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The Residual Behavior of Chlorothalonil and Metalaxyl in The Egyptian Cucumbers Fields Infested with Downy Mildew Using The High-Performance Liquid Chromatography (Hplc) and Quechers Method.

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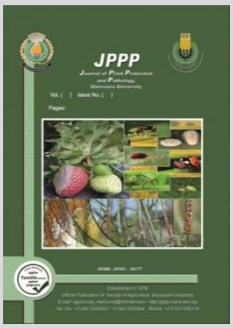

ABSTRACT

The dissipation rate of the commercial fungicide with two active ingredients (Chlorothalonil 64% + Metalaxyl 8%) that was approved by the agriculture pesticides committee was evaluated in the cucumber fields infested with downy mildew in Egypt using HPLC analytical system. The experiment was designed in randomized block design. After spraying at the recommended rate of applications, the samples of cucumber were chosen randomly from treated and un-treated plants after the interval of zero time(2hours), 1, 3, 7,10, and 14 days. The technique was validated at different fortification levels (0.01, 0.5, and 0.1 µg mL^{-1}). The recoveries of both fungicides Chlorothalonil and Metalaxyl were 87.6 and 95 %. The results showed that the initial residues (zero time) were 4.38 and 3.21 mg/kg^{-1}. All detected residues significantly decreased upon time elevated. The residues extracted by an optimized QuEChERS coupled with HPLC analytical system were accurate and acceptable The limit of quantitation was 0.05 for both fungicides. The dissipation curves of both Chlorothalonil and Metalaxyl followed the first-order kinetics. The half-life's (t½) values were 0.89 and 1.1days in, respectively. Under the optimized condition, the residues of Chlorothalonil and Metalaxyl in cucumber were below the codex maximum residue limit (0.5 and 3 mg/kg^{-1}). Consequently, the pre-harvest intervals (PHI) in cucumber were seven days for the fungicide.

Keywords: Cucumber, Downy mildew, Chlorothalonil, Metalaxyl, QuEChERS.

INTRODUCTION

Downy mildew, the disease caused by *Podosphaera xanthii* (Castagne), is widespread in glasshouse and cultivation of cucumber plants and it can lead to heavy damage and economical loss worldwide (Sarhan *et al.*, 2020). Symptoms of downy mildew started in vegetation as yellow spots which turned upon time necrotic. Fungicides are the basic recommended application strategies for cucumber downy mildew (Bagi *et al*., 2009). While pesticides play an important role in the effort to overcome plant losses caused by different types of pests to increase food production, at the same time, they can cause several environmental hazards due to their toxicities and accumulation in the environment (Hamama and Fergani, 2019). To avoid high levels of pesticides persistence and residues in crops, modern insecticides should be created with a deep perception of their dissipation behavior in the environment. One of the newly recommended commercial fungicides with two active ingredients (Chlorothalonil 64% + Metalaxyl 8%) against downy mildews was evaluated. Chlorothalonil is a non-systemic foliar fungicide that destroys the germinating fungal cells, leading to disruption of glycolysis and energy production known causing fungicidal action. While, Metalaxyl is known as a systemic fungicide that inhibits protein synthesis in the pathogen cells thereby preventing or reducing new infections and reduce the disease severity of the infected crop (The Pesticide Manual, 2012). Various factors affect the behavior of the fungicide and regulate their fate in the environment that directly affects human health (Cabras *et al*.1990).

The of pesticide residues and estimation of the degr adation for any used in cultivation for a several crops in the field should be legally planted as the most important regist ration requirements in Egypt . The pre harvest interval (PH I) is known as the timebetween the application and collecti ng the cultivation (Abd Al-Rahman *et al*., 2012). Recently the QuEChERS method as well as one of the most distinctive AOAC (AOAC, 2000) official protocols for quantitation of most of pesticide residues in a many food matrices (Lehotay, 2007). Also, choosing the most appropriate methodology for sample preparation methods greatly influences the reliability and accuracy of food analysis (Seddik *et al*., 2012). This study focused mainly to estimate the residue levels of the main active ingredient of newly recommended commercial product (Chlorothalonil and Metalaxyl) in cucumber cultivation infected with downy mildew in the field using the QuEChERS method coupled with (HPLC) coupled with photodiode array detector (DAD) analysis technique.

MATERIALS AND METHODS

Chemicals:

The fungicide (Fol chrois® 72%WP) composed of two different groups (Chlorothalonil 64%+Metalaxyl 8%) was purchased locally and used for a field experiment in this work at the recommended dose:

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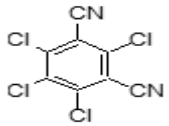
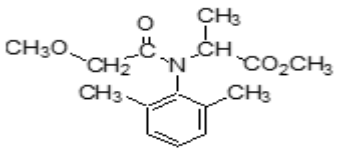
A- Chlorothalonil (Fungicide) ; multi-site: chloronitrile IUPAC name tetrachloroisophthalonitrile , 2, 4, 5, 6-tetrachloro-1, 3-benzenedicarbonitrile



B- Metalaxyl (Fungicide) ; phenylamide: acylalanine **IUPAC name** methyl N-(methoxyacetyl)-N-(2,6-xylyl)-DL-alaninate; methyl 2-{[(2,6 dimethylphenyl)methoxyacetyl]amino}propionate .methyl *N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl)-DL-alaninate .



Preparation of standard solutions.

The stock solution (100µg/ml) reference standard of Chlorothalonil and Metalaxyl was done in acetonitrile in a 100 ml flask. The successive dilution and spiking solution for HPLC analysis were done daily.

Field Trials

The field experiments were carried out at Mahilat Firnawaa village, Shubrakhit Province, El-Beheira, Egypt, on 22 April 2021. Cucumber plants (*Cucumis sativus*) were cultivated in lines consisting of eight rows. Plots were arranged as randomly design with three replications for each application. Fungicides treatments were treated with an adjusted backpack motorized system. Agricultural practices were made according to the recommended crop schedule. Before application, samples of cucumber plants of similar ripening stages, sizes, and shapes were labeled. Mature plants were sprayed by Fol Chrois®72%WP (Chlorothalonil 64%+Metalaxyl 8%) at rate of 250 gm. /100L W. The control plots were left unsprayed. Samples, one kilogram for each treatment were collected at intervals of initial (two hours after application), 1, 3, 7, 10, and 14 days. Control samples were also collected after each sampling time interval during the experiment. Immediately after collecting cucumbers. Commercial formulations, the doses employed, ADI, and MRL are summarized in Table 1.

Table 1. Fungicides formulation, rate, MRL and ADI.

Common name	Trade name	Rate [g or ml/100(LW)]	MRL* (mg/kg)	ADI** (mg/kg)
Chlorothalonil	Fol chrois® 72%WP	250 gm. / 100LW	3	0.03
Metalaxyl			0.5	0.03

***MRL = Maximum residual limits at Codex committee on pesticide residues**
****ADI = Acceptable daily intake.**

Residue analysis:

Sampling, extraction, and clean-up

Homogenization of cucumber fruits (one kilogram for each sample) was carried out using a food processor (Thermomix, Vorwerk) for five minutes vigorously. The homogenate of each sample was then placed into 50-ml polypropylene tubes and cooled at −20 °C until further analysis. Extraction takes place according to Lehotay *et al*., 2010. Ten grams of each homogenized sample was weighed into a 50-ml centrifuge tube. Extraction and cleaned-up were done extracted and optimized according to Anastassiades *et al*,. 2003 by blending with ten milliliters of 1.0% acidified acetonitrile with acetic acid and shake for one minute using a vortex mixer at maximum speed. Afterward, four grams of anhydrous MgSO4, one gram of NaCl, one gram of sodium citrate dihydrate, and 0.5 g disodium hydrogen citrate sesquihydrate were added, then extracted by shaking vigorously on vortex for two minutes and centrifuged for ten minutes at 3000 rpm. Afterward, centrifugation was adjusted at 5,000 rpm for 5 min. Two milliliters were filtered and analyzed by Agilent 1100 HPLC-DAD.

Residue analysis of fungicides

Both active ingredients of the tested fungicide (Chlorothalonil and Metalaxyl) was analyzed using (HPLC) (USA), with a quaternary pump, manual injector (Rheodyne), thermostat compartment for the column, and photodiode array detector. The separation was achieved on a chromatographic column ODS H optimal (150 mm × 4.6 mm, 5 µm film thicknesses). The column was kept at room temperature. the mobile phase, flow rate, and the detection wavelength of Chlorothalonil and Metalaxyl were summarized in Table (2).

Table 2. HPLC system conditions for detecting both Chlorothalonil, and Metalaxyl.

Pesticides	Mobile phase	Flow rate	Detection wavelength(nm)
Chlorothalonil	(acetonitrile/water) (70:30,v/v)	1 ml/min	230
Metalaxyl	Acetonitrile/methanol/ water (40:20:40v/v/v)	0.8ml/min	225

Recovery studies

According to SANCO/1257/2013 (SANCO, 2013) method validation was performed for the extraction and determination of Chlorothalonil and Metalaxyl in cucumber plants. The method was validated following a conventional validation procedure that included the following parameters: (Linearity) multilevel calibration of Chlorothalonil and Metalaxyl was diluted either with a pure solvent in series at (10, 2.5, 1, 0.5, 0.2, and 0.01) µg/ml, (Matrix effect) comparing the response produced from the Chlorothalonil and Metalaxyl in a pure solvent solution with the samples were first extracted and then spiked with Chlorothalonil and Metalaxyl in the same solvent at the same concentration level, (Selectivity and Sensitivity). Determining (LOQ), (LOD), Trueness (bias) using five replicates to check the recovery at the levels (1, 0.5, and 0.01) mg/ml and Repeatability Precision relative standard deviation (RSD).

Statistical analysis

Half-life times calculation ($t_{1/2}$) of the Recovery of Chlorothalonil and Metalaxyl residues were calculated mathematically according to Moye *et al.* (1987). The dissipation kinetics of both fungicides residues were determined by the first-order kinetics equation: $C_t=C_0e^{-kt,}$ Where C_t represents the concentration of the fungicide residue at the time of t, C_0 represents the initial deposits after

application, and *k* is the constant rate of fungicide disappearance per day. From this equation, the dissipation half-life periods ($t_{1/2} = ln\ 2/k$) of the studied fungicide. The analytical determinations were made in triplicate for each sample. Data were subjected to analysis of variance (ANOVA) followed by the least significant difference (CoStat Statistical Software, 1998-2005).

RESULTS AND DISCUSSION

Method validation and recoveries.

Recovery calculations were done on control cucumber fruits with three treatments of each fungicide (0.01, 0.5 and 1.0 mg kg-1). To confirm the validity of the method the analytical method was evaluated in three replicates considering different parameters including a (LOD), a limit of quantification (LOQ), recoveries of both fungicides, and repeatability expressed as RSD. The linear range was from 0.1 to 0.01 μg mL^{-1}. Good linearity was obtained for Chlorothalonil and Metalaxyl. The limits of quantification were 0.05 and 0.01 mg/kg for Chlorothalonil and Metalaxyl respectively that were far below the MRLs established for Chlorothalonil and Metalaxyl, respectively. The mean recoveries of Chlorothalonil and Metalaxyl were 87.6% and 95 %; during method development fully comply with the acceptable recovery range ranged between 70 and 120% while the RSD was also feasible with this method ≤20% according to EU method validation guidelines (SANTE/11813/2017, 2017). These results proved that the QuEChERS method for sample preparation coupled with, HPLC-DAD analysis is a good method for residue quantifications of the tested fungicides in cucumber fruits (Table 3).

Table 3. Recoveries and relative standard deviations for Chlorothalonil and Metalaxyl in cucumber fruits at various fortification levels.

Spike level	Chlorothalonil			Metalaxyl		
(mg/kg) (n*=3)	Recovery±SD	Average% ±SD	RSD%	Recovery±SD	Average%±SD	RSD%
0.10	89.0b ±7.07		6.86	85.0 b ±5.66		5.0
0.50	93.0 a±7.54	92.30± 6.11	7.0	90.0 a ±9.40	87.60 ±5.03	9.03
0.01	95.0a ±11.31		10.50	88.0 a ±1.41		1.30
LSD 5%	2.75			2.26		

*** Number of replicates**
***Least significant difference**

Dissipation of Chlorothalonil and Metalaxyl in cucumbers fruits under field condition

The residues and dissipation rates of Chlorothalonil and Metalaxyl in cucumbers fruits were tested after three applications at the rates recommended on cucumbers fruits under field conditions Table (4) and Fig(1). The initial residues at zero time (two hours after application) were 4.38 and 3.21 mg/kg. The obtained results indicated that both fungicides decreased significantly with different day intervals after application. Chlorothalonil and Metalaxyl dissipate rapidly after application. The residues amount decreased significantly to 2.98 and 1.59 mg/kg in cucumbers fruit for Chlorothalonil and Metalaxyl, respectively, within the first 24 h after application. Following that period, Chlorothalonil residues decreased significantly to 1, 0.5, and 0.01mg/kg, while, Metalaxyl decreased significantly to 0.73, 0.26, and 0.04 at 3, 7, and 10 days after treatment, respectively. Both fungicides residues in samples were undetectable after 14 days after treatment. The same pattern for dissipation was recorded for Chlorothalonil and Metalaxyl in tomato fruits (Abd Al-Rahman *et al*., 2012). The residue analysis of fungicides in cucumber fruits exhibited first order kinetics.

Table 4. Determination of Chlorothalonil and Metalaxyl in cucumbers fruits at different time intervals from the application.

Days after treatment	Chlorothalonil		Metalaxyl	
	Residues (mg/kg^{-1})	Dissipation %	Residues (mg/kg^{-1})	Dissipation %
Z	4.38 ^{a} ± 1.84	0.00	3.21 ^{a} ± 1.45	0.00
1	2.98 ^{b} ± 0.85	31.96	1.59 ^{b} ± 0.30	50.46
3	1.00 ^{c} ± 0.55	77.16	0.73 ^{c} ± 0.31	77.25
7	0.51 ^{d} ± 0.30	88.35	0.26 ^{d} ± 0.21	91.90
10	0.01 ^{e} ± 0.06	99.77	0.04 ^{e} ± 0.04	98.75
14	ND		ND	
LSD	0.30	9.91	0.19	10.61
MRL(mg/kg^{-1})	3		0.5	
$t_{1/2}$ (day)	0.89		1.1	
PHI	3		7	

Z: two hours after the insecticide application (zero time). MRL: acceptable maximum residue limits Rec: Mean recovery t1/2: Half-time PHI: Pre-harvest interval ND: Not detected.
***Values within the same row having the same letters are non-significant, p>0.05.**

The half-life times for Chlorothalonil and Metalaxyl were 0.89 and 1.1 days respectively. The pre-harvest interval (PHI) of Chlorothalonil and Metalaxyl were 3 and 7 respectively, the same findings were obtained by Abd Al-Rahman *et al*., 2012 while, different dissipation behavior for Chlorothalonil in different vegetables as tomato fruits were obtained by Gambacorta *et al*. (2005), and in cabbages Zhang *et al*. (2007). Metalaxyl was known to be a broad-spectrum activity fungicide and registered for use on a wide range of crops and in the world with different climatic conditions (Urech *et al*., 1977). In addition to its ability to enhance plant growth and crop yield Metalaxyl was found to be moderately stable under normal environmental conditions (Metalaxyl, 1993).

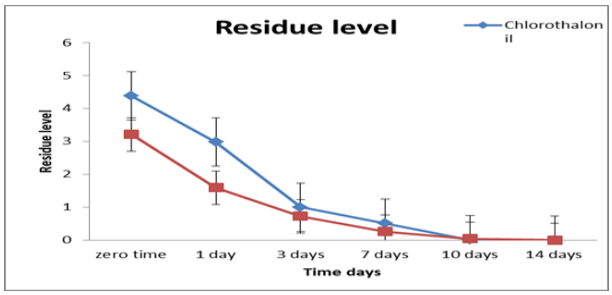


Figure 1. Decline rate of Chlorothalonil and Metalaxyl in cucumber fruits after different time intervals of application

The half-life values and variable dissipation rates of Metalaxyl residues in cucumber were comparable with those previously reported from Talebi, 2002. Different dissipation rates of both fungicides might due to the difference between their chemical structure and physiochemical characters, photolysis, biotic and abiotic process (US EPA, 1999) under the field conditions that almost affect their persistence in the field with different climatic parameters. Cucumber (*C. sativus*) is a widely-cultivated creeping vine plant in Egypt. It is considered a high nutrients fruit rich in antioxidants. The integrated pest management (IPM) strategies focused mainly on the goal of good agricultural practices including the reduction of pesticide usage to reduce the most environmentally dangerous pesticides. Monitoring of pesticide residues is one of the main targets of IPM to predict adequate concentrations and estimate the PHI values. To certify the residual behavior of both Chlorothalonil and Metalaxyl results should align with standard limits. The (LOQ) values in both insecticides were lower than MRLs established by Codex Committee Codex Alimentarius Commission for Pesticides residues, 2013) and Switzerland (EU) (EU, 2009). The QuEChERS method showed perfect recoveries, and the used systems showed excellent separation of the tested fungicides. Based on the previous results the residue levels of both Chlorothalonil and Metalaxyl will be suitable when used for cucumber fruits in Egypt. The obtained results suggest that if cucumber fruits are destined to be sold as a fresh product, it may be advisable to lower the dose of the treatments and don't used before a safe period of seven days that were in line with (Gambacorta *et al* 2005).

CONCLUSION

The dissipation rate of the newly recommended fungicide Fol Chrois®72%WP (Chlorothalonil 64%+Metalaxyl 8%)) in cucumber fruits were evaluated under the field condition. The results have been characterized with excellent recoveries and minimum LOQs values. The MRLS of Chlorothalonil and Metalaxyl were lower than MRLs, fulfilling the Codex Committee and (EU) criteria. The results obtained in this study confirm that the proposed methods are easy and reliable for the determination of the analyzed Chlorothalonil and Metalaxyl fungicide residues in cucumber fruits. Safe consumption of cucumber fruits could be after a waiting period of seven days to prevent any hazards to customers.

ACKNOWLEDGMENTS

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سلوك متبقيات الكلوروثالونيل والميتالاكسيل فى حقول الخيار المصابه بالبياض الزغبى بإستخدام جهاز الكروماتوجراف السائل عالى الأداء (HPLC) بإستخدام طريقة الـQUECHER.

هانم محمود عرفه سليمان

قسم بحوث متبقيات المبيدات وتلوث البيئة-المعمل المركزي للمبيدات -مركز البحوث الزراعية ـ الجيزة ـ مصر

في خلال الدراسة تم تقدير معدل إختفاء المبيد الفطرى اللذى يحتوي على المادتين(كلوروثالونيل 64%- ميتالاكسيل 8%) المستخدم لمكافحة البياض الزغبى على نبات الخيار باستخدام جهاز (HPLC) وتم أخذ عينات من الخيار المعاملة على فترات زمنية بعد ساعتين من الرش, 1,3,7,10,14 أيام من بعد التطبيق يوم وتم الإستخلاص والتنقية باستخدام طريقة الQUECHERS والتقدير على جهاز(HPLC) السائل. وتم التحقق من دقة الطريقة عند مستويات تلوث مختلفة (0.01,0.5,0.1) ميكرو جرام/كجم وكان متوسط معدل الاسترجاع لمبيدي الكلوروثالونيل والميتالاكسيل 87.6% و 9%على التوالى. كما أظهرت النتائج أن كمية المتبقى البدائي بعد ساعتين من الرش 4.38 و 3.2 ميلجرام/كجم لمبيدي كلوروثالونيل و ميتالاكسي. كم لوحظ تراجع منطقي لمستويات المتبقيات مع مرور الوقت. وكان الحد الكمى للطريقه هى 0.05 ميلجرام /كجم. كما أثبتت النتائج أن طرق الإستخلاص والتقدير تتميز بالدقة العالية التي تناسب معدلات المتبقيات للمبيدات الفطرية لمحاصيل الخيار المتفق عليها. وكان منحنى اختفاء مبيدي الكلوروثالونيل والميتالاكسيل يتبع معادلة الخط المستقم من الدرجة الاولى حيث كانت فترة نصف العمر هى 0.89 و1.1 أيام لمبيدي الكلوروثالونيل والميتالاكسيل على التوالى.وكانت فترة ماقبل الحصاد 7 يوم لهذا المبيد وفقا للحدود القصوى المسموح بها للإتحاد الأوربى.

الكلمات الدالة : خيار-بياض زغبى – كلوروثالونيل-الميتالاكسيل- معدل إختفاء- QuEChERS