J. Plant Prot. and Path., Mansoura Univ., Vol. 3 (3): 227 - 238, 2012

GENETIC VARIABILITY STUDY OF *Phytophthora infestans* ISOLATES BASED ON MATING TYPE MOLECULAR MARKERS AND METALAXYL RESISTANCE

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ABSTRACT

Isolates of Phytophthora infestans (Mont.) de Bary were collected from potato fields located in main cultivation regions in Egypt during two successive seasons, 2006 and 2007. The ITS4 and ITS5 primers were used to study the genetic variation among the five isolates. Resulted pattern cleared out the differences among isolates concerning the density of the bands and the number in bands for each isolate. A molecular approach was used in order to identify the mating types. Genomic DNA of P. infestans obtained from mycelium grown in pure culture was amplified with the specific primers W16-1/2 and subsequently digested with HaeIII restriction enzyme. The results revealed the presence of the A2 mating type in 1 isolate (Salhia, Behira) among 5 isolates obtained from commercial fields in Egypt. Also, since it is observed that fungicides containing metalaxyl are loosing their efficacy in many fields, when in vitro tests for metalaxyl resistance in P. infestans isolates were performed. Results revealed strong presence of the metalaxyl tolerant and resistant phenotypes in Egyptian P. infestans population. Importance of the genetic studies to follow pathogenicity and population structure of P. infestans was noticed.

Keywords: Phytophthora infestans, mating type, molecular markers, metalaxyl resistance.

INTRODUCTION

Phytophthora infestans is an Oomyceteous pathogen, causes late blight disease on potato and tomato. It has two mating types, A1 and A2, which form oospores by sexual reproduction (Kamoun and Smart, 2005). A single genotype, designated US-1, dominated *P*. *infestans* populations outside Mexico between 1840 and the mid-1970s (Goodwin *et al*., 1997). This situation changed in the early 1980s, when A2 mating type isolates appeared in Europe. Currently, A2 mating type isolates are present almost everywhere that potatoes are grown (Erwin and Ribero, 1998; Seishi *et al*., 2008). *P. infestans* is a diploid, heterothallic Oomycete with two mating type, designated A1 and A2, which are found with equal frequency in some regions of Mexico, the presumed center of origin (Goodwin *et al*., 1992). Until the 1980s, the A2 mating type was confined to Mexico, and earlier worldwide spread of the pathogen was attributed to a single A1 isolate of the pathogen (Goodwin *et al*., 1994). In the early 1980s, A2 mating type isolates appeared in Europe and precisely in Switzerland. Currently, A2 mating type isolates are present all over northern Europe, in Asia, in several African countries, in South America and in North America (Fry and Goodwin, 1997; Govers *et al*., 1997). This had led to increased virulence and genetic variation worldwide,

suggesting that it was the result of sexual reproduction. A1 and A2 mating types are believed to represent compatibility mating types that differ in hormone production and response (Judelson *et al*., 1997). Development of male and female gametangia (antheridium and oogonium) is stimulated by the hormones within a mating type zone. The haploid A1 and A2 nuclei fuse to form a diploid nucleus within the oospore which develops a thick wall, allowing survival for many years in soil. Germination of the oospore releases progeny of either A1 or A2 mating type that are able to infect newly planted tubers, stems, or leaves which are in contact with soil (Drenth *et al*., 1995).
Metalaxyl is belonging to phenylamide compounds which were released at the end of the 1970s and, initially, they were very effective systemic fungicides with both protectant and curative effects against Oomycetes (Gisi and Cohen, 1996). Metalaxyl was one of the most effective phenylamides and has been applied intensively to control downy mildews in many crops and late blight in tomato and potato (Cohen and Coffey, 1986). Due to its specific mode of action and continuous usage, high selection pressure could be exerted and insensitive subpopulations might predominate in the field (Williams and Gisi, 1992). As early as 1980s, metalaxyl-insensitive isolates of *P*. *infestans* were reported in Europe and confined to specific areas with no reports of widespread distribution (Dowley and O'Sullivan, 1981 and Reis *et al*., 2005).

It was reported that metalaxyl resistance is mediated by a single gene (Cooke, 1981). Cases of resistance have evolved where this fungicide was used as a single product in curative or eradicant treatments and because of that, Metalaxyl-resistant genotypes emerged rapidly worldwide. In Egypt, the use of metalaxyl in many potato fields was associated with loss of its efficacy. The genetic structure of *P*. *infestans* populations in Egypt has not been characterized so far. The aim of this study was to characterize a sample representing the pathogen population in Egypt by identifying the presence of different mating types and the level of resistance to the fungicide metalaxyl. This might help in developing better management strategies against this pathogen in Egyptian potato fields.

MATERIALS AND METHODS

Isolation of *P. infestans* from infected potato leaves. Samples of blighted potato leaves were collected from different potato growing regions in Egypt, especially from the reclaimed land fields in Nubaria, Shabab (Behira Governorate) and Salhia (Ismailia Governorate) in 2006 and 2007 growing seasons. Small pieces of infected tissue from the sporulating border of the lesion were cut and placed under thin potato slices (2-3 mm) in a Petri dish. Dishes were incubated at 20°C for 5-7 days till there was abundant sporulation appeared on the upper side of the slice. The clean pure mycelium was then transferred onto green peas medium (boiling extract of 120 g of green peas, 9 g of phytoagar and 30 g of dextrose in 1l of distilled water) amended with 12 mg/l of rifampicin in Petri dishes. Plates were incubated for at least two weeks at 18 ± 1C.

Extraction of Genomic DNA.

Different isolates were grown for 14 days on green pea's broth medium. Mycelium were then harvested and washed twice with sterile distilled water. DNA was extracted from 50 mg of mycelium using Qiagen Kit for DNA extraction. The extracted DNA was dissolved in 100 µl of elution buffer. The concentration and purity of the obtained DNA was determined by using "Gen Qunta" system from Pharmacia Bio-tech. The purity of the DNA for all samples was between 90-97% and the ratio between 1.7 - 1.8. Concentration was adjusted at 6 ηg/ µl for all samples using TE buffer (pH 8.0).

Amplification of ITS region and digestion with restriction enzyme. For PCR amplification of the ITS region of *P*. *infestans* ,10 ηg of genomic DNA of each isolate was added to 24 µl of reaction mixtures containing 200 µM dNTPs, 0.1µM each of primers ITS4 (5-TCCTCCGCTTATTGATATGC-3) and ITS5 (5-GGAAGTAA AAGTCCTAACAA-3), 1unit of Taq DNA polymerase (Promega, Germany), 1x PCR buffer and dH_2O. The PCR reaction conditions consisted of 2 min at 96 °C (pre-heating), 1 min at 96 °C,1 min at 58 °C, 2 min at 72 °C for 35 cycles and final extension 10 min at 72 °C followed by storage at 4 °C. The amplified products were separated on 1.5% agarose gel to check product size and purity using 1X TBE buffer. Produced gel was stained with ethidium bromide solution (1µg/ml), at 75 constant voltage and determine with UV transilluminator.

The obtained PCR product was cleaved with *Hae*III restriction enzyme (5'-GG/CC-3') by adding 10 Units of the restriction enzyme to the mixture and incubating it for 2 hr at 37°C in 1X buffer. The digestion product was separated by agarose gel electrophoresis and visualized on UV light after staining with ethidium bromide.

DNA amplification (W16-1 & W16-2) for mating type and digestion with restriction enzyme. Study was carried out as described by Jamour and Hamada (2006). The PCR reaction mixture consisted of 20 ηg of genomic DNA, 0.2 mM dNTP, 1Unit Taq polymerase (0.25 µl), 1X buffer (2.5 µl) and 0.5 µM of each selected primer (W16-1/W16- 2). For the caps marker amplification, the following PCR profile was used: 30 s at 94°C, 30 s at 57°C and 30 s at 72°C for 30 cycles. The obtained PCR product of 600 pb was cleaved with *Hae*III restriction enzyme (5'-GG/CC-3') by adding 10 Units of the restriction enzyme to the mixture and incubating it for 2 hr at 37 °C in 1X buffer. The digestion product was separated by agarose gel electrophoresis and visualized on UV light after staining with ethidium bromide.

Sensitivity of *P. infestans* isolates to metalaxyl. Agar test and detached leaflet test were used to assess metalaxyl sensitivity of different isolates.

Agar test. The sensitivity of five *P*. *infestans* isolates was assessed based on radial growth on metalaxyl-amended green pea's culture medium as described by Sozzi and Staub (1987) with modifications. Petri plates containing green pea's medium amended with metalaxyl fungicide to a final concentration of 0, 5, 10 or 100 µg/ml were centrically inoculated with 5-mm-diameter mycelial plugs from a 10-day-old *P*. *infestans* colony. Plates were incubated at 18±0.5 C in the dark. After 14 days, the colony diameter of all isolates was measured in two perpendicular directions previously marked at

the bottom of each plate. Final radial growth was corrected by subtracting 5 mm of the mycelial plug from measured colony diameter. Mean colony diameters in culture medium amended with metalaxyl at 5, 10 or 100 µg/ml were divided by the mean colony diameter of control plates and multiplied by 100 to determine relative growth (RG). Metalaxyl sensitivity of isolates was compared to the control on the basis of Matuszak (1994), Goodwin (1996) and Sedigui (1999) criteria as shown in Table (1).

Table (1): Basis for sensitivity estimation of *P. infestans* isolates to metalaxyl using relative growth (RG) as proposed by Goodwin (1996) Sedigui (1999) and Matuszak (1994).

Criteria	Sensitivity level	5 ppm	10 ppm	100 ppm
Goodwin	S[a]	<40 [b]	–	–
	R	>40	–	–
Sedigui	S	–	<10	–
	I	–	10-60	–
	R	–	>60	–
Matuszak	S	<40	<40	<40
	I	>40	–	<40
	R	>40	>40	>40

[a] **S, sensitive; I, intermediate and R, resistant.**
[b] **Relative growth (Percentage of growth compared to the control).**

Detached Leaf test. Metalaxyl sensitivity of the five isolates was also assessed using a perceptive detached potato leaf assay modified after Sozzi *et al*. (1992). Potato leaflets of susceptible cv. Lady Rosetta were put in Petri plates, abaxial side up, in contact with filter paper saturated with metalaxyl preparation at 0, 0.01, 0.1, 1, 10, or 100 µg/ml. Each leaflet was inoculated in a central point with 20-µl droplet of a 5×10^3 sporangia/ml and incubated at 18±1.0 C with a 16-h photoperiod. Three replicates (leaflets) were assigned for each concentration. After 6 days, the area infected or covered by fungal growth in each leaflet was roughly categorized under a dissecting microscope according to a five-grade scale in which 0 = no apparent symptoms, 1 = up to 25%, 2 = up to 50%, 3 = up to 75% and 4 = >75% of the leaflet surface was covered with pathogen growth (Sozzi *et al*., 1992). Disease severity was calculated according to the equation suggested by Townsend and Heuberger (1943) as follows:

Disease severity (%) = sum (n x r) x100 / 4N.

Whereas: (n) is the number of leaves in each numerical grade (r) and (N) is the total number of inoculated leaves multiplied by the maximum numerical grade. Disease severity (%) was then transformed into corrected fungicide efficacy percentage using Abbott formula (Busvine, 1971).

$$\text{Abbott Corrected Efficacy (CE\%) \%} = \left(1 - \frac{Ds \,.\, T_n}{Ds \,.\, Co_n}\right) * 100$$

Where: *Ds* = Percentage of disease severity, T = treatment, Co = control, *n* = assigned time for recording data.

The effective dose for 50% growth inhibition (ED_{50}) was estimated for each isolate via a linear regression model for dose-response relation that fitted on

growth inhibition as a function of fungicide concentration (Reis *et al*., 2005). An isolate was rated as sensitive, intermediately sensitive, or resistant if ED50 was >0.001 and <0.01 µg/ml, >0.01 and <10 µg/ml, or ≥10 µg/ml, respectively.

RESULTS

The ITS4 and ITS5 primers were used in order to amplify universal fragments of *Phytophthora* genotype to study the genetic variation between the five isolates. Polymerase chain reaction amplification products spanning approximately 900 bp of the ITS regions as shown in Fig. (1) in all isolates.

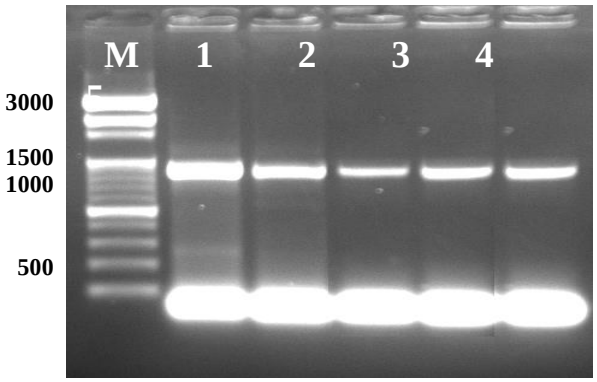

Fig. 1. Ethidium bromide stained gel (1.5%) of cleaved amplified polymorphic sequences within ITS - PCR amplified fragments resulting from. *P. infestans* Isolates: 1, Sal7a; 2, Sal9b; 3, Shb2b; 4, Nub10a and 5, Beh6a, M: marker (Amresco 100 bp Ladder, USA).

The amplified product from ITS4 and ITS5 was digested with *HealII* enzyme. The obtained results after digestion were shown in Fig. (2) and the pattern cleared out the differences among isolates concerning the density of the resulted bands and the number in bands for each isolate.

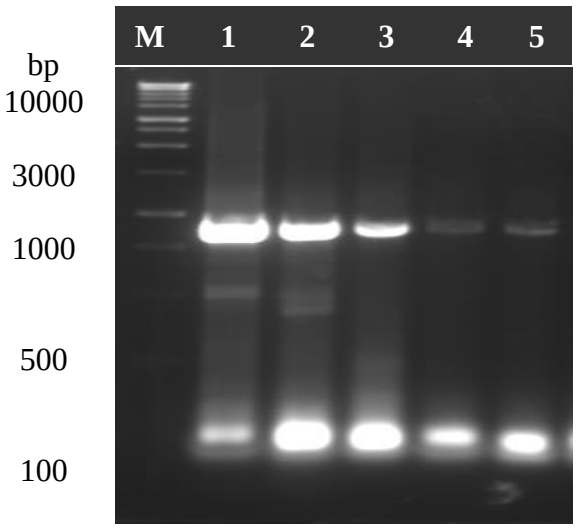

Fig. 2. Ethidium bromide stained gel (1.5%) of cleaved amplified polymorphic sequences within ITS PCR amplified fragments resulting from single digestion with *Hae*III. *P. infestans* Isolates: 1, Sal7a; 2, Sal9b; 3, Shb2b; 4, Nub10a and 5, Beh6a, M marker (Amresco 1K bp Ladder, USA).

Identification of A1 and A2 mating type in *P. infestans* isolates. In order to identify the mating types, the genomic DNA of 5 isolates were PCR amplified using the primers W16-1 and W16-2. Subsequently, the amplified product was digested with *Hae*III restriction enzyme, revealing two different patterns as described by Jamour *et al*. (2006). The one with two bands of 600 pb and 550 bp linked to the A1 mating type while the other one with a unique 550 bp band linked to the A2 mating type (Fig. 3).

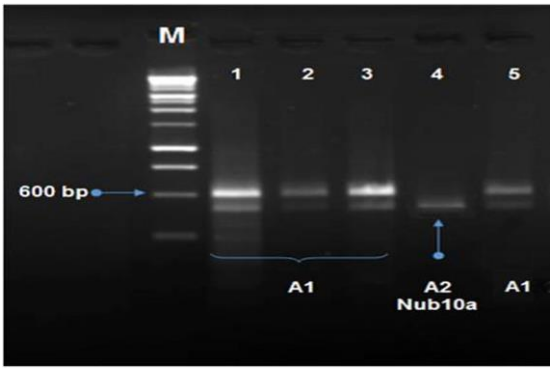

Fig. 3. Ethidium bromide stained gel (2%) of cleaved amplified polymorphic sequences within W16 PCR amplified fragments resulting from single digestion with *Hae*III. *P. infestans* Isolates: 1, Sal7a; 2, Sal9b; 3, Shb2b; 4, Nub10a and 5, Beh6a.

These CAPS markers were able to precisely differentiate the A2 mating type which is homozygote for this marker from the A1 mating type which is heterozygote. The molecular analysis showed that only one isolate, Nub10a, among 5 tested isolates belonged to A2 mating type. Since the population used for the assay was quite limited, no A2 : A1 ratio could be obtained out of current work.

Heterothallic fertilization: In dual cultures between the A2-Nub10a and each of the A1-type isolates on peas medium, positive result was obtained only with Salh7a (Fig. 4). The picture and the illustration show the attachment of the thick wall oogonia with the small antheridia coating the base of the oogonial stalk in a heterrothalic fertilization process. No mature oospore was observed in the intermediate area between the two isolates.

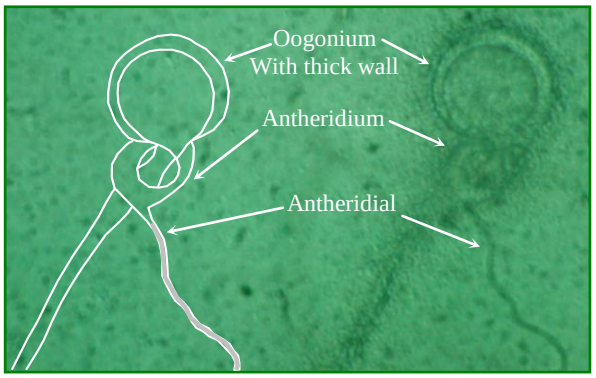

Fig. 4: ***Heterothallic fertilization of an oogonium by an antheridium belonging to isolate Nub10a (A2) and isolate Salh7a (A1) on peas medium.***

Table (2): Metalaxyl sensitivity of *P. infestans* isolates using relative growth (RG) according to Goodwin (1996) Sedigui (1999) and Matuszak (1994).

Isolates	RG			Phenotype		
	5 ppm	10 ppm	100 ppm	Goodwin	Matuszak	Sedigui
Sal7a	46	31	8	R[a]	I	I
Nub10a	32	26	5.5	S	S	I
Sal9b	52	42	26	R	I	I
Shb2b	65	57	41	R	R	I
Beh6a	21	8	–	S	S	S

[a] **R, Resistant; S, Sensitive; I, Intermediate.**

Metalaxyl sensitivity. Two methods were used to study the effect of metalaxyl on *P. infestans* as follows:

Agar test. Categorization of the five *P. infestans* isolates varied according to the used criteria. While Goodwin criteria considered 3 isolates as resistant to metalaxyl (Sal7a, Sal9b and Shb2b), only one of them, Shb2b, was considered resistant under Matuszak criteria and all of them were intermediate under Sedigui one. Sedigui criteria was the most conservative one as it required the isolate to have RG >60 at 10 ppm to be considered as

resistant. Thence, none of the test isolates passed this frontier. It is noteworthy to mention that all isolates were collected from fields where farmers used phenylamide fungicides application on a regular base.

Detached leaf test. Data presented in Table (3) show that according to the ED50 categorization (Reis *et al*., 2005); none of the tested isolates was insensitive to metalaxyl. All isolates were either sensitive (4 isolates, 80%) or intermediately insensitive (1 isolate, 20%). Effective Lethal Doses 50% calculated upon linear regression model for dose-response relation that fitted on growth inhibition as a function of fungicide concentration for isolates Nub10a, Sal9b, Beh6a and Shb2b were 0.0086, 0.0057, 0.0081 and 0.0068 µg/ml, respectively (>0.001 and <0.01 µg/ml) while it was 0.068 µg/ml for Sal7a (>0.01 and <10.0 µg/ml). Meanwhile, when data expressed as Corrected Efficacy % of metalaxyl, the EC% values of all isolates except Sal7a, the intermediately insensitive isolate, was above 50% at 0.01 µg/ml.

Table (3): Metalaxyl sensitivity of *P. infestans* isolates expressed as Disease Severity (D.S. %) and Corrected Efficacy (CE %).

Isolate	Sensitivity measure	0.0 (Control)	0.01	0.1	1.0	10	100	ED_{50} µg/ml
Sal7a	DS%	83.3	50.0	29.2	33.3	16.7	0.0	0.068
	CE%	0.0	40.0	65.0	60.0	80.0	100.0	
Nub10a	DS%	91.6	37.5	29.2	20.8	16.7	8.3	0.0086
	CE%	0.0	59.1	68.2	77.3	81.8	90.9	
Sal9b	DS%	83.3	37.5	25.0	29.2	8.3	8.3	0.0057
	CE%	0.0	55.0	70.0	65.0	90.0	90.0	
Beh6a	DS%	91.6	25.0	33.3	20.8	8.3	8.3	0.0081
	CE%	0.0	72.7	63.6	77.3	90.9	90.9	
Shb2b	DS%	91.6	41.7	41.7	33.3	16.7	16.7	0.0068
	CE%	0.0	54.5	54.5	63.6	81.8	81.8	

DISCUSSION

The use of CAPS markers allowed the identification of the presence of A2 mating type among some Egyptian isolates of *P. infestans* collected from different potato fields. PCR amplification of DNA from pathogen mycelium with specific primers W16-1/2 linked to the mating type locus allowed the amplification of a 600bp fragment in all tested isolates. Digestion of the PCR product with restriction enzyme *Hae*III revealed two distinct patterns. The first, which is a heterozygote, with two bands of 600bp and 550bp was characteristic of the A1 mating type. This is the most common mating type of *P. infestans* described all over the world (Jamour and Hamada, 2006). The second pattern, with a single band of 550bp was obtained for the A2 mating type which is present all over northern Europe, in Asia, in several African countries, in South America and in North America (Govers *et al*., 1997). These results allowed us to confirm the presence of the A2 mating type in Egypt. The A2 mating type was detected only in Nubaria region, where potato cultivated mainly for export to European Union countries, in addition to field-grown and plastic house tomatoes. Previously, it was assumed that *P. infestans* may survive on potato seeds or on potato and

tomato debris and may be transmitted from one crop to another all the year long, but current results supported the hypothesis that the perfect stage of the pathogen may contribute to it's annual flourishing in Egyptian fields with the beginning of winter where climatic conditions favors it's activity (high humidity and cool temperatures). To conclude, we can not now exclude the possible formation of oospores which can enhance primary infection sources of the disease, since these sexual spores can be dormant on plant debris and soil for such long periods.

Because the Egyptian climate favors late blight epidemics in most years and susceptible potato cultivars, such as Spunta, Lady Rosette and Cara, are widely grown, control depends mainly on fungicide application and on the development of resistant or tolerant *P. infestans* strains following frequent use of different fungicides became a major limitation for disease management.

Mutation and subsequent selection could have resulted in variation in metalaxyl sensitivity in Egyptian isolates of *P*. *infestans*. Even though migration contributes to introducing insensitive isolates into an area from a founder population (Goodwin *et al*., 1996). In the Egyptian case, continuous annual import of foreign potato seeds substitute regular immigration as a main source of new isolates since part of the seeds are carrying *P*. *infestans* propagules.

More of a concern is the variation in ranking isolates that was found when different tests were used. Even though reports of a correlation between agar and leaf disc methods were found (Goodwin *et al*., 1996), we couldn't establish correlation between same methods using metalaxyl fungicide. Our results were in agreement with data obtained by Reis *et al*. (2005). Considering that leaf-disc tests are more closely related to what happens in the field, this indicates that practical resistance to metalaxyl may occur in *P*. *infestans* populations in Egypt. Additional trials specifically planned to address the practical resistance issue might be needed.

Our data showed higher level of metalaxyl resistance when test isolates were exposed directly to fungicide incorporated in solid media compared with leaf detached technique. There was a highly significant difference among assay methods and the isolates used in this study. However, sensitivity and insensitivity responses of different isolates to phenylamide were consistent in different experiments. These results confirm other findings (Goodwin *et al*., 1998).

The higher effect of the fungicide in detached leaf test compared with agar test may be explained by the production of certain metabolites of the fungicide in the presence of living cells that might be more toxic to the pathogen compared with the original formula itself. Obtained metalaxyl metabolite included aryl hydroxylation, ester cleavage, ether cleavage (*O*-dealkylation), *N*-dealkylation (side-chain cleavage) Metalaxyl, CGA 329351 (N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-D-alanine methyl ester) and ring methyl hydroxylation (Owena and Donzel, 1986) EC50 = 36 mg/l and its metabolites: GA62826, EC50 > 1000 mg/l; CGA 67868, EC50 = 195.4 mg/l; CGA 108906, EC50 = 74 mg/l; CGA 107955 and CGA 108906 (Anonymous, 1997).

The occurrence of resistant isolates in fields where metalaxyl fungicides were used suggests that resistant isolates were developed over time under the selection pressure. Extensive use of metalaxyl fungicides leads to rapid increase of the resistant population produced by sensitive mutations all over the world (Reis *et al*., 2005). Therefore, to limit selection of resistant isolates, it is recommended to apply systemic fungicides in the early stage of the crop production, mainly as preventive strategy, with minimal number of applications during the crop season to minimize selection of resistant isolates.

Our current approach looked into the genetic characterization of the population of *P. infestans* by identifying both the mating type and the resistance to fungicide. These two markers were helpful aids in determination of the genetic variability of the pathogen. Genetic shifts in pathogen populations can lead to reassessment of management strategies for controlling the disease. For late blight, this is of particular importance because (i) disease control is heavily based upon fungicide application, (ii) *P. infestans* is a high-risk pathogen for development of resistance to fungicide, and (iii) the high value of tomato or potato crops together with the destructiveness of late blight make yield losses a more serious issue. Monitoring activities should be continued to assess efficacy of fungicide resistance management. Nevertheless, we propose to use other genetic markers that could help us more to distinguish between different isolates with various agronomic traits and to characterize the *P. infestans* population. The use of more specific and neutral molecular markers will be considered in a future work.

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دراسة التباين الوراثى لعزلات الفيتوفثيرا انفستنس بناءا" على المعلمات الجزيئية للنمط التزاوجى ومقاومة الميتالاكتسيل .

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تم تجميع عزلات المسبب المرضي فيتوفثورا انفستنس من حقول البطاطس بمناطق الزراعة الرئيسية في مصر خلال موسمي الزراعة ٢٠٠٦ و ٢٠٠٧. وقد استخدمت البادئات ITS4 and ITS5 في التمييز بين العزلات المختلفة حيث تباينت فيما بينها من حيث كثافة الباندات (شظايا الحمض النووي) وعددها، أيضاً استخدم المدخل الجزيئي في تعريف المسبب وأنماطة التزاوجية. أجريت عملية التضاعف للحمض النووي دنا المعزول من ميسيليوم المسبب النامي في المزارع النقية الناتجة عن عينات نباتية مصابة بواسطة البادئات W16-1/2 ثم تلى ذلك هضمه بانزيم *Hae*III المحدد. أظهرت النتائج وجود النمط التزاوجي A2 في عزلة واحدة (صالحية، بحيرة) من بين خمس عزلات تم التحصل عليها من الحقول التجارية بمصر. أيضا حيث انه لوحظ أن المطهرات الفطرية المحتوية علي الميتالاكسيل تفقد فعاليتها في الكثير من الحقول فقد أجري اختبار معملي لمقاومة الميتالاكسيل في عزلات الفيتوفثورا انفستنس. أوضحت النتائج وجود تحمل ومقاومة عاليين للميتالاكسيل في مجتمع عزلات المسبب المرضي. وقد لوحظت من نتائج البحث أهمية إجراء دراسات وراثية لمتابعة التطور في تركيب مجتمع الفيتوفثورا انفستنس وتغير قدراته المرضية.

قام بتحكيم البحث

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