that *G. ciliata* has been introduced to Greece since some time ago and is already established in the country while *S. spinosa* has recently entered the country and is now spreading and acclimatized. Results of a field survey through other important agricultural areas of the country are needed before a sound conclusion on the distribution and importance of the two species can be drawn.

Other important weed species additions to the Greek flora during the last years and the need for measures to effectively prevent their spread have been reviewed in a previous article (1).

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ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Galinsoga ciliata (Raf.) S.F. Blake και *Sida spinosa* L. Πρώτη καταγραφή των δύο ζιζανίων στην Ελλάδα

Σ. Λυμπεροπούλου και Κ.Ν. Γιαννοπολίτης

Περίληψη Δύο είδη ζιζανίων αναφέρονται ότι βρέθηκαν για πρώτη φορά στην Ελλάδα: Το *Galinsoga ciliata* (Raf.) S.F. Blake (Asteraceae) βρέθηκε να απαντάται σε υψηλές πυκνότητες φυτών μέσα σε καλλιέργειες λαχανικών στην περιοχή του Μαραθώνα Αττικής. Το *Sida spinosa* L. (Malvaceae) βρέθηκε σε λίγα διάσπαρτα φυτά μέσα σε καλλιέργειες βαμβακιού στην περιοχή Λούρου Πρέβεζας και Παλαμά Καρδίτσας. Και τα δύο θεωρούνται ως εισβάλοντα ξενικά είδη, μη περιλαμβανόμενα μέχρι σήμερα στη χλωρίδα της ηπειρωτικής Ελλάδας (το πρώτο) και της Ελλάδας (το δεύτερο). Παρουσιάζονται, επίσης, τα χαρακτηριστικά που επιτρέπουν την ασφαλή διάκριση των δύο ειδών.

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Activity of pyriproxyfen, an insect growth regulator, on *Culex pipiens* (Diptera: Culicidae)

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Summary In order to find an adequate replacement of temephos an insect growth regulator (pyriproxyfen) was evaluated as agent that can keep water bodies free from mosquito larval development for a period of up to 6 days. Bioassays were conducted under laboratory condition against *Culex pipiens* (Diptera: Culicidae) larvae. Furthermore, the attractiveness or repellency of the water containing each of these two killing agents was estimated as oviposition substrate for this mosquito species. Results indicated that both temephos and pyriproxyfen were highly effective against mosquito larvae although they act in a different way, and they can eliminate the mosquito production for the period that they were tested. As it is indicated by the larvicidal bioassays, pyriproxyfen showed very good activity causing complete adult emergence inhibition and its effectiveness is almost equal to those of the organophosphate compound in terms of total mosquito mortality. The presence of temephos in the water had no effect on the attractiveness or repellency of *Cx. pipiens* oviposition substrate in contrast with pyriproxyfen which acted as repellant.

Additional keywords: Culex pipiens biotype *molestus*, temephos, mosquito larvae, mosquito mortality, mosquito oviposition

Introduction

Culex pipiens is a mosquito species widespread in Europe causing many nuisance problems. Especially its biotype *molestus* prefers to feed mainly on mammals (2) and occurs more frequently in human environments. Females have been reported to bite man indoors and outdoors (in Latin *molestus* means nuisance). Except nuisance, their role as disease vectors is another important matter and Lundström (10) suggests that *Cx. pipiens* biotype *molestus* Forskal 1775 should be collected and processed for isolation of West Nile virus in order to evaluate the occurrence of the virus in an area.

For these reasons there is often a necessity to implement an integrated mosquito control program against this mosquito species.

The research on the development of semiochemical products has created an intelligent approach, from the standpoint of drawing the insect to the poison rather than bringing the poison to the insect, the so-called "attract-and-kill strategy" (21). This new strategy has many advantages, such as intelligent combination of pheromone and insecticide, species specific, targeted application, protection of beneficial organisms and minimisation of the risk of resistance development (17, 21).

Agents such as oviposition pheromones (8), extracts from plants (7) and skatole waters (16) which act as oviposition attractants could be valuable tools in applications of the attract-and-kill strategy for the control of *Culex* mosquitoes.

For many years the organophosphate insecticide temephos used to be the most common larvicide in the mosquito control programs and its efficacy has been well documented (3, 15). Nevertheless, after its not

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inclusion in Annex I of the Directive 98/8/EC, European Union banned the use of this active substance in the member states. After that there is a pressing need of finding other efficient insecticides to replace temephos in mosquito control programs and in the attract-and-kill strategy as well.

Nowadays, the main tendency for the control of vectors without the presence of disease is to use more environmentally friendly chemicals such as insect growth regulators (IGRs) (14).

For that reason, the IGR pyriproxyfen was tested for its residual effect over a 6-day period and compared with temephos in order to assess it as a possible control agent for the attract-and-kill strategy, in combination with the above mentioned oviposition attractant agents.

Although pyriproxyfen is a rather new insect growth regulator, its mode of action has already been well studied on mosquitoes as well (5, 9). As a member of the IGR family it has a remarkable larvicidal activity and good efficacy against many mosquito species in a variety of mosquito breeding sites (5, 18). Additionally, it has been reported that pyriproxyfen appears to be highly selective for mosquitoes and causes the minimum undesirable effects on the environment and public health (13).

Furthermore, as it is known that some IGRs or other larvicides have a negative effect on oviposition activity (1, 11, 19) the attractiveness of the water as an oviposition site when pyriproxyfen or temephos is added was also examined.

Biological control agents such as *Bacillus thuringiensis* subsp. *israelensis* (B.t.i.) were not used in this study as according to the literature, the registered in Greece products have virtually no residual effect against mosquito larvae beyond application (4).

Materials and Methods

Mosquito rearing

The *Cx. pipiens* biotype *molestus* colony used was maintained at the Benaki Phytopathological Institute, for more than two decades. Adults were kept in wooden framed cages (33×33×33 cm) with 32×32 mesh at 25±2°C, 80±2% relative humidity and a photoperiod of 14:10 (L:D) h. Cotton wicks saturated with 10% sucrose solution were provided to the mosquitoes as food source. Females laid eggs in round, plastic containers (10 cm diameter × 5 cm depth) filled with 150 ml of tap water. Egg rafts were removed daily and placed in cylindrical enamel pans in order to hatch (35 cm diameter × 10 cm depth). Larvae were reared under the same temperature and light conditions and were fed daily with baby fish food (TetraMin®, Baby Fish Food) at a concentration of 0.25 g/l of water until pupation. Pupae were then collected and introduced into the adult rearing cages (6).

Insecticide formulations

Formulated products that are commonly marketed in Greece of 0.5% pyriproxyfen (Sumitomo Corporation Hellas S.A., SUMILARV) and 50% temephos (Basf Agro Hellas S.A., ABATE 50 EC) were tested at the doses of 2 mg/l and 0.15 ml/l, respectively. The dosages were equivalent to the lowest recommended label rates for each active substance.

Larvicidal bioassays

The bioassay method followed was based on the standard test for determining the susceptibility or resistance of mosquito larvae to insecticides (22). However, in the present study, besides the typical bioassay where larvae of 3rd and early 4th instars are used, we carried out bioassays with one-day egg rafts as well. Aqueous insecticide stock solutions were prepared in conical flasks as follows: Four to six consecutive dilutions were prepared as working solutions in a 3-litre glass jar, depending on the active ingredient, to obtain the desirable concentration. Before their use, glass jars were stored uncovered under similar conditions as with mosquito rearing. Glass jars filled with tap water were used as controls. Bioassays were performed for 6 days, after the preparation

of dilutions (day 0). Every 2 days the jars were weighted and tap water was added up to initial volume to supplement water loss due to evaporation.

For the typical larval mortality bioassay, twenty larvae of 3rd and early 4th instars were placed in a glass beaker with 100 ml of stock solution of each insecticide. Five replicates were made per concentration and a control treatment with tap water was included in each bioassay. Beakers with larvae were placed at 25±2ºC, 80±2% relative humidity and a photoperiod of 14:10 (L:D) h.

For the bioassays with the egg rafts, 100 ml of each stock solution were added in a 250 ml glass beaker and one newly laid egg raft (less than 20 h old) was transferred by means of a wooden stick on the water surface (70±5 eggs per egg raft). In addition, 1 ml of baby fish food solution (TetraMin®, Baby Fish Food) was added to each beaker every 2-days to provide larvae with food.

Oviposition bioassays

Two-choice oviposition experiments were set in sieve covered wooden framed cages (33x60x33 cm). Two to three days old male and female adult mosquitoes were removed daily from the maintenance cages (not containing oviposition beakers) and introduced into the bioassay cages. The bioassay cages were kept under the above-mentioned rearing conditions. Two glass beakers (10 cm diameter x 5 cm depth), one containing 100 ml distilled water and the other 100 ml distilled water plus the larvicidal, were placed into the cages in approximately 40 cm distance between each other as more centrally as possible in order to provide oviposition sites. Each oviposition bioassay lasted six days.

Data recording and analysis

Larval mortality was assessed by counting the number of dead larvae every 24 h. In the cases where 100% larval mortality did not occur, pupal mortality was assessed too. Percentage mortality was calculated for each treatment and replicate by dividing the number of dead and moribund larvae to the total (dead and alive). Dead larvae were considered those that could not be induced to move when they were gently touched with a glass pipette in the siphon or the cervical region. Moribund larvae were those who were incapable of rising to the surface, within a reasonable period of time, or those not showing the characteristic diving reaction when the water was disturbed; they could also show discolorations or unnatural positions (22).

Efficacy of each insecticide was assessed as the mortality noted at each treatment compared to the mortality of the controls. In addition, the percentage of larvae that pupated was estimated for the evaluation of pyriproxyfen effect.

For the oviposition bioassays the number of egg rafts was counted and removed every 24 h after the introduction of the oviposition beakers into the bioassay cages. The number of egg rafts in the treated beaker was converted to percentages of the total number of egg rafts in both beakers for each cage. These results refer to three experiments for each case.

Results and Discussion

Pyriproxyfen and temephos were bioassayed against egg rafts of *Cx. pipiens* biotype *molestus* at a concentration of 0.2 gr/l and 150 μl/l respectively. The bioassay solutions were stored from 1 to 6 days under constant conditions before use (post treatment days).

None of the tests were discarded because control mortality was lower than 20% in all cases.

In the bioassays with the larvae of 3rd and early 4th instars all the larvae were dead within a 24 h period (mortality 100%), at the doses tested. These results for both insecticides are quite expected as a general rule the recommended by the producer application rate usually gives the maximum effectiveness against susceptible strains of the target organisms.

The efficacy of both insecticides when

egg rafts are used is shown in Figure 1 where mortality percentage is presented for every larval instar and pupa stage. From the results it is clear that temephos killed all the first and second larval instars (100% mortality) whereas pyriproxyfen did not significantly differ from the control for the first to the fourth larval instars. In Table 1 adult stage emergence is presented for the control and each insecticide.

Pyriproxyfen was also found to be statistically highly effective in the stage of pupa with a mortality ranging from 80% to 95% and proved to be a useful tool for the control of *Cx. pipiens*. The results are in agreement with the already known mode of action of pyriproxyfen, even though egg rafts were used instead of larvae of 3rd or late 4th instar (20).

Regarding the oviposition bioassays, results for a period of 6 days are shown in Figure 2. For the first two days pyriproxyfen showed a rather repelling action but the rest four days of the experiment the attraction level reached almost control levels. However, as similar effect of pyriproxyfen on the gravid females mosquitoes is not known further study needs to be conducted. On the contrary, temephos did not seem to affect oviposition during the 6-day period.

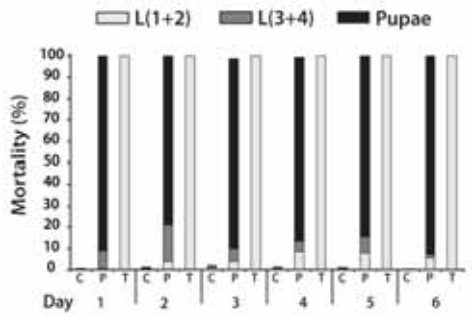

Figure 1. Percentage mortality for the control (C), pyriproxyfen (P) and temephos (T) at the 1st and 2nd larval instar [L(1+2)], 3rd and 4th larval instar [L(3+4)] and at pupal stage.

Table 1. Adult emergence of Control, pyriproxyfen (P) and temephos (T) against hatched larvae of *Cx. pipiens* biotype *molestus* for every post treatment day.

Day	Treatment		
	Control	P	T
1	82.7%	0.0%	0.0%
2	86.1%	0.0%	0.0%
3	85.1%	1.2%	0.0%
4	83.5%	0.6%	0.0%
5	94.0%	0.0%	0.0%
6	86.5%	0.0%	0.0%

In conclusion, pyriproxyfen and temephos, as shown in Table 1, revealed the same results for a period of 6 days and the only difference was the mode of action of each larvicidal. Pyriproxyfen residual activity with egg rafts for at least one week period was very hopeful for the aims of this study, which was to investigate if pyriproxyfen could be used instead of temephos in integrated control programs with other means of mosquito control, such as oviposition attractants. While a simple contact with temephos was enough to kill the larvae and oviposition pattern did not affected, pyriproxyfen needs more time and is also repellant for gravid mosquitoes.

Previous work indicated that the combination of temephos with the pheromone could result in the implementation of the attract-and-kill strategy (12). Further research is needed to evaluate the effectiveness of pyriproxyfen and its utility as larvicidal agent. Moreover, additional knowledge when pyriproxyfen combine with pheromone would allow the effectively practical application in larval breeding sites such as rain water collection areas, artificial contain-

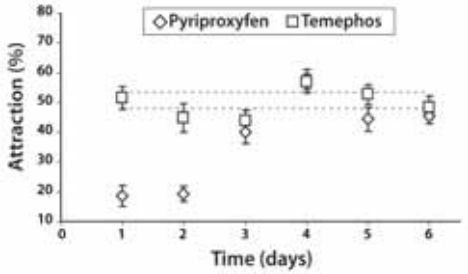

Figure 2. Oviposition effected by temephos and pyriproxyfen. The dashed lines represent the upper and lower values of the control mean ± SE (50.1 ± 2.1%, n=10).

ers etc., in rural and urban localities.

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Επίδραση του pyriproxyfen, ενός ρυθμιστή ανάπτυξης εντόμων, σε προνύμφες κουνουπιών του είδους *Culex pipiens* (Diptera: Culicidae)

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Περίληψη Μετά την απόφαση της Ευρωπαϊκής Ένωσης για την απαγόρευση των βιοκτόνων με δρων συστατικό το temephos και τον αποκλεισμό τους από τα προγράμματα καταπολέμησης κουνουπιών στην Ευρώπη υπάρχει επιτακτική ανάγκη να αντικατασταθεί η χρήση τους από άλλα βιοκτόνα εξίσου ή περισσότερο αποτελεσματικά.

Στην παρούσα εργασία μελετήθηκε δράση ενός βιοκτόνου με δρων συστατικό το pyriproxyfen εναντίον προνυμφών κουνουπιών του είδους *Culex pipiens* (Diptera: Culicidae). Το εν λόγω βιοκτόνο που ανήκει στην κατηγορία των ρυθμιστών ανάπτυξης εντόμων δοκιμάστηκε ως προς την αποτελεσματικότητά του σε προνύμφες 3ης και 4ης ηλικίας καθώς και ως προς την υπολειμματική του δράση για μια περίοδο έως 6 ημέρες. Στόχος ήταν η διαπίστωση της καταλληλότητας του pyriproxyfen για χρήση σε προγράμματα ολοκληρωμένης αντιμετώπισης κουνουπιών όπου με συνδυασμό ενός ή περισσοτέρων ελκυστικών ωοθεσίας θα μπορούσαν να διατηρήσουν μια πιθανή εστία ανάπτυξης κουνουπιών καθαρή από τα έντομα αυτά.

Οι βιοδοκιμές έγιναν σε ελεγχόμενες συνθήκες και χρησιμοποιήθηκαν προνύμφες και σχεδίες ωών του κοινού είδους κουνουπιού *Cx. pipiens* biotype *molestus* από εργαστηριακή εκτροφή. Η επίδραση του pyriproxyfen μελετήθηκε και συγκρίθηκε με αυτή του temephos τόσο στις αναπτυγμένες προνύμφες όσο και στις προνύμφες που προέκυψαν από τις σχεδίες ωών που χρησιμοποιήθηκαν στις βιοδοκιμές. Επιπλέον μελετήθηκε κατά πόσο η παρουσία ενός από τα δύο αυτά βιοκτόνα μπορεί να επηρεάσει τη συμπεριφορά των θηλυκών κουνουπιών ως προς την επιλογή της συγκεκριμένης εστίας για ωοθεσία.

Από τα αποτελέσματα προέκυψε ότι το pyriproxyfen είναι εξίσου αποτελεσματικό με το temephos εναντίον των προνυμφών των κουνουπιών παρά το γεγονός ότι λόγω του διαφορετικού τρόπου δράσης του δεν θανατώνει άμεσα τα έντομα. Τα τελικά ποσοστά θνησιμότητας ήταν παρόμοια με αυτά του οργανοφωσφορικού εντομοκτόνου (temephos) ενώ η παρεμπόδιση εμφάνισης ακμαίων κουνουπιών ήταν πλήρης ακόμη και στις επεμβάσεις που είχε χρησιμοποιηθεί διάλυμα pyriproxyfen ηλικίας 6 ημερών.

Η παρουσία όμως του pyriproxyfen στο νερό είχε ως αποτέλεσμα μια απωθητική δράση ως προς την ωοθεσία των θηλυκών κουνουπιών σε αντίθεση με το temephos που δεν εμφάνισε ούτε απωθητική αλλά ούτε και ελκυστική δράση.

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