Plant Pathology Journal

ISSN 1812-5387

science alert

ANSInet
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Plant Pathology Journal 13 (1): 28-36, 2014
ISSN 1812-5387 / DOI: 10.3923/ppj.2014.28.36

Species Determination of Cross-Reacting Bacteria in Immunofluorescens Diagnosis of Potato Brown Rot

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Abstract: Several advanced tests for detection of *Ralstonia solanacearum* phylotype II, sequevar I (race 3 biovar 2) in different habitats are nowadays developed. The immunofluorescent antibody staining techniques (IFAS), along with enzyme linked immunosorbent assay (ELISA) have been widely practiced. Using IFAS, however, under certain circumstances produced non-specific reactions. In the present study, a total of suspected 711 bacterial isolates recovered over four years period (2009-2012) of routine testing of potato crop in PBRP laboratories, Egypt, were subject to further determinations. All these isolates were tested by IFAS using internationally approved antiserum brand to detect *R. solanacearum*. These isolates were preliminary screened on Semi Selective Media of South Africa (SMSA) medium for presumptive detection of *R. solanacearum*. Colonies showing characteristics morphology of the pathogen in concern were scored for 206 isolates on SMSA medium. These characteristically screened isolates were physiologically and biochemically tested to confirm identity to *R. solanacearum*. A sum of 505 bacterial isolates devoiding the described characteristics of the pathogen were categorized into 7 groups according to their gram-staining reaction, cell morphology and cultural characteristics. Each group embraces a tentatively similar isolates according to motility, catalase and oxidase test(s) in principal. The results of Biolog identification revealed the following species of the cross-reacting bacteria that being affiliated to different families: *Acinetobacter calcoaceticus*, *Aeromonas caviae*, *Moraxella osloensis*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas corrugata*, *Pseudomonas mendocina*, *Pseudomonas tolaasii*, *Salmonella typhimurium*, *Serratia proteamaculans*, *Shewanella algae*, *Sphingobacterium multivorum* and *Bacillus maroccanus*. The aforementioned identified bacteria were tentatively tested for the possible pathogenic potential to plant as shown by causing soft rot in potato tubers and inducing Hypersensitive Reaction (HR) in leaves of Burley tobacco plants.

Key words: Brown rot, cross-reacting bacteria, hypersensitive reaction, immunofluorescence antibody stain (IFAS), pectinolytic potentials, potato bacterial wilt, polyclonal antiserum, *Ralstonia solanacearum*, real-time PCR

INTRODUCTION

Successful disease management and control practices greatly depend on an understanding of the ecology of the pathogenic organism in the environment. The ability of *R.solanacearum* to survive in field soil is subject to controversy due to co-extensiveness of strains and diversity of host plants for certain races (Kelman, 1953; Buddenhagen and Kelman, 1964), latent infection (s), re-infestation of soil through irrigation water (Farag *et al*., 1999) and last but not the least the lack of sensitive methods, at the time of the majority of studies had been carried out, for the detection of the pathogen at different strata of the soil profile. More recent studies, based in principal on molecular biology, have eliminated in part the difficulties related to the accurate sensitive detection.

R. solanacearum is the causal organism of Potato Bacterial Wilt (PBW) or potato brown rot (PBR) a disease that, after late blight, is the second major constraint to potato production in tropical and subtropical regions worldwide (French, 1994). Regardless of the place of origin the movement of seed and/or commercial potato stocks either diseased showing symptoms or latently infected tubers is the main way for disease spreads (Graham *et al*., 1979) and strict quarantine regulations have been applied to avoid dissemination of the disease in the member states of the European Union Directive No.98/57EC. Several detection methods, including isolation, indirect immunofluorescence antibody staining (IFAS) analysis, enzyme-linked immunosorbent assays (ELISAs) and bioassays, though the apparent constraint for each of them, have been used for examination of

potato lots (Elphinstone *et al*., 1996, 2000). It could be concluded, however that ELISA, IFAS, immunofluorescence colony staining (IFC) may be considered as a good compromise between sensitivity and specificity of detection and between ease and expense of application (Robinson-Smith *et al*., 1995; Elphinstone *et al*., 1996).

The present study was undertaken to determine the most predominant genera involved in the cross-reaction with the IFAS techniques.

MATERIALS AND METHODS

Tuber samples processing: 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. The stolon ends of the processed tuber sample (200 tubers) were extracted in phosphate buffer at pH 7.0. The sample was shaken (100 rpm) under controlled temperature (15°C) for 2 h. The supernatant was centrifuged (10,000×g) under cool (6°C) for 15 min and the supernatant was discarded. The pellet was resuspended in 1 mL of a second phosphate buffer at pH 7.2 and subjected to laboratory examination(s).

Immunofluorescence antibody stain (IFAS): IFAS as a presumptive serological identification of *R. solanacearum* was carried out. The polyclonal antibodies was provided by Loewe Biochemica GmbH, Germany and produced in goats against *R. solanacearum* race 3, biotype II, cat. No.07356. The anti-rabbit anti-goat (RAG/IgG (H+L) (FITC) antiserum is conjugated with fluorescin isothiocyanate cat. No. 07200, provided by Nordic Immunological Laboratories B.V., Netherlands and being used in IFAS testing.

Tested isolates: A total of 711 fluorescence emitting isolates during IFAS testing, of the samples under examination were used to check the possible range of cross-reaction(s).

Isolation of IF positive bacteria: Buffer resuspended pellets, after being used in IFAS testing, were streaked on King's medium B (King *et al*., 1954) and Semi Selective Media of South Africa (SMSA) medium (Elphinstone *et al*., 1996) and were incubated at 28°C for three days. The developed colonies were streaked on nutrient agar slants and used in further tests.

Real-time PCR assay: The Real-time PCR (Taq-Man) or Fluorogenic PCR for DNA amplification was made in PBRP laboratories, Egypt according to Weller *et al*. (2000). The reaction mixture consisted of sixteen μL of master mix (1 μL primer forward, 1 μL primer reverse, 1 μL probe, 12.5 μL master mix and 0.5 μL BSA was added to each well, of a 96-well PCR plate. The wells were filled enough with all samples and control reactions in duplicate. Nine microlitter of nucleic acid extract was added to each tube as appropriate, ensuring that the sample was pipetted directly into the master mix. Positive controls of DNA extracts and water was used for negative control. After the filled wells were capped, the plate was transferred to the machine Applied Biosystem 7500 real time thermo-cycling and detection of fluorescence.

Pathogenicity test and pectinolytic potentials: The seven groups represented by 13 isolates were tested for producing soft rot symptoms in potato tubers (Fawzi, 1980) and producing Hypersensitive Reaction (HR) in tobacco leaves (Boucher *et al*., 1985).

Class E Inova healthy seed tubers used in this study were kindly provided by the Potato Brown Rot Project (PBRP), Agric. Res. Center, Giza, Egypt. Pure cultures were tested for ability to induce soft rot symptoms in potato tubers. Bacterial suspensions were prepared by adding approximately 5 mL of sterile tap water to each culture; the growth was washed out and used for inoculation after standardization of optical density to give 0.3 (610 nm) to obtain approximately 1×10^{6} CFU mL^{-1}. Alcohol flamed standard tubers were used for testing rotting ability. A hole was made in each tuber, in mid distance between stolon and rose ends, by using sterilized cork borer and the bacterial suspension (0.5 mL) was pipetted in. The hole was then closed with the respective removed cylinders and sealed with sterile paraffin wax. Inoculated tubers were incubated for 6 days at 28-30°C and examined for rot development. Control tubers were prepared in the same way using sterile water instead of bacterial suspensions. Rotting ability was determined by tuber inoculation technique used by Fawzi (1980) taking the calculated amounts of rot in cm^3 as criteria for rotting ability (Hollis and Goss, 1950).

Hypersensitivity (HR) on Tobacco: Hypersensitivity produced was tested in tobacco leaves cv. Burley, infiltrated as previously described by Boucher *et al*. (1985). Reactions correspond to delay and/or partial necrosis of the infiltrated area was considered.

Identification of the isolates: A total of 505 isolates causing cross-reaction were categorized into 7 different groups according to macro-morphological characteristics. Isolates representing the seven groups were selected for complete identification in Cairo MIRCEN by using Biolog GEN III DB.15 G.

Statistical analyses: One way ANOVA was conducted to distinguish between the effects of the different used organisms in the amount of rot produced by cm^3 (SPSS version17).

RESULTS

IF examination of samples throughout 2009-2012: In a trail to provide a solid answer on the value of IF testing, for screening out latent infection(s) with *R. solanacearum* in exported potato crop, a four years monitoring assay was followed. In the first year of the study in 2009, 21958 symptomless samples were subject to IFAS testing that showed 21758 negative IF reaction and 200 samples, ranging between positive and false positive reaction. The confirmed IF positive samples in this year were 33% out of 200 IF suspected samples (Table 1). In the second year of the study, in 2010, out of 22649 symptomless samples, 22458 showed negative IF reaction and 191 suspected positive, though the confirmed positive was determined to be 27.2%. Similar trend could be recognized in the year 2011and the recorded confirmed positive results was 34.1%. It is interesting to recognize the very low number of the suspected positive samples (150 samples) in 2012 compared to those recorded in the years 2009, 2010 and 2011 being 200, 191 and 170 samples, respectively. Moreover, it is worth to note that the lowest confirmed percentage of positive IF was recorded in 2012 being 20%.

It could be concluded in general that out of 101599 symptomless tubers samples tested only 206 sample were confirmed positive. They represent 29% of the total suspected positive (Table 1). Figure 1(a-b) shows different patterns of cross-reacting bacteria ranging from low frequency of fluorescent cells (Fig. 1a) to high frequency/microscopic field (Fig. 1b).

Results of identification cross-reacting bacteria: Data in Table 2 show the Biolog identification of the IF cross-reacting bacteria explored in this study. The majority of the identified species was found affiliated to the family Pseudomonadaceae (79.2%) that embraces fluorescent pigment producers and non pigmented ones. The fluorescent pseudomonads were identified as *Pseudomonas fluorescence*, *Pseudomonas putida* and non fluorescent pseudomonads as *Pseudomonas mendocina*, *Pseudomnas tolaasii* and

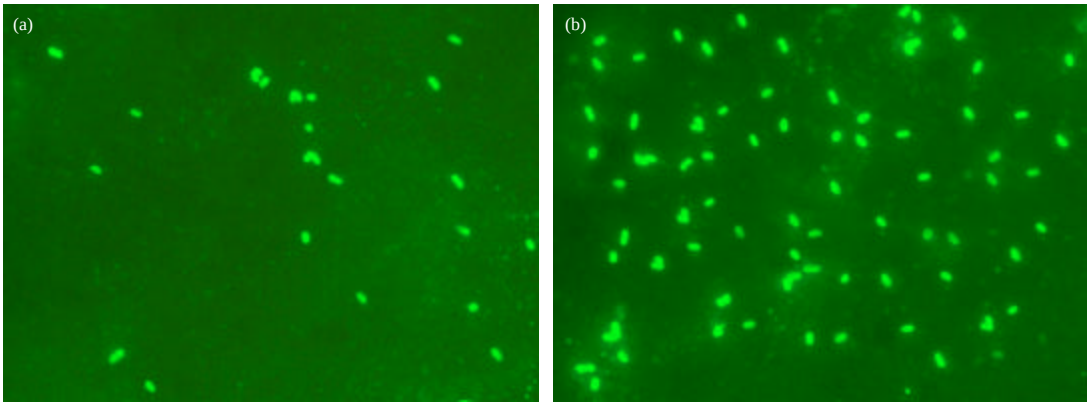

Fig. 1(a-b): Cell morphology of a cross-reacting bacteria (a) Low frequency of fluorescing cells per microscopic field and (b) High frequency of fluorescing cells per microscopic field

Table 1: Showing No. of samples in four years (2009-2012) examination, No. of IF suspected positive, confirmed positive and the percentage of latent infection

			Positive and false positive samples			
Seasons	No. of tests	No. of IF negative samples	No. of IF suspected positive samples	No. of IF confirmed positive samples	Confirmed positive (%)	Latency (%)
2009	21958	21758	200	66	33.0	0.3
2010	22649	22458	191	52	27.2	0.2
2011	34126	33956	170	58	34.1	0.2
2012	22866	22716	150	30	20.0	0.1
Total	101599	100888	711	206	29.0	0.2

Pseudomonas courrugata. The latter species is known to be enlisted among plant pathogens (Kudela *et al*., 2010).

It is interesting to note that about 7.3% of the isolated bacteria were found to belong to the family Enterobacteriaceae, being *Serratia proteamaculans* and *Salmonella typhimurium*. The latter species is known to be enlisted among animal pathogens (Santos *et al*., 2001).

Moraxella osloensis and *Acinetobacter calcoaceticus* belonging to Moraxellaceae 3% of the total were found as IF cross-reacting. These species are known to be human pathogens (Tan and Grewal, 2001; Doughari *et al*., 2011).

With regard to the spore-forming bacteria affiliated to Bacillaceae only *Bacillus maroccanus* (18 isolate and 3.6% of the total) were found IF cross-reacting.

It could be concluded in general that bacterial families of different habitats as Sphingobacteriaceae (3%), Shewanellaceae (2%) and Aeromonadaceae (2%) were also found to be among the IF cross-reacting bacteria during *R. solanacearum* IF polyclonal testing.

Table 2: Families embracing cross-reacting bacteria and the percentage of incidence

Family	Species
Pseudomonadaceae (400) isolates = 79.2% of the total	fluorescent pseudomonads: *Pseudomonas fluorescence* *Pseudomonas putida* Non-fluorescent pseudomonads: *Pseudomonas corrugata* *Pseudomonas mendocina* *Pseudomonas tolaasii*
Enterobacteriaceae (37) isolates = 7.3% of the total	*Serratia proteamaculans* *Salmonella typhimurium*
Bacillaceae (18) isolates = 3.6% of the total	*Bacillus maroccanus*
Moraxellaceae (15) isolates = 3% of the total	*Acinetobacter calcoaceticuss* *Moraxella osloensis*
Shewanellaceae (10) isolates = 2% of the total	*Shewanella algae*
Sphingobacteriaceae (15) isolates = 3% of the total	*Sphingobacterium multivorum*
Aeromonadaceae (10) isolates = 2% of the total	*Aeromonas caviae*

Pathogenicity and hypersensitivity (HR) of cross-reacting bacteria: Pathogenicity test of the isolated bacteria showed a considerable difference in severity of rot produced by various bacterial groups under investigation. Therefore, it was found interesting to determine precisely the ability of each group to induce tuber rot under controlled well defined conditions (Table 3). It is interesting to note that the most predominant genera that caused soft rot, was fluorescent pseudomonads such as *Pseudomonas fluorescence, Pseudomonas putida.* Also, the non fluorescent pseudomonads such as *Pseudomonas corrugata* and *Pseudomonas mendocina* caused soft rot. On the other hand, *Serratia proteamaculans*, *Shewanella algae* and *Sphingobacterium multivorum* also cause soft rot in potato. *Pseudomonas tolaasii*, *Aeromonas caviae*, *Acinetobacter calcoaceticus*, *Moraxella osloensis*, *Salmonella typhimurium* and *Bacillus maroccanus* gave negative results with the pathogenicity test. The highest decay zone was recorded for *Pseudomonas corrugata*. The lowest decay zone was recorded for *Serratia proteamaculans* and *Sphingobacterium multivorum*. No significant difference between *Pseudomonas fluorescence*, *Pseudomonas mendocina* and *Shewanella algae* (Table 3).

In hypersensitive reaction, the bacteria *Pseudomonas corrugata* gave positive result on tobacco leaves but bacteria *Serratia proteamaculans* gave weakly positive result on tobacco leaves (Fig. 2a-c). On the other hand, the rest of the others bacteria did not give any positive results with this test.

Table 3: Preliminary determination of bacteriological and pathological characteristics of the cross-reacting bacteria

Species	G. staining	Catalase test	Oxidase test	Motility	Pathoginicity test	Amount of rot cm^3	HR test
Pseudomonas fluorescence	-	+	+	+	+	9.9^{e}	-
Pseudomonas putida	-	+	+	+	+	7.5^{b}	-
Pseudomonas corrugata	-	+	+	+	+	14.9^{d}	+
Pseudomonas mendocina	-	+	+	+	+	10.6^{c}	-
Pseudomonas tolaasii	-	+	-	+	-	0.0	-
Acinetobacter calcoaceticus	-	+	-	-	-	0.0	-
Moraxella osloensis	-	+	+	-	-	0.0	-
Salmonella typhimurium	-	+	-	+	-	0.0	-
Serratia proteamaculans	-	+	-	+	+	5.4^{a}	-/+
Shewanella algae	-	+	-	+	+	11.6^{c}	-
Sphingobacterium multivorum	-	+	+	-	+	5.7^{a}	-
Aeromonas caviae	-	+	-	+	-	0.0	-
Bacillus maroccanus	+	+	+	-	-	0.0	-

Species with the same letter is not significantly different either at 0.005 or 0.001

Fig. 2(a-c): Hypersensitive reaction of cross-reacting bacteria, (a) *Serratia proteamaculans* and (b-c) *Pseudomonas corrugata*

DISCUSSION

Serological techniques for diagnosis plant pathogens have been used during the last few decades (Cambra and Lopez, 1978). These methods with polyclonal antisera were described as rapid and inexpensive but lacked sensitivity and commonly gave false positive results due to non-specific reaction with other bacteria (Calzolari *et al*., 1982; De Boer, 1982; Elphinstone *et al*., 1998) and limited sensitivity (De Boer *et al*., 1994, 1996) that being described as a serious drawback for differentiating pathogenic bacteria from saprophytic bacteria with such method of detection (Griep *et al*., 1998). Lipopolysaccharide (LPS) molecules from different bacterial species have the same general chemical composition, comprising three major parts covalently bound to one another (Nikaido and Nakae, 1979; Rietschel *et al*., 1982). Four monoclonal antibodies against *Escherichia coli* j5 reacted with antigens from some different gram negative bacteria (*Salmonella minnesota* Re595, *Agrobacterium tumefaciens* and *Pseudomonas aeruginosa*) these bacteria affiliated to different families (Enterobacteriaceae, Pseudomonadaceae, Vibronaceae and Rhizobiaceae). The simplest explanation of this extensive antigenic cross-reaction is that all lipid A moieties in gram-negative bacteria have a common evolutionary origin and have been strongly conserved over time. Also, lipid A may be an essential component of these bacterial cells (Mutharia *et al*., 1984).

According to Miller (1984) and also from experience gained in this study with potato bacteriology, *P. fluorescens* is recognized as the most frequently cross-reacting bacterium in serological tests (Kokoskova and Pankova, 1998). In a similar other groups study, antibodies for *C. michiganensis* subsp. *sepedonicus* cross-reacted with other bacteria of genus Clavibacter, such as *C. michiganensis* subsp. *michiganensis* and *C. michiganensis* subsp. *insidiosus* (De Boer, 1982; De Boer *et al*., 1988). The cross-reactivity confirms a strong serological relationship between these two subspecies and *C. michiganensis* subsp. *sepedonicus*. Cross-reactivity is a problem of all serological diagnostic tests but the specificity of tests with monoclonal antibodies that react with a single antigenic determinant is potentially much better than tests with polyclonal antibodies that react with a large number of different determinants.

It is well documented that, the testing protocol of the EU directive No. 98/57/EC for potato brown rot diagnosis is based on five steps. Among these steps the streaking on Semi Selective Media of South Africa (SMSA) medium is an essential steps, though the development of atypical colony forms, or saprophytic bacteria were recognized (Pradhanang *et al*., 2000).

***Pseudomonas putida*:** It is a Gram-negative, rod shaped affiliated to Pseudomonadaceae. It is a plant growth-promoting rhizobacterium originally isolated from the rhizosphere of potato. The bacterium was reported as a potential pathogen antagonist through siderophore mediated competition for iron and/or inducing systemic resistance (Meziane *et al*., 2005). It induced soft-rot in potato tubers as well.

***Pseudomonas corrugata*:** Its Development on SMSA medium is recognized in this study. It is described as Gram-negative, rod shaped non-sporulating bacterium

and is known to produce a serious disease in greenhouse-grown tomatoes and recovered from healthy roots of greenhouse-grown alfalfa plants (Lukezic, 1979). The bacteria caused external and internal dark brown lesions, collapse of pith, vascular browning and wilting of inoculated tomato plants leaves (Kudela *et al*., 2010). In the present study it showed pathogenic potentials to potato tubers and induced Hypersensitive Reaction (HR) on tobacco leaves. The association of this bacterium with the sampled potato and the origin of this bacterium developed on SMSA medium are difficult to be discussed in terms of methodologies used in this study.

***Pseudomonas mendocina*:** It is a Gram-negative, rod shaped, non fluorescent bacteria of pseudomonadaceae. It is motile, non-sporulating, oxidase and catalase positive. Colonies are flat, smooth butyrous and produced yellow pigment. It can be isolated from soil and water (Palleroni *et al*., 1970). Moreover, *P. mendocina* was reported to stimulate plant growth and inhibited the reproduction of the root knot nematode, *Meloidogyne incognita* in tomato plant (Robin *et al*., 1999).

***Pseudomonas tolaasii*:** It was identified in this study, it is a Gram-negative, rod shaped and found in soil (Brodey *et al*., 1991). This bacterium is described as the causal agent of brown blotch disease of cultivated mushroom (Kim *et al*., 2011). According to this study, it did not cause soft rot symptoms on potato tubers or induced HR reaction in tobacco leaves.

***Salmonella typhimurium*:** It is a pathogenic Gram-negative, non-sporulating rod shaped bacteria of the Enterobacteriaceae. It is pathogenic to animals and predominately found in the intestinal lumen. The origin of this species as a cross-reacting bacterium may be due to farmyard manure application in potato farming practices followed in Egypt.

Contaminated manure and manure slurry may pose a risk when used as fertilizer. Several conditions may influence the survival of pathogens in manures and/or its slurry. They include temperature, pH, microbial content, Oxidation-Reduction Potential (ORP) and time. However, few reports attempting to quantify the impact of these factors on survival of *Salmonella* in manure were found (Himathongkham *et al*., 1999). Contrary to *Serratia proteamaculans* isolated in this study it neither produced soft rot symptoms in potato tubers nor induced HR in tobacco leaves.

***Serratia proteamaculans*:** It is a Gram-negative, non-sporulating, motile bacteria affiliated to Enterobacteriaceae. It can be isolated from natural environments, from plant roots, soil and water. Also, this bacterium was isolated as a root endophyte from *Populus trichocarpa* and has been found to promote plant growth. It helps the host to overcome toxic effect of environmental pollution. On the other hand, this bacterium produces a cold-active low molecular bacteriocin-like compound named serraticin A. The latter is different from other microcens assayed with wide inhibitory spectrum and can be applied as a control agent against pathogenic bacteria (Sanchez *et al*., 2010). It produced, however, soft rot symptoms in potato tubers in the present study, indicating a possible degree of pathogenic potential.

***Acinetobacter calcoaceticus*:** It is strictly aerobic non-fermentative, Gram-negative, non motile, short rods, oxidase negative and nitrate reductase negative (Doughari *et al*., 2011). The genus Acinetobacter is widely distributed in nature and is commonly occurs in soil. However, this bacterium is siderophoregenic and highly efficient plant growth promoter (Sarode *et al*., 2009). It survives on moist and dry surfaces including a hospital environment and foodstuffs. It is however, thought to be an allergy-protective in man due to skin colonization (Debarry *et al*., 2010).

***Moraxella osloensis*:** It is a Gram-negative aerobic bacterium that is coccal or rod shaped but which tends to be pleomorphic. This bacterium is associated with slug parasitic nematodes so it is used in biological control of slugs (Tan and Grewal, 2001). The bacterium did not produced soft rot in potato tuber or induced hypersensitivity to tobacco and the development of this bacterium on SMSA medium is questioned.

***Sphingobacterium multivorum*:** It was identified with the cross-reacting bacteria. It is a Gram-negative, strictly aerobic, non-motile short rod (Yabuuchi *et al*., 1983) and another *Sphingobacterium composti* can be isolated from cotton-waste compost (Yoo *et al*., 2007). The origin of these bacteria, however, may be due to the used brand of peat moss for potato packing during exportation.

***Shewanella algae*:** It is a Gram-negative, motile rod which in general is non-fermentative, non-sporulating although the ability to ferment glucose has been reported in a few species and is classified with Shewanellaceae

(Ivanova *et al*., 2001) and most strains reduce (TMAO) trimethylamine-N-oxide (Brettar *et al*., 2002). *Shewanella* species can be isolated from a wide range of habitats. *Shewanella algae* is a pathogenic bacteria for humans most commonly involve ears, skin and soft tissues with or without bacteraemiae. In the present study it produced soft rot in potato tubers indicating pectinolytic ability and had no ability to induce hypersensitivity to tobacco.

***Aeromonas caviae*:** it is enlisted with the IFAS cross-reacting bacteria. The occurrence of enteric pathogens and *Aeromonas* species in organic vegetables was reported by McMahon and Wilson (2001). The development of *Aeromonas caviae* on isolation plates in this study may be attributed to the older practices, of raising potatoes in Egypt, under heavy dressing with farmyard manures.

***Bacillus maroccanus*:** It is a Gram-positive; formed white colonies, rod shape and lack of flagella. Normally distributed in quite common habitats such as soil, water and foods. The standard *Bacillus maroccanus* was first isolated from warm arid soil (Delaporte and Sasson, 1967), it was also reported to be isolated from rotten zucchini (Garcia *et al*., 2004) and from mural painting (Heyrman *et al*., 2005). The strain *Bacillus maroccanus* surprisingly shows remarkable tolerance towards low temperature, high salinity and variable pH conditions. It was assigned as extreme halotrophic and facultative alkalophilic. In this study it did not produce soft rot in potato tubers and had no ability to induce hypersensitivity to tobacco leaves.

CONCLUSION

The IF method is useful for rapid primary screening of a large number of samples but lacked sensitivity and possibly leading a false positive results due to non-specific reaction with other bacteria. Also, lipid A-portion of Lipopolysaccharide (LPS) may be responsible for the cross-reaction from a variety of Gram-negative bacteria.

In retrospect, it could be concluded that the problem of cross-reacting bacteria in IFAS diagnosis of potato brown rot is largely dependent on the conditions of raising potatoes. The heavy dressing of farmyard manures may be the original cause of such phenomenon along with the use of low graded peat-moss for potato packing. The use of the SMSA medium, did not strictly eliminate the development of such contaminant is either soil saprophytes and or/human and plant pathogens. The plant inoculation, according the EU protocols of tomato seedlings as well may be misleading due to the development of certain plant pathogens or endophytes as *Pseudomonas corrugata* that causing serious disease problems in tomatoes under green-house conditions. Therefore, careful consideration must be taken during the application of EU protocols to avoid such pitfalls. Moreover, the strict dependency on Polymerase Chain Reaction (PCR) as a final decision for potato brown rot diagnosis may be advised.

ACKNOWLEDGMENTS

I wish to express my deepest gratitude to Dr. Nabil S. Farag, Head of Research Emeritus, Plant Pathology Research Institute, ARC, for constructive criticism, continuous encouragement, fruitful scientific discussions, valuable advises, guidance and sincere help. Thanks are extended to Dr.Abou-Serie M. Ismael, Director of Potato Brown Rot Project, for providing every possible facilities and sincere encouragement.

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