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Effect of Pesticide(s) Carryover on Results of Polymerase Chain Reaction and Multiplex-PCR for Diagnosis of Potato Bacterial Pathogens

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ABSTRACT

The possible carryover effect of three fungicides and one insecticide on results of PCR and Multiplex-PCR for detection of bacterial pathogens was studied. The potato brown rot caused by phylotype II, sequevar I of *Ralstonia solanacearum* and the potato ring rot bacteria *Clavibacter michiganensis* ssp. *sepedonicus* were considered in this study. Under optimized conditions for PCR activity, the potato extract prepared from the Cara potato cultivar did not interfere with band development of DNA's extracted from the organisms in concern. Regardless of their mode(s) of action, the studied pesticides exerted different effect(s) on band development according to their chemical grouping. The dimethomorph (Acrobat), systemic fungicide, caused complete inhibition of band development of DNA extracted from either potato brown rot or potato ring rot bacteria which may be considered as false negative reaction(s). The propamocarb carbamate (Previcur N), systemic fungicide, did not inhibit band development of the organisms in concern. The dithiocarbamate plus zinc and manganese (Diathane M45), contact fungicide however, caused a complete band inhibition of ring rot DNA contrary to that being reported for brown rot, indicating a preferential polymerase activity. The organophosphate (Malathion), penetrative non-systemic insecticide, did not inhibit DNA band development for both organisms. Multiplex-PCR of mixed DNA's of *R. solanacearum* and *C. michiganensis* ssp. *sepedonicus* caused a false negative reaction through inhibition of band development of *R. solanacearum* but not for *C. michiganensis* ssp. *sepedonicus*, under the effect of propamocarb carbamate (Previcur N) fungicide and organophosphate (Malathion) insecticide. It could be concluded that the pesticides carryover effect on PCR results of potato brown rot and ring rot bacteria are being variable according to the pesticide grouping. The dimethomorph (Acrobat) fungicide caused false negative effect with both organisms under study followed by dithiocarbamate (Diathane) fungicide in case of ring rot bacteria. The false negative multiplex-PCR is being evident only in case of *R. solanacearum* under the stress of both propamocarb carbamate (Previcur N) fungicide and on the organophosphate (Malthion) insecticide. The mode of action of the used pesticide, either contact or systemic, had no effect on PCR or multiplex-PCR results. Further investigations are needed with different groups of pesticide.

Key words: Bacterial ring rot, band inhibition, false negative multiplex-PCR, fungicides and insecticides, PCR technique, potato brown rot bacteria, residues of certain pesticides, *R. solanacearum* and *C. michiganensis* ssp. *sepedonicus*

INTRODUCTION

Potato ring rot caused by *Clavibacter michiganensis* ssp. *sepedonicus* and potato brown rot caused by *Ralstonia solanacearum* are tuber-borne quarantine diseases (Kudela, 2007; Kabeil *et al*., 2008). The latent infection produced by these bacterial pathogen, under certain circumstances, has complicated the usual quarantine inspection decision, hence the dependency on more sophisticated laboratory methods have been described (Caruso *et al*., 1998). Among these protocols, polymerase chain reaction, PCR and its variant multiplex-PCR were tried (Seal *et al*., 1993; Farag *et al*., 2010). The multiplex-PCR as a modification of the original PCR testing is subject to certain reaction difficulties as PCR drift and PCR selection (Wagner *et al*., 1994) in addition to other difficulties related to the availability of primers for various plant pathogens (Martin *et al*., 2000; Schaad and Fredrick, 2002), the formation of primer dimer (Brownie *et al*., 1997) and the carryover of certain inhibitory factors in the samples (Rossen *et al*.,1992; Wilson, 1997). The most inhibitory realized factors being plant polysaccharides (Demeke and Adams, 1992), humic acid in plant and soil (Tsai and Olson, 1992; Watson and Blackwell, 2000) and certain groups of antibiotics (Farag *et al*., 2010). Therefore, the correct sampling and optimization of reaction conditions must be strictly considered to minimize such non-specific reactions and avoid false results (De Lomas *et al*., 1992; Polz and Cavanaugh, 1998).

The concept of this study stems from the fact that potato raised in the temperate parts of the world is subject to an array of foliar disease and insect attacks that are being routinely controlled with pesticides (Samoucha and Cohen, 1990; Powelson and Inglis, 1999; Gafar and Dagash, 2011).

The present study aimed to determine the possible carryover effect of potato pesticide (s), with different mode (s) of action, on the results of multiplex-PCR for detection of bacterial pathogens.

MATERIALS AND METHODS

Optimization of reaction conditions: The entire adjustment of multiplex-PCR conditions is carried out with consideration of the following:

Bacterial pathogens: Bacterial cultures of the targeted pathogens and their origin are shown in Table 1. The Bacterial Ring Rot (BRR) pathogen was grown on yeast extract glucose mineral salt agar, YGMA, [g:L Bacto-Yeast-Extract (Difco)2.0; D (+) Glucose (monohydrate) 2.5; K_2HPO_4 0.25; KH_2PO_4 0.25; $MgSO_4.7H_2O$ 0.1; $MnSO_4H_2O$ 0.015; NaCl 0.05; $FeSO_47H_2O$ 0.005; Bacto-Agar (Difco) 18] at 20°C (Anonymous, 1993). The bacterial brown rot (BBR) was grown on Kelman's Tetrazolium medium [g:LCasamino acids (Difco) 1.0; Bacto-Peptone (Difco) 10.0; Dextrose 5.0; Bacto-Agar (Difco) 15.0, after sterilization and Cooling to 50°C, a filter-sterilized solution of 2,3,5-triphenyl tetrazolium chloride (Sigma) is added to obtain a final concentration of 50 mg L^{-1}, (Kelman, 1954).

Primers: The sequence of primers used in this study is shown in Table 2. The primers were provided by OPRON, USA.

Table 1: Source of bacterial pathogens and disease(s) produced

Bacterial pathogen	Source	Disease
Clavibacter michiganensis ssp. *sepedonicus*	*PD221	BRR
Ralstonia solanacearum (race 3, biovar 2)	**PBRP300	BBR

BRR: Bacterial ring rot, BBR: Bacterial brown rot, *PD: Plant protection service, Wageningen NL., **PBRP: Potato brown rot project, Dokki, Egypt

Table 2: Sequence of primers used with BRR and BBR (phylotype II, sequevar I, i.e., race 3, biovar 2)

Bacterial pathogen	Abbreviation	Sequence (5'-3')
Clavibacter michiganensis ssp. *sepedonicus*	Cms 50 R	5'TCCTGAGCAACGACAAGAAAA-3'
	Cms 50 F	5'GAGCGCGATAGAAGAGGAACTC-3'
Ralstonia solanacearum race 3, biovar 2 (phylotype II, sequevar I)	R.sol flic R	5'GGCGGCCTTCAGGGAGGTC-3'
	R.sol flic F	5'GAACGCCAACGGTGCGAACT-3'

Table 3: Name of pesticides used with multiplex-PCR

Pesticide used	Active component	Mode of action
Fungicides trade name		
Acrobat	Dimethomorph	Systemic
Diathane M45	Dithiocarbamate plus zinc and manganese	Contact
Previcur N	Propamocarb carbamate	Systemic
Insecticide trade name		
Malathion	Organophosphate	Penetrative, non-systemic

Procedure: Multiplex-PCR amplification was run by PTC-200 Peltier (USA) thermal cycler for primers listed in Table 2. The reaction mixture (25.0 μL) consisted of 12.5 μL Qiagen Taq PCR master mix, 2 μL of each of solanacearum primers and 2 μL of sepedonicus primers along with 2 μL of DNA extract. Supplement of pesticides in potato extract was added separately as 2.5 μL solution to the reaction mixture and being replaced by the same quantity of sterile UPW in the check treatment.

The initial denaturation was made at 96°C for 3 min followed by 35 reaction cycles at 94°C for 30 sec, then 52°C for 30 sec and 72°C for 30 sec. The mixture was then kept at 72°C 10 min^{-1} and stored at 4°C. Aliquots (13 μL) of the reaction mixture were resolved by electrophoresis in agarose gel (2%) and DNA fragments were visualized by ethidium bromide (o.5% μg mL^{-1}).

Potato extract: Potato extracts were made according to the protocols of the European Union Directive No. 98/57 EC and routinely used in Potato Brown Rot Project (PBRP), Egypt. Thin minced slices of the stolon ends of tubers (200 tubers) for processing were extracted in phosphate buffer [4.26 g of Na_2HPO_4 and 2.72 g of KH_2PO_4 (50 mM PO_4) L^{-1} of distilled water, pH 7.0]. The sample was shaken (100 rpm) under controlled temperature (15°C) for 4 h. Potato extract was used in different pesticide treatments.

Pesticides: The carryover effect of a group of fungicides and widely used insecticide at 1000 ppm of the commercial products on the results of multiplex-PCR was determined. Table 3 shows the trade name, active component and mode of action.

RESULTS

Multiplex-PCR optimization: Under the afore-mentioned conditions of the experiment the bands of *R. solanacearum* (phylotype II, sequevar I), previously called potato race 3, biovar 2, were recognized at 400 bp and those for *C. michiganensis* ssp. *sepedonicus* at 200 bp, being compatible with the master mix bands (Fig. 1). The figure also shows that potato extract cv. Cara did not interfere with band development for DNA extracted from either *R. solanacearum* and *C. michiganensis* ssp. *sepedonicus* (Fig. 1).

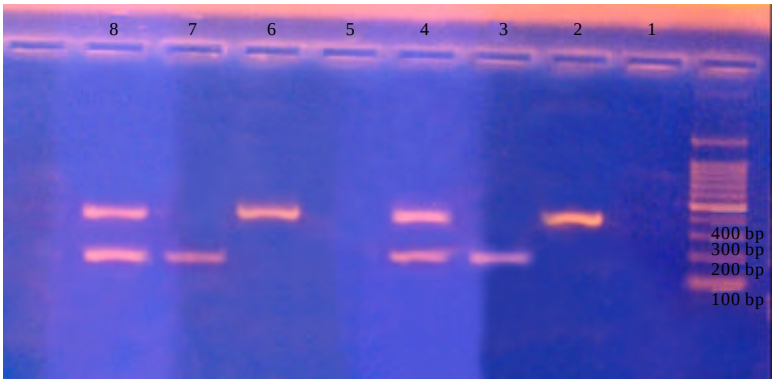

Fig. 1: Potato extract did not affect band development; Treatments from 6 to 8 with potato extract; Treatments from 2 to 4 without potato extract; Negative controls (1 and 5); 2 = *R. solanacearum* without potato extract; 3 = *C. michiganensis* ssp. *sepedonicus* without potato extract; 4 = *R. solanacearum*+*C. michiganensis* ssp. *sepedonicus* without potato extract; 6 = *R. solanacearum* with potato extract; 7 = *C. michiganensis* ssp. *sepedonicus* with potato extract; 8 = *R. solanacearum*+*C. michiganensis* ssp. *sepedonicus* with potato extract

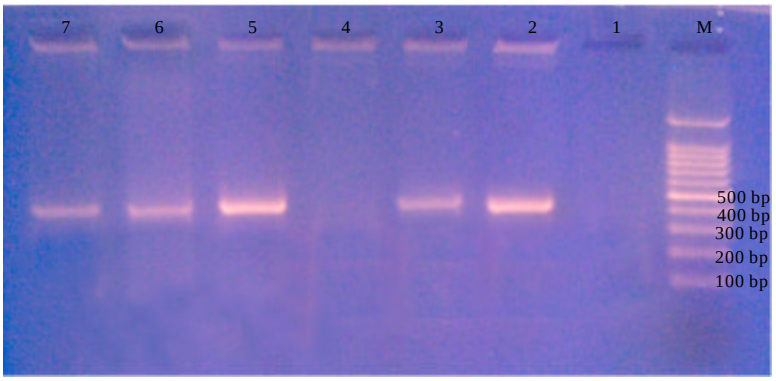

Fig. 2: Effect of pesticides on band development for *R. solanacearum* at 400 bp; M = Molecular size of marker (100 bp DNA); 1 = Negative control, 2 = Positive control, 3 = *R. solanacearum*+potato extract, 4 = *R. solanacearum*+potato extract+Acrobat, 5 = *R. solanacearum*+potato extract+Previcur N, 6 = *R. solanacearum*+potato extract+Diathane, 7 = *R. solanacearum*+potato extract+Malathion. Complete inhibition with acrobat pesticide (treatment 4)

Effect of pesticide(s) carryover on multiplex-PCR results: The possible carryover effect of fungicides used for control of the fungal pathogens, along with an insecticide used for control of potato aphids on multiplex-PCR results, are shown in Fig. 2-4.

Figure 2 shows that the carbamate fungicides, regardless of their mode of action, either contact or systemic, did not affect band development for DNA extracted from *R. solanacearum*. The

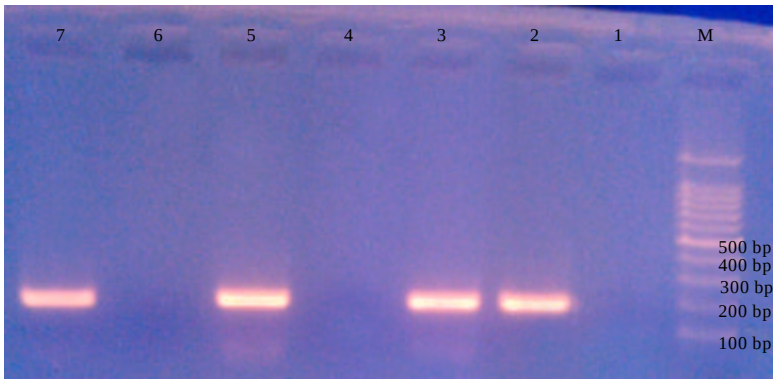

Fig. 3: Effect of pesticides on band development for *C. michiganensis* ssp. *sepedonicus* at 200 bp; M = molecular size marker (100 bp DNA), 1 = Negative control, 2 = Positive control, 3 = *C. michiganensis* ssp. *sepedonicus* +potato extract, 4 = *C. michiganensis* ssp. *sepedonicus*+potato extract+Acrobat, 5 = *C. michiganensis* ssp. *sepedonicus*+potato extract+Previcur N, 6 = *C. michiganensis* ssp. *sepedonicus*+potato extract+Diathane, 7 = *C. michiganensis* ssp. *sepedonicus*+potato extract+Malathion. Complete inhibition with Acrobat and Diathane pesticides (treatment 4, 6)

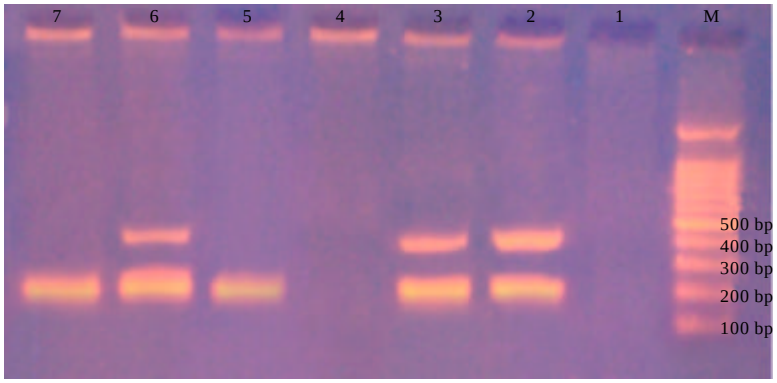

Fig. 4: Effect of pesticides on band development for *R. solanacearum*+*C. michiganensis* ssp. *sepedonicus* at 400, 200 bp; M = Molecular size marker (100-bp DNA), 1 = Negative control, 2 = Positive control, 3 = *R. solanacearum*+*C. michiganensis* ssp. *sepedonicus*+potato extract, 4 = *R. solanacearum* +*C. michiganensis* ssp. *sepedonicus*+potato extract+Acrobat, 5 = *R. solanacearum*+*C. michiganensis* ssp. *sepedonicus*+potato extract+Previcur N, 6 = *R. solanacearum*+*C. michiganensis* ssp. *sepedonicus*+potato extract+Diathane, 7 = *R. solanacearum*+*C. michiganensis* ssp. *sepedonicus*+potato extract+Malathion. Complete inhibition with acrobat pesticide (treatment 4)

propamocarb carbamate (systemic, previcur N,) and the bisdithiocarbamate (contact, diathane,) did not inhibit band development. The dimethomorph group (acrobat, systemic), however, caused a complete inhibition of band development.

Table 4: Summary on the effect of pesticides on the two organisms

	Band development for the targets			
			Mixed	
Pesticide	*R. solanacearum* (400 bp)	**C.ms (200 bp)	*R. solanacearum* (400 bp)	**Cms (200 bp)
Dimethomorph (Acrobat)	-	-	-	-
Dithiocarbamate plus zinc and manganese (Diathane M45)	+	-	+	+
Propamocarb carbamate (Previcur N)	+	+	-	+
Organophosphate (Malathion)	+	+	-	+

**Ralstonia solanacearum*, ***Clavibacter michiganensis* ssp. *sepedonicus*, +: No inhibition, -: Inhibition of bands

Figure 2 also shows that the organophosphate insecticide (penetrative but non systemic, malathion) did not inhibit band development for DNA of *R. solanacearum*.

With regard to the effect of fungicides carryover on *C. michiganensis* ssp. *sepedonicus*, Fig. 3 shows that the carbamate fungicide has exerted different effect on band development. The systemic propamocarb carbamate (previcur N) did not inhibit band development, contrary to the contact bisdithiocarbamate (diathane) that caused complete inhibition of banding. The systemic dimethomorph group (acrobat) caused complete inhibition of DNA banding as well. The penetrative non-systemic organophosphate insecticide (malathion) did not inhibit band development of sepedonicus DNA which is similar to that being reported for solanacearum DNA extracted.

The combined interaction of DNA's extracted from both *R. solanacearum* and *C. michiganensis* ssp. *sepedonicus* in multiplex-PCR trails is shown in Fig. 4. The systemic propamocarb carbamate (previcur N), caused, under the conditions of the experiment, an unexpectedly band inhibition for *R. solanacearum* at 400 bp which is being different from that reported in the usual PCR testing (Fig. 2). Such inversion in result is not evident in the usual PCR trial with C. *michiganensis* ssp. *sepedonicus* at 200 bp (Fig. 3).

The contact bisdithiocarbamate (diathane), however, did not inhibit banding of DNA of the mixed organisms, though the reported inhibitive effect on banding of *C. michiganensis* ssp. *sepedonicus* at 200 bp (Fig. 3). The systemic dimethomorph (Acrobat) fungicide has inhibited DNA banding of both organisms under investigation (Fig. 4). The latter effect is similar to that being reported in the usual PCR trials individually for both of them (Fig. 2 and 3).

The non-systemic penetrative organophosphate insecticide (malathion) caused apparent band inhibitions for *R. solanacearum* and distinct production for *C. michiganensis* ssp. *sepedonicus* band at 200 bp was detected (Fig. 4) similar to that being detected Fig. 3.

Table 4 summarizes the effect of pesticides on the both organisms.

DISCUSSION

Early studies on multiplex-PCR have highlighted the obstacles that can jeopardize the production of sensitive and specific multiplex assays but more recent studies have provided systematic protocols and technical improvements for simple test design (Elnifro *et al*., 2000). Multiplex-PCR as a modification of the original PCR testing is subject to a certain difficulties as PCR drift and PCR selection (Wagner *et al*., 1994). PCR drift is a bias assumed to be due to fluctuation in the interaction of PCR reagents particularly in the early cycles which could arise in the presence of a very low template concentrations (Dieffenbach *et al*., 1993; Mutter and

Boynton, 1995); variations in the thermal profiles of a thermo cycler, resulting in an unequal ramping temperatures; or simple experimental error. PCR selection on the other hand, is defined as a mechanism which inherently favors the amplification of certain templates, due to the properties of the target, the target flanking sequences, or the entire target genome (Wagner *et al.*, 1994).

The present study aimed to study the effect of the possible carryover of four potato pesticides on the results of PCR and the results of its variant multiplex-PCR for the detection of *R. solanacearum* and *C. michiganensis* ssp. *sepedonicus*.

The results showed that under optimized reaction condition, the potato extract exerted no interference with band development of the brown rot pathogen at 400 bp and the ring rot pathogen at 200 bp, with either PCR or multiplex-PCR. In this regard, Farag *et al.* (2010) reported that the multiplex-PCR showed a high degree of reliability to detect these bacterial pathogens along with *Erwinia carotovora* ssp. *atroseptica*, the causative agent of potato blackleg.

The selected pesticides are of common use on potato worldwide (Samoucha and Cohen, 1990; Powelson and Inglis, 1999; Chaurasia, 2005; Gafar and Dagash, 2011). Regardless, of their mode(s) of action, either contact or systemic, the propamocarb carbamate (previcur N) and bisdithiocarbamate (diathane) did not inhibit the band development of the DNA target of the brown rot pathogen. Under similar conditions, however, the bisdithiocarbamate caused a complete inhibition of DNA band of the other target pathogen i.e., the bacterial ring rot. Therefore, it could be concluded that the carbamate fungicides, according to their chemical nature and the attached side groups, were reacting differently. Such reported differences may be attributed as well to certain factors preventing optimal annealing and/or the extension rates of the polymer in case of ring rot pathogen (Ruano *et al.*, 1991). Therefore, the bisdithiocarbamate (diathane) carryover may interfere with PCR reaction, thus producing false negative results and producing considerable difficulties during ring rot diagnosis. The non-systemic penetrative organophosphate insecticide (malathion) did not inhibit the band development of the targets in concern, indicating no interference with enzyme activity under both circumstances. The systemic dimethomorph fungicide (acrobat), under the conditions of the experiment, caused a complete inhibition of band development of DNAs extracted from either *R. solanacearum* or *C. michiganensis* ssp. *sepedonicus* which may be considered as false negative reaction. The effect may be attributed to direct interference of the fungicide with enzyme activity, due to possible changes in the optimal reaction conditions (Stephenson and Abilock, 2012). In this regard the multiplex-PCR with certain medical bacteria showed 2 false-negative in many positive samples but no false-positive was detected (Kim *et al.*, 2010). It is well established recently that the rate at which primers anneal to their target and the rate at which annealed primers are extended along the desired sequence, during different cycles of the amplification, are among the most important factors controlling the successful detection. Factors preventing optimal annealing rates include poorly designed primers and suboptimal buffer constituents and annealing temperature. Such conditions are not apparently evident in the present study as shown by the positive controls for both organisms (Fig. 1-3). Moreover, the bias in template-to-product ratios may be considered in multiplex-PCRs that are designed to amplify more than one target simultaneously (Walsh *et al.*, 1992; Mutter and Boynton, 1995; Polz and Cavanough, 1998).

In retrospect, it could be concluded that potato extract did not inhibit band development of DNA extracted from *R. solanacearum* and *C. michiganensis* ssp. *sepedonicus* either in PCR or multiplex-PCR. The dimethomorph (Acrobat) fungicide caused a false negative PCR and

multiplex-PCR results, for both organisms under investigation. Multiplex-PCR, for mixed DNA's of *R. solanacearum* and *C. michiganensis* ssp. *sepedonicus*, caused false negative results in propamocarb carbamate (Previcur N) and organophosphate (Malathion) fungicides treatments.

Further investigations are needed to study in greater depth an additional numbers of pesticides practically used with potatoes to overcome the possible carryover effect on PCR and multiplex-PCR results during bacterial disease diagnosis.

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