International Scholars Journals

African Journal of Parasitology Research ISSN 2343-6549 Vol. 7 (3), pp. 001-006, March, 2020. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

First detection of *Pseudomonas viridiflava*, the causal agent of blossom blight in apple by using specific designed primers

Mahsa Alimi[1]*, Heshmatollah Rahimian[2], Nader Hassanzadeh[1], Meysam Taghinasab Darzi[3], Asadollah Ahmadikhah[3], Asghar Heydari[4] and Giorgio Mariano Balestra[5]

[1]Department of Plant Pathology, Faculty of Agriculture and Natural Resources, Science and Research Branch, Islamic Azad University, Tehran, Iran.
[2]Department of Plant Pathology, Sari Agricultural Sciences and Natural Resources University, Sari, Iran.
[3]Department of Plant Pathology, Gorcan Agricultural Sciences and Natural Resources University, Gorgan, Iran.
[4]Iranian Research Institute of Plant Protection, Tehran, Iran.
[5]Department of Science and Technology for Agriculture, Forestry, Nature and Energy, University of Tuscia, Viterbo, Italy.

Accepted 09 March, 2019

This study was conducted for detection and identification of the causal agent of blossom blight of apple, which results in blast of whole tree. During spring 2011, a newly occurring disease was observed in 4 to 5 years-old *Malus domestica* (cv. Mutsu) trees in Northern area of Iran. A bacterial population was repeatedly isolated from the infected plants. Koch's postulate (pathogenicity test) was fulfilled on potted plants under controlled environmental conditions (greenhouse). Based on morphological, physiological, biochemical and pathological tests, the causal agent was identified as *Pseudomonas viridiflava.* Polymerase chain reaction (PCR) identification of the bacterial isolates was done based on newly designed consensus primer pair (PsV-F and PsV-R). The consensus primers were achieved by alignment of *P. viridiflava* 16S rRNA gene sequences available in nucleic acid data bank, National Centre for Biotechnology Information (NCBI) database. This primer set was successful for detection of *P. viridiflava* strains. Deoxyribonucleic acid (DNA) fragments amplified by this primer set gave a specific amplification band of ~180 bp. Sequencing was done directly (PCR products). Single bands of two isolates were extracted and sequenced for molecular characterization and compared with sequences available in Gene Bank (NCBI), using BLAST search tool. The results showed complete identity of isolates with those of the *P. viridiflava* strains in the databases. This to our knowledge is the first report of the occurrence of *P. viridiflava* on apple.

Key words: Blossom blight, apple, *Pseudomonas viridiflava*, specific primer.

INTRODUCTION

Pseudomonas viridiflava has not been isolated from apple yet, but *Pseudomonas syringae* pathovars have been reported as causal agent of important disease of apple trees. *P. syringae* pv. *papulans* (PSP) was reported as the causal agent of the apple blister canker, it was first described in the Missouri state of USA by Rose (1916). During the late 1970's, Dhanvantari (1977), and Burr and Hurwitz (1979) described heavy damages in orchards as the most important disease of apple (cv. Mutsu) due to PSP in Canada (Ontario) and USA (New York and Michigan), respectively. Recently, blister spot disease was reported in Germany (Moltmann, 2000) and France (Kerkoud et al., 2000). In South Africa another pathovar *Pseudomonas syringae* pv. *syringae* van Hall has been reported to cause bacterial blister bark of apple and blossom blast of apple (Mansvelt and Hattingh, 1987).

In Iran, a new disease on apple trees was observed during spring 2011. Symptoms were not completely

Corresponding author. E-mail: Mahsa_a5@yahoo.com.

similar to those described in previous studies. This disease was detected in 6 to 7 years-old *Malus domestica* trees in Northern area of Iran (Golestan province). First symptoms were observed in spring as blossom blight; flowers shown necrosis of anthers and filaments and dark brown color of sepals. Brown spots were then spread to whole buds and blossom (Figure 1). In summer, the trees showed some advanced symptoms including large brown scorch on margin of leaves; heavily shoot dieback that led to decline of whole tree (Figures 2 and 3). The objective of this study was to identify and characterize the causal agent of the disease occurred on Malus plant trees in north provinces of Iran, using novel molecular techniques including specific primer that was designed for the first time for the detection of *P. viridiflava*.

MATERIALS AND METHODS

Morphological and biochemical tests

Affected plant parts were collected and stored in plastic bags at 6°C until isolation procedure was performed. Samples showing various stages of disease development were mostly collected from infected trees. In sterile condition in lab, they were washed using sterile distilled water at least twice. Small pieces taken from the margin of brown spots were triturated and suspended in tubes containing 5 ml sterile distilled water. One loop full of the each suspension was streaked onto Nutrient Agar (NA) plates. The plates were then incubated for 48 h at 28°C. Suspected single colonies were subcultured and checked for purity, then cultured on King's medium B (KMB) to verify eventual capacity to produce fluorescent pigments (King et al., 1954). LOPAT determinative tests were applied to screen all fluorescent Pseudomonads (Lelliott et al., 1966). Ice nucleation activity (INA) was also investigated using a test-tube technique at -5°C (Young et al., 1987). Five *P. viridiflava* strains representative strains were further characterized using differential diagnostic tests (Schaad et al., 2001).

For further characterization, the following additional tests were performed; methods had been previously described by Schaad et al. (2001). Each test was repeated at least twice. Gram test in 3% KOH (Suslow and McCain, 1981), urease, gelatin liquifaction, litmus milk, and salt tolerance (5 and 7%) and gas formation from glucose. In addition, tests for arginine dehydrolase, hydrogen sulfide production from peptone, reduce substance from sucrose, tyrosinase, casein hydrolase, nitrate reduction, indole production, 2-ketogluconate oxidation, lecithinase, MR-VP tests, starch hydrolysis, phenylalanine deaminase, aesculin and tween80 hydrolysis. Carbohydrate utilization tests using Ayers basal medium were also performed and the results were recorded daily up to 28 days (Hildebrand et al., 1988).

*Corresponding author. E-mail: Mahsa_a5@yahoo.com. Tel: +98 911 3712794. Fax: +98 0171 4420982.

Abbreviations: PCR, Polymerase chain reaction; **NCBI,** national centre for biotechnology information; **KMB,** King's medium B; **INA,** ice nucleation activity; **OD,** optical density; **CFU,** colony forming units; **DNA,** deoxyribonucleic acid; **TBE,** tris-borate buffer; **HR,** hypersensitive reaction; **PSP,** *Pseudomonassyringae* pv. *papulans*.

Pathogenicity test

Bacterial inocula of five representative isolates consisted of a suspension made from a 24 h-old colony grown on King's B medium and adjusted to 1 x 10^7 colony forming unit (cfu)/ml. The bacterial suspensions were inoculated to leaves of *M. domestica* (cv. Mutsu) by using insulin syringe. Each injection was made in triplicate. Plants were maintained in pots under greenhouse conditions during pathogenicity test. Control plants were injected with sterile distilled water and handled similarly. Symptoms were daily evaluated after 48 to 72 h up to 30 days.

Genomic deoxyribonucleic acid (DNA) preparation

Representative *P. viridiflava* strains were grown at 26 to 28°C on KMB for 24 h. Cells were washed with sterile distilled water and suspension prepared. Optical density (OD) of 1000 µL of bacterial suspension was adjusted to 0.1 at 600 nm using spectrophotometer to reach a final concentration of bacterial cells approximately 10^8 colony forming units (CFU)/ml. In the next step, 100 µL KOH was added to suspensions and the mixtures and were boiled for 1 min at 100°C. At the end, suspension was centrifuged for 5 min at 1000 rpm. Supernatant of centrifuged suspension was removed as DNA of bacteria and stored at -20°C for utilization (Arabi et al., 2006).

Polymerase chain reaction (PCR) procedure for deoxyribonucleic acid (DNA) target amplification with specific primer

Primers were selected from conserved regions of 16SrRNA regions. Primers PsV-F (5_GTAGGTGGTTTGTTAAGTTGAA_3) and PsV-R (5_ACCTCAGTGTCAGTATGAGC_3) are located in 16SrRNA region (Table 1). Oligonucleotides were synthesized by local company (CinnaGen Co, Iran). PCR reactions were carried out in a 20 µl reaction volume Master kit (Bioneer, Korea). A volume of 1.5 µl of Total genomic bacterial DNA was added to 20 µl of PCR PreMix, consisting of 10 mM Tris-HCl (pH 9); 30 mM KCl; 30 mM MgCl2; dATP, dCTP, dGTP and dTTP (0.25 mM each); and 1 units of *Taq* DNA polymerase) and 10 pmol of each primer in 16.5 ml sterile distilled water at 25°C.

PCR amplification was performed in a CG1-96 thermocycler (Corbet Reaserch, Australia) as follows: an initial denaturation step at 95°C for 4 min and then 35 cycles of 95°C for 1 min, 52°C for 1 min, 72°C for 1.30 min; and a final extension at 72°C for 10 min. Amplified DNA fragments were examined by horizontal electrophoresis in 1.5% agarose gel (Bioneer, Korea) in tris-borate buffer (TBE) buffer (Maniatis et al.,1982; Schaad et al., 1995) with 10 µl aliquots of PCR products (Sambrook and Russell, 2001). PCR products were stained with ethidium bromide (EtBr 1. 25 mg/L), visualized and photographed under UV light (312 nm). 1 kb DNA ladder (Bioneer, Korea) was run alongside DNA samples on the electrophoresis gel. All amplifications procedures were performed at least twice in separate assays to ensure the reliability of the patterns.

Deoxyribonucleic acid (DNA) sequencing

The isolates were tested with using our own designed specific primer of *P. viridiflava* (Mahsa and Asadollah, unpublished), were identified by sequencing 184 bp of their 16S rRNA region (Moore et al., 1996) and BlastN (Altschul et al., 1990) for the most similar sequences in GenBank. DNA fragments obtained from the agarose gel after PCR and purified by Ultra-Clean purification kit of CinnaClon as described by the manufacturer (CinnaClon, Iran).

Figure 1. Bacterial blossom blight caused by *P. viridiflava* on *M. domestica*; early disease symptoms including brown spots and necrosis of blossom.

Figure 2. Brown scorches of apple leaves caused by *P. viridiflava.*

Figure 3. Dieback of infected trees caused by *P. viridiflava* that led to the whole tree decline in summer.

Table 1. Accession numbers in Genebank used for nucleotide alignment.

Accession	author	Host	Reference
EU834638	Weinert, N.	Rhizosphere of potato	Plant Soil 326 (1-2), 437-452 (2010)
EU834454.1	Weinert, N.	potato	Plant Soil 326 (1-2), 437-452 (2010)
DQ294984.1	Weinert, N.	potato	(15-NOV-2005) Plant Pathology Research Unit, INRA, BP 94. Allee des Chenes, Montfavet 84140, France
EU434634.1	Long, H.H.	Solanum nigrum	Molecular Ecology, Max-Planck-Institute for Chemical Ecology, Hans-Knoell-Strasse 8, Jena 07745, Germany
GQ398130.1	Martin-Sanz, A.	Pisum sativum	Molecular Biology, Universidad de Leon, Campus de Vegazana, Leon 24071, Spain
GQ398129.1	Martin-Sanz, A.	Pisum sativum	Molecular Biology, Universidad de Leon, Campus de Vegazana, Leon 24071, Spain
GQ398128.1	Martin-Sanz, A.	Pisum sativum	Molecular Biology, Universidad de Leon, Campus de Vegazana, Leon 24071, Spain
HM190224.1	Myung, I.-S.	Rape	Agricultural Microbiology, National Academy of Agricultural Science, 245 Seodun, Kwonsun, Suwon 441-707, Korea
|HM190229.1	Myung, I.-S.	Rape	Agricultural Microbiology, National Academy of Agricultural Science, 245 Seodun, Kwonsun, Suwon 441-707, Korea
HM190223.1	Myung, I.-S.	Rape	Agricultural Microbiology, National Academy of Agricultural Science, 245 Seodun, Kwonsun, Suwon 441-707, Korea
HM190222.1	Myung, I.-S.	Rape	Agricultural Microbiology, National Academy of Agricultural Science, 245 Seodun, Kwonsun, Suwon 441-707, Korea
HM190221.1|	Myung, I.-S.	Rape	Agricultural Microbiology, National Academy of Agricultural Science, 245 Seodun, Kwonsun, Suwon 441-707, Korea
HM190220.1	Myung, I.-S.	Rape	Agricultural Microbiology, National Academy of Agricultural Science, 245 Seodun, Kwonsun, Suwon 441-707, Korea
HM190219.1	Myung, I.-S.	Rape	Agricultural Microbiology, National Academy of Agricultural Science, 245 Seodun, Kwonsun, Suwon 441-707, Korea
HM190218.1	Myung, I.-S.	Rape	Agricultural Microbiology, National Academy of Agricultural Science, 245 Seodun, Kwonsun, Suwon 441-707, Korea
EU863636.1	Warmink, J.A.	mycosphere soil	Microbial Ecology, University of Groningen, Kerklaan 30, Haren, Groningen 9750RA, Netherlands
EU045448.1	Andriopoulos, C.F.	Not mentioned	Department of Botany, Faculty of Biology, National and Kapodistrian University of Athens, Athens 15781, Greece
EU045447.1	Andriopoulos, C.F.	Not mentioned	Department of Botany, Faculty of Biology, National and Kapodistrian University of Athens, Athens 15781, Greece
DQ512797.1	Amato, P.	tropospheric cloud water	UMR 6504 CNRS-Universite Blaise Pascal, Laboratoire de Synthese et Etudes de Systemes a Interet Biologique, 24, Avenue des Landais, Aubiere 63177, France

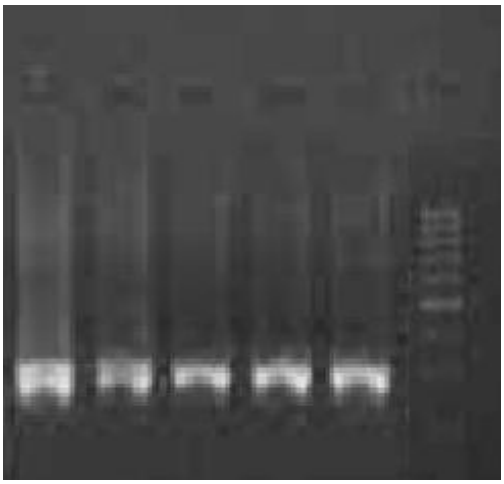

Figure 4. Electrophoretic analysis of PCR-amplified 16S rRNA gene of *P. viridifalvae* strains using the designed specific primer pair.

RESULTS

Morphological and biochemical tests

Bacterial colonies which were consistently isolated from infected samples on NA medium for 48 h were 2 mm in diameter, white, convex and with smooth to undulate margins, fluorescent on King s medium B. All five *P. viridiflava* strains caused hypersensitive reaction (HR) on tobacco leaves (cv, Virginia Bright). They were Gram-negative and catalase positive. According to the LOPAT tests, the isolates were negative for levan production, oxidase test and arginine dihydrolase. Potato soft rot was positive for all strains; they also showed INA at -5°C. All strains were catalase positive and capable of hydrolyzing gelatin, aesculin, tween80 and casein. None of the strains were able to produce indole, reduce nitrate and oxidize 2-keto-gluconate. Strains were able to produce urease. Litmus milk test became alkaline. All strains showed ability to utilize arginine, ascorbate, L-asparagine, biotin, butyrate, citrate, D (-) fructose, fumarate, D (+) galactose, galacturonate, gluconate, D-glucose, Lglutamic acid, glycerol, histidine, lactate, leucine, malonate, D-mannitol, D-mannose, D (+) Melezitose, Meso-erythritol, Myoinositol, D-raffinose, D-bitol but they did not use sucrose as a sole carbon sources. Based on the aforementioned standard biochemical, physiological and pathological tests (Schaad et al., 2001), representative isolates of the causal agent of blossom blight of apple in Iran was identified as *P. viridiflava* on the determinative schemes proposed by Schaad (2001).

Pathogenicity tests

Upon artificial inoculations, typical symptoms of the disease were induced in apple seedlings. The symptoms induced by the strain were similar to those observed in natural infections. Water soaked spots appeared 5 to 6 days after inoculation. The necrotic lesions were developed at the inoculation sites and expanded to 4 cm in a week. The centre of the lesions later became dry and tan and led to hollow spots. In some cases, the lesions coalesced and leaves appeared blighted. Similar symptoms of the disease were observed 2 weeks after inoculation. This was followed by re-isolation and identification of the bacterium from the inoculated plants. No other than inoculation-sites damaged, were evident on control plants. Re-isolations made from the artificially infected plants yielded pure cultures identical to those inoculated. Bacterial identification as of *P. viridiflava* was confirmed by LOPAT tests. All strains of *P. viridiflava* isolated from the apple host resulted pathogenic.

Molecular tests

Validation by polymerase chain reaction (PCR) and sequencing

Molecular analysis of the strains showed that designed specific primer set amplify a single band, in comparison with ladder this fragment was ~180 bp size (Figure 4). The DNA fragment obtained by performing PCR tests on strains PVap3 and PVap4 of *P. viridiflava* which were isolated from the agarose gel and purified using a silica-based procedure followed by Ultra-Clean DNA purification and sequenced by CinnaClon (CinnaClon, Iran)

PCR products were sequenced and compared with the corresponding sequences available at NCBI by BLAST search software (Altschul et al., 1990). The sequences of both strains PVap3 and PVap4 were identified as *P. viridiflava* with 97% homology with the analogous sequences of *P. viridiflava* available in the database. As mentioned earlier, this to our knowledge is the first report of the occurrence of *P. viridiflava* on apple in Iran as in the world.

DISCUSSION

Overall results of this study suggest that the bacterium consistently isolated from diseased flower of apple trees is *P. viridiflava*. The bacterium which usually identified as an opportunistic and epiphytic pathogen (Billing, 1970; Burkholder, 1930; Wilkie et al., 1973, Balestra and Varvaro, 1997) on a wide variety of plants (Bradbury, 1986), but this species has not been reported on apple yet. In previous studies in Germany, North America and Italy apple blister damage has been reported by *P. syringae* pv. *syringae* mainly on apple cv. Mutsu (Kerkoud et al., 2000). However, other cultivars, such as

fuji, golden delicious, and gala have also been reported to be susceptible when young leaves or fruit is inoculated with *P. syringae* pv. *papulans* (Kerkoud et al., 2000)

The outbreak of this disease has been reported to be associated with favorable environmental conditions that promote invasion by the pathogen. Since rainy, cool and humid conditions occurred during spring, it was concluded that it might be related to the occurrence of disease. Wet prolonged conditions, combined with the absence of copper compound applications created ideal conditions for disease outbreak.

In addition to the above issues, a reliable and sensitive PCR assay was developed in this study for the detection of *P. viridiflava* on diseased flower and leaves; it is a potentially useful tool for epidemiological studies on blossom blast of apple. Parts of 16S rRNA genes displayed a nucleotide sequence diversity similar to that observed in genes encoding housekeeping functions, such as *gyrB* and *rpoD* (25), suggesting that this part of gene is a good candidate for analysis of the evolutionary course of *P. viridiflava*. Developing these two primer sets, it was possible to identify *P. viridiflava.*

Due to wide distribution and cultivation of apple in other regions of Iran, the epidemiology of this disease should be studied for improving the control strategies to prevent any further spread of the disease. It is also important to determine the main sources of infection. Adequate cultural practices and the application of preventive copper based compounds have so far been highly effective in reducing disease damages. An epidemiological study of blossom blight on different *M. domestica* cultivars in Iran and of new strategies to control *P. viridiflava* has been initiated. In general, the results of this study may have practical application in the etiology, epidemiology and management of *P. viridiflava* which seems to be a very important and destructive pathogenic bacterium on apple trees in most apple growing regions of the world including Iran.

ACKNOWLEDGEMENTS

This research project was based on PhD assertion in Science and Research Branch, Islamic Azad University, Tehran. The authors are particularly grateful to Agricultural Science and Natural Resources University of Gorgan for their fundamental technical assistance.

REFERENCES

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990). Basic local alignment search tool. J. Mol. Biol., 15(3):403–410.

Arabi F, Nikravesh Z, Babaeezad V, Rezaeean V, Rahimian H (2006).Occurrence of bacterial leaf spot of garden beet caused by *Pseudomonas syringae* pv. *aptata* in Iran. Iran J. Plant Pathol., 42: 655-671.

Balestra GM, Varvaro L (1997). Epiphytic survival and control of *Pseudomonas viridiflav*a on *Actinidia deliciosa*. Acta Horticulturaen 444(2): 745-749.

Billing E (1970) *Pseudomonas viridiflava* (Burkholder, 1930; Clara, 1934). J. Appl. Bacteriol., 33: 492–500.

Bradbury JF (1986). Guide to Plant Pathogenic Bacteria. Farnham House, Slough, UK, Mycological Institute, CAB International, pp 332.

Burkholder WH (1930). The bacterial disease of the bean. A comparative study. Cornell University Agric. Exp. Stat. Mem., 127: 1–88.

Burr TJ, Hurwitz B (1979). The etiology of blister spot of 'Mutsu' apple in New York State. Plant Dis. Rep., 63: 157–160.

Dhanvantari BN (1977). A taxonomic study of *Pseudomonas papulans* Rose. NZ. J. Agric. Res., 20: 557–561.

Hildebrand DC, Schroth MN, Sands DC (1988). *Pseudomonas* In: Schaad ND (ed) Laboratory Guide for Identification of Plant Pathogenic Bacteria. The American Phytopathological Society. St. Paul. Minnesota USA, pp 60–80.

Kerkoud M, Manceau C, Gardan L, Samson R, Paulin JP (2000). Epiphytic occurrence of *Pseudomonas syringae* pv. *Papulans* (Rose) in France, where blister spot has never been seen. Eur. J. Plant Pathol., 106:481-485.

King EO, Ward MK, Raney DE (1954). Media for the demonstration of pyocyanin and fluorescein, J. Lab. Clin. Med., 44: 301.

Lelliott RA, Billing E, Hayward AC (1966). A determinative scheme for the fluorescent plant pathogenic Pseudomonads. J. Appl. Bacteriol., 29: 470–489.

Maniatis T, Fritsch EF, Sambrook J (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory. NY., pp. 545

Mansvelt EL, Hattingh MJ (1987). *Pseudomonas syringae* pv. *syringae* associated with apple and pear buds in south Africa. Plant Dis., 71: 789-792.

Moltmann E (2000). Die blasenfleckenkrankheit an 'Delbarestivale'-verursacht durch *Pseudomonas syringae* pv. *papulans.* Obstbau., 8:457- 458.

Moore ERB, Mau M, Arnscheidt A, Böttger EC, Hutson RA, Collins MD, Van De Peer Y, De Wachter R, Timmis KN (1996). The determination and comparison of the 16S rRNA gene sequences of species of the genus *Pseudomonas* (*sensu stricto*) and estimation of the natural intrageneric relationships. Syst. Appl. Microbiol., 19:478–492.

Rose DJ (1916). Blister spot of apples. Phytopathology, 6: 110.

Sambrook J, Russell DW (2001). 'Molecular cloning. A laboratory manual', 3rd. ed. Cold Spring Harbor, NY.Laboratory Press, pp. 235.

Schaad NW, Jones JB, Chum W (2001). Laboratory Guide for Identification of Plant Pathogenic Bacteria. American Phytopathological Society Press, St. Paul, MN., pp.373.

Schaad NW, Cheong, SS, Tamaki E, Hatziloukas E, Panopoulos NJ (1995). A combined biological and enzymatic amplification (BIOPCR) technique to detect *Pseudomonas syringae* pv. *phaseolicol*a in bean seed extracts. Phytopathology, 85:243-248.

Suslow TV, McCain AH (1981). Greasy canker of poinsettia caused by *Pseudomonas viridiflava*. Plant Dis., 65: 513–514.

Wilkie JP, Dye DW, Watson DRM (1973). Further hosts of *Pseudomonas viridiflava*. New Zealand J. Agric. Res., 16: 315–322.

Young JM, Dye DW, Bradbury JF, Panagopoulos CG, Robbs CF (1987). A proposed nomenclature and classification for plant pathogenic bacteria. New. J. Agric. Res., 21: 153–77.