

Detection and Identification of some *Pseudomonas* Species causing Soft Rot using *TUF* Gene

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Abstract

Introduction: Soft rot caused by pectolytic bacteria makes annual production and storage losses throughout the world. This study aimed to detect and identify pathogenic *Pseudomonas* causing soft rot in some of the vegetables and ornamentals.

Materials and methods: During the growing seasons of 2015–17, plant samples of eggplant, maize, radiator plant, sweet pepper and tomato with water soak and soft rot symptoms were collected. Biochemical and morphological features were characterized according to the standard bacteriological criteria. The *tuf* encoding gene from these representatives was amplified using Bac-tuf-F and Bac-tuf-R primers, subjected to sequencing and aligned in the NCBI.

Results: Total of 120 isolates were recovered from the samples. Based on synthesizing pectic enzymes, five putative *Pseudomonas* strains were selected. Based on the DNA sequence-based phylogeny, in combination with biochemical and morphological characteristics, these soft rot *Pseudomonas* were identified as *Pseudomonas aeruginosa*, *P. entomophila*, *P. mosselii* and *P. putida*. Koch's postulates were verified by re-isolating the strains from inoculated plant segments.

Discussion and conclusion: To the best of our knowledge, this is the first evidence of the pathogenicity of *P. aeruginosa* on tomato, *P. entomophila* on sweet pepper and *P. putida* on eggplant. Furthermore, this study firstly reports the association of *P. aeruginosa* and *P. mosselii* with soft rot in maize and radiator plant, respectively.

Key words: Eggplant, Maize, Radiator Plant, Sweet Pepper, Tomato

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Introduction

Vegetables are considered as increasingly important crop of Khuzestan province in the southwest of Iran. There is growing concern that soft rot disease annually reduces up to 20% of vegetable yields in this province (unpublished data). Studies over the past two decades have provided important information on soft rot disease due to their importance of causing severe economic loss in crop yield and quality throughout the world (1-3). Bacterial soft rot causal agents are one or a combination of soft rotting pectolytic bacteria in tropical and subtropical climates. These bacterial pathogens belongs to several genera in different families including *Pectobacterium*, *Dickeya*, *Pseudomonas*, *Xanthomonas* and *Bacillus* (4-8). Consequently, *Pectobacterium* and *Dickeya* belonging to the family *Enterobacteriaceae*, cause soft rot disease in most crops as the most common plant cell wall-degrading enzymes-producing pathogens (6-8). Previous studies have reported some species of other bacterial genera as the causal agent of soft rot in fleshy fruits, vegetables, and ornamentals throughout the world (5). In the same way, *Pseudomonas*, belongs to the family *Pseudomonadaceae* of the order *Pseudomonadales* known as soft rot causal agent in plants (9), i.e. *P. cichorii*, *P. marginalis*, and *P. viridiflava* (5, 10, 11, 12, 13). This genus is a Gram-negative and aerobic bacterium, saprobes that can commonly be found in soil, marshes and living or dead tissues of plants and animals (14, 15); and some of them also act as important pathogens (9, 16, 17, 18). Previous studies have based their criteria for selecting *Enterobacteriaceae* as the common cause of soft rot. However, far too

little attention has been paid to *Pseudomonas* as the causal agent of soft rot disease; therefore, this survey aims to identify and characterize pathogenic *Pseudomonas* causing soft rot in some of the vegetables and ornamentals on the basis of morphological, biochemical, pathogenicity and phylogeny features.

Material and Methods

Collection of Specimens: During the growing seasons of 2015–17, the samples were collected from Khuzestan province (the townships of Ahvaz, Dezful, Gotvand, Izeh, Shoosh, and Shooshtar) in the southwest of Iran. The stems, fruits and leaves of plants with typical soft rot were sampled throughout the growing seasons from both fields and agricultural products market. Total of 25 symptomatic plants were collected including the species of eggplant (*S. melongena*, Solanaceae), maize (*Zea mays*, Poaceae), radiator plant (*Peperomia magnoliifolia*, Piperaceae), sweet pepper (*Capsicum annuum*, Solanaceae), and tomato (*Solanum lycopersicum*, Solanaceae). The diseased organs were stored at 4°C and processed within 3 days of collection.

Isolation and Purification: Infected organs was washed under running tap water and dried. The small pieces from the margin of infected tissue were surface-sterilized by dipping them in 2–5% sodium hypochlorite (30 sec), and followed by two to three rinses in sterile distilled water. These sections were placed in petri dishes containing 5 mL of sterilized peptone water and macerated for 10 min. A loopful of the resulting suspension was streaked onto nutrient agar (NA) media as described by Schaad *et al* (19). From each medium, one colony per colony type was isolated and

purified on nutrient agar. Pure cultures of the resulting bacterial isolates were obtained by colony sub-culturing. The single discrete colonies were transferred to fresh NA plates as purified culture for short-term storage and 15% glycerol at -80°C for long-term conservation. The isolates were deposited in the Collection of Bacterial Cultures, Department of Plant Protection, Shahid Chamran University of Ahvaz, Iran.

Physiological and Biochemical Tests:

Purified colonies of each strain were subjected to physiological and biochemical tests which are routinely used to differentiate *Pseudomonas* species. The tests were performed according to protocols established by Lelliott and Stead (20), Suslow *et al* (21) and Schaad *et al* (19). The tests were as follow: Gram reaction, KOH test, fluorescence on King's B, colony shape, anaerobic growth (O/F), salt tolerance with growth in 5-7% NaCl, growth at 37°C , oxidase and catalase activity, production of yellow pigment on YDC, levan production from sucrose, hydrolysis of gelatin, starch, aesculin and Tween 80, citrate utilization and production of acid from D-glucose, D-maltose, D-mannose, D-sucrose and Dextrose.

Potato Soft Rot Test: Pectolytic activity of the isolates under study on potato was tested according to modified method described by Lelliott and Stead (20). The healthy potato tubers were dipped in 70% Ethanol (15 sec), flamed and cut into 1-cm-thick slices under sterile condition. A 1-mm-deep and 5-mm-diameter hole was made in the center of each slice placed on sterile filter paper into petri dishes using a glass rod. A small quantity of inoculum taken from 48-hours cultures was spread into holes. Free culture media was used for the control tubers. The

tubers were then incubated at 28°C and 95 % relative humidity for 72 h before the diameter of the rotting tissue in each tuber was measured. The rot symptom extending through most of the diameter of the potato slice was considered as a positive reaction, while slight rot at the point of inoculation was considered as a negative reaction. As a control, pipette tips inserted into tubers contained sterile water rather than a bacterial suspension. Potato soft rot tests were conducted twice.

Pathogenicity Test: All petrified isolates were evaluated for pathogenicity on healthy plant organs. Plant segments inoculated in pathogenicity tests included the fruits of eggplant (isolate SCUC-43) sweet pepper (isolate SCUC-Dol), and tomato (isolate SCUC-36), stems of maize (isolate SCUC-44) and leaves of radiator plant (isolate SCUC-45). The segment of each plant with similar diameter and vigor were surface-sterilized by submersion in 2% sodium hypochlorite for 2–6 min, washed two times with sterile distilled water (2 min) and then allowed to air dry. Holes of different diameters (1–3 mm diameter depend on the organ types) were stabbed into the plant segments and inoculated with 300 μl of the bacterial suspension ($\text{OD}_{600}=1.0$, equivalent to 1×10^8 cfu/ml), followed with covering the wounds by removed tissue plugs. As a control solution, sdH_2O was used. Four replicates of each treatment were covered with plastic bags to maintain high humidity for 48 h in growth chamber at 24°C and evaluated for symptoms up to 21 days after inoculation. The plant segments were daily inspected for the appearance of the symptoms of water-soaking, soft rot and maceration. The pathogenicity tests were conducted twice.

DNA Extraction and Gene Amplification:

Genomic DNA was extracted using the modified method of boiling lysis (22). The DNAs were qualified and quantified using spectrophotometer (Eppendorf BioPhotometer plus) and loading on the gel and used at ~500 ng/ μ L for molecular investigations. A product of approximately 960 bp corresponding to the elongation factor *Tu* gene (*tuf*) was amplified using specific primer pair Bac-tuf-F (5'-ACHATCGGYCmCGTTGACCA-3') and Bac-tuf-R (5'-TCDGTDGTRCGGAAGTAGAACTG-3') (23). PCR amplification was performed in a thermal cycler (MJ MiniTM Gradient Thermal Cycler) with 50 μ L total volume containing 2 μ L of each primer (10 μ M), 5 μ L of 10x prime Taq Reaction Buffer (GenBio, South Korea), 2 μ L of dNTP (2.5mM each), 6 μ L of 25mM MgCl₂, 0.6 μ L of Prime Taq DNA Polymerase (5 units/ μ L, GenetBio), 2 μ L of template DNA (~500 ng/ μ L in concentration) and 30.4 μ L sterile purified water (Mili-Q Water). The PCR conditions consisted of an initial melting step of 5 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 90 s at 72 °C, and a final extension step of 10 min at 72 °C.

Sequencing and Phylogenetic Analyses:

The PCR products were run on 1% agarose gels in 1.0 \times Tris-acetic acid-EDTA (TAE) buffer and the target fragments (expected-size bands) were excised and cleaned according to the manufacturer's protocol of GF-1 AmbiClean Kit (Vivantis, Malaysia). The PCR products were sequenced in both directions using the PCR primers in Macrogen Company (Humanizing Genomics, Macrogen, South Korea).

Electropherogrammes were edited where necessary using BioEdit Sequence Alignment Editor v. 7.0.9.0 (24), and consensus sequences were obtained from forward and reverse sequences using DNA Baser Sequence Assembler v4 programs (2013, Heracle BioSoft, www.DnaBaser.com). All generated sequences were deposited in GenBank (Accessions numbers: MF804917-19 and MF566065-66). The consensus sequences were compared to those of reference strains (<https://www.ncbi.nlm.nih.gov>) using the basic local alignment search tool (BLAST) search algorithm. Phylogenetic analyses and construction of phylogenetic tree based on the *tuf* sequences were performed using Maximum Likelihood (ML) algorithm in MEGA version 6 (25). Sequence data belonging to the reference strains which have been fully sequenced (Total-genome-sequenced bacteria) were also included in these analyses. The *Pectobacterium wasabiae* species from family *Enterobacteriaceae* were used as outgroup taxon to root phylogenetic trees. DNA sequences were singularly aligned with ClustalW algorithm using the software MEGA version 6 (25), and manually adjusted for errors. The best-fitting ML nucleotide substitution model was selected according to Bayesian Information Criterion (BIC). The phylogenetic trees were constructed by Subtree-Pruning-Regrafting (SPR) algorithm and following options: gaps (insertion/deletions) were complete deletion, bootstrap (BP) replicates were 1000, initial trees for ML analysis were formed by NJ/BioNJ algorithm and branch swap filter was set very strong.

Result

Isolation and Identification of the Causal Bacteria: Based on colony morphology and color, total of 120 bacteria were isolated from symptomatic plant samples in seven different regions in the southwest of Iran (Fig 1). In total, 11 isolates were identified as *Pseudomonas* spp. Among 11 strains obtained, only five isolates were identified as soft rot *Pseudomonas* with showing the pectolytic activity on potato slices *in vitro* and subjected for further characterization.

DNA sequence analysis, in combination with biochemical and physiological features, identified these strains as *Pseudomonas aeruginosa*, *P. entomophila*, *P. mosselii* and *P. putida*. Two strains of *P. aeruginosa* SCUC-36 and SCUC-44 were obtained from the tomato fruit (*Solanum lycopersicum*) and stem of maize (*Zea mays*) showing typical maceration in Izeh and Gatvand,

respectively. The strain *P. mosselii* SCUC-45 were isolated from the leaf of radiator plant (*Peperomia magnoliifolia*) in Ahvaz, and then subsequently, *P. entomophila* SCUC-Dol from the fruit of sweet pepper in Shooshtar and *P. putida* SCUC-43 from the fruit of eggplant (*Solanum melongena*) in Shoosh.

Physiological and Biochemical Tests: The detailed results of general physiological and biochemical tests mentioned in Table 1. All of the five strains were fluorescent on King's B medium, mucoid colony, negative in Gram reaction and levan production, positive in catalase and citrate utilization. All the strains were not able to grow under 7% NaCl. They utilized D-glucose and maltose and hydrolyzed starch. These strains did not produce yellow pigment on YDC medium.

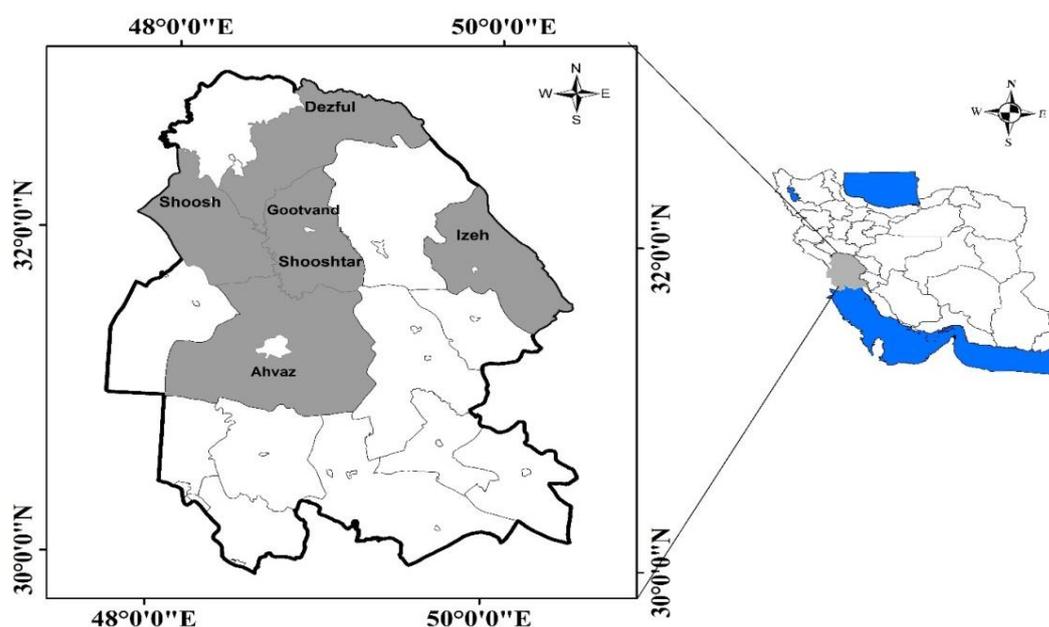


Fig. 1- Map of Iran showing sampling regions

Table 1- Biochemical and physiological characterization of isolates surveyed.

Test	<i>Pseudomonas aeruginosa</i> SCUC-36	<i>P. aeruginosa</i> SCUC-44	<i>P. entomophila</i> SCUC-Dol	<i>P. mosselii</i> SCUC-45	<i>P. putida</i> SCUC-43
Gram reaction	-	-	-	-	-
5% NaCl tolerance	-	-	+	+	+
7% NaCl tolerance	-	-	-	-	-
Catalase	+	+	+	+	+
Mucoid growth	+	+	+	+	+
Yellow pigment on YDC	-	-	-	-	-
Growth at 37°C	+	+	-	+	-
40 C	+	+	-	+	-
Hydrolysis of gelatin	+	+	+	+	-
Hydrolysis of starch	-	-	-	-	-
Hydrolysis of aesculin	+	-	-	-	+
Hydrolysis of Tween 80	+	+	+	-	-
levan production	-	-	-	-	-
Citrate utilization	+	+	+	+	+
Oxidase	+	+	+	+	-
Amylase	-	-	-	-	-
Utilization from Glucose	+	+	+	+	+
Maltose	+	+	+	+	+
Mannose	+	+	+	-	+
Dextrose	-	-	+	-	+

+, representing the positive reaction and -, shows the negative reaction. The experiment was repeated twice for each representative isolate.

DNA and Phylogenetic Analysis: PCR using Bac-tuf-F and Bac-tuf-R primers resulted in the expected size bands of approx. 960 bp from all the strains surveyed (Fig 2). Using a BLASTn search of *tuf* partial regions, the strains of *P. aeruginosa* (SCUC-36 and SCUC-44), *P. mosselii* (SCUC-45), *P. putida* (SCUC-43)

and *P. entomophila* (SCUC-Dol) showed the closest match (99–100% sequence identity) to their reference strains in the NCBI GenBank database, i.e., *P. aeruginosa* Ocean-1155 (CP022526.1), *P. mosselii* SJ10 (CP009365.1), *P. putida* KT244 (AE015451.2) and *P. entomophila* L48 (CT573326.1).

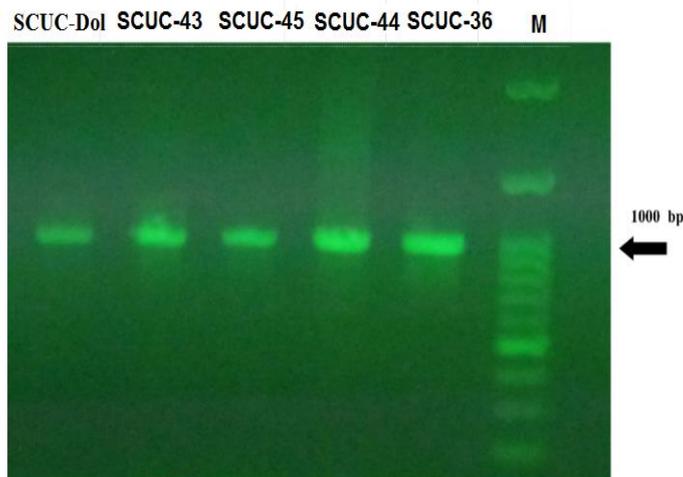


Fig. 2- Agarose gel electrophoresis of PCR-products of representative strains with Bac-tuf-F and Bac-tuf-R primers. M: 1500 bp ladder.

Fifty two taxa, including very close relative species of bacteria under survey, were included in phylogenetic analysis (Fig. 3). The sequence alignment of the *tuf* generated a total of 755 characters including gaps after alignments, 448 (59%) of which were constant, 66 (8.7%) were parsimony uninformative and 240 (32%) were parsimony informative. The Tamura-Nei model of evolution, including estimation of invariable sites and assuming a discrete gamma distribution with five rate categories (TN93+G+I) were used for the phylogenetic analysis. Based on phylogenetic tree, two strains of the species *P. aeruginosa* SCUC-36 and SCUC-44 were clustered to their reference strains (including strains of NHmuc, S86968 and

W36662) and generated supportive monophyletic clade with strong Bootstrap 100% (Fig 3). *P. putida* SCUC-43 were associated to their reference strains (including strains of W619, GB-1, H8234 and F1) and generated supported clade with strong Bootstrap 99%, (Fig 3). Furthermore, *P. entomophila* SCUC-Dol was grouped to its reference *P. entomophila* strain L48 and formed a weakly supported clade (BS 82%). The phylogram constructed based on *tuf* region could not provide sufficient resolution to distinguish the strains of *P. mosselii* from other species, and clustered them with *P. putida* group, i.e. *P. alkylphenolia*, *P. entomophila*, *P. monteilii*, *P. parafulva*, *P. plecoglossicida* and *P. putida*.

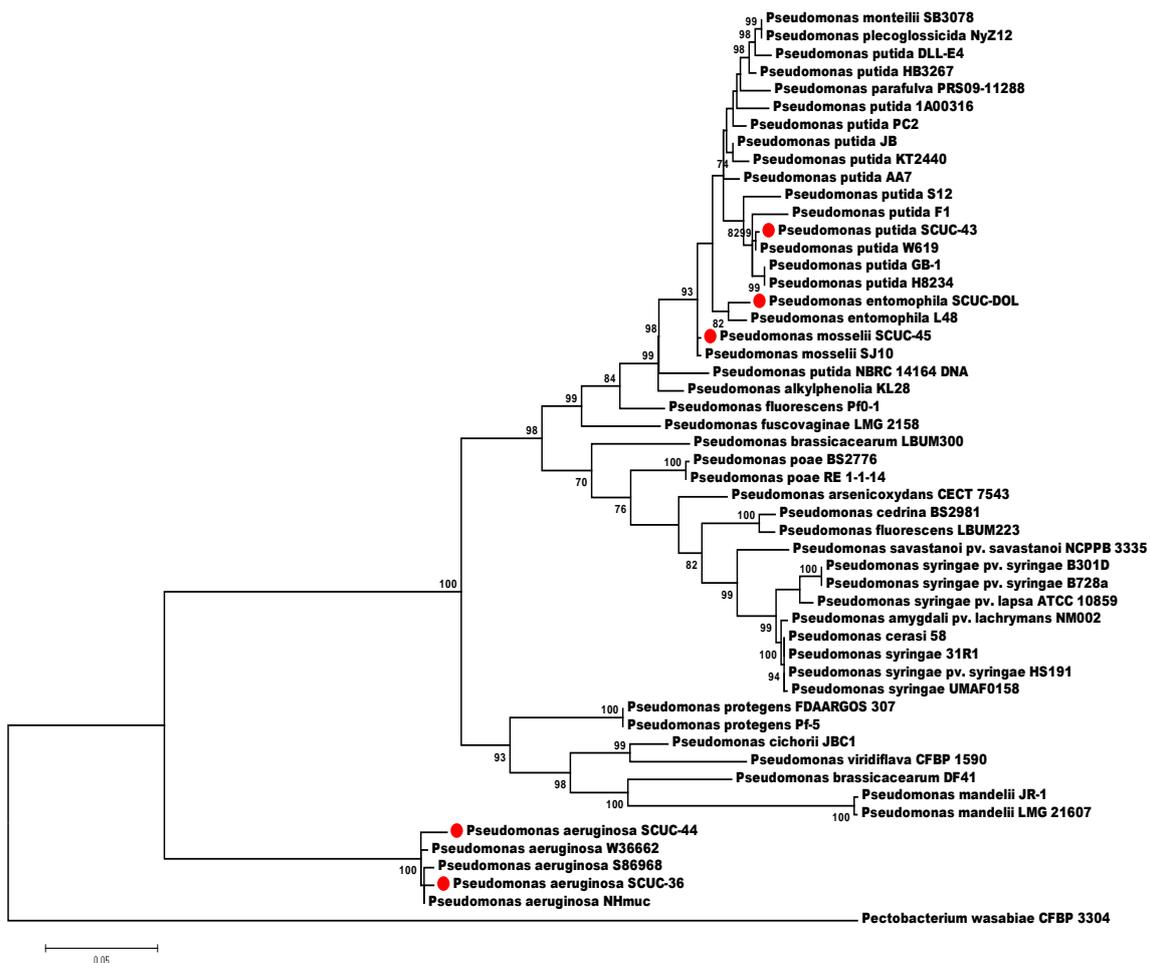


Fig. 3- *tuf* gene tree

The *tuf* gene tree was constructed from a maximum likelihood analysis based on *tuf* sequences of 52 bacterial taxa, including our isolates and reference strains representing very close relative species. Bootstrap values greater than 50% (expressed as percentages of 1000 replications) are shown at the nodes. The tree was rooted with *Pectobacterium wasabiae* CFBP 3304. Scale bar indicates the number of substitutions per site.

Pathogenicity Tests: The ability of the strains under study, which were positive in potato soft rot tests, to cause soft rot was investigated on the same plant organs that were isolated from them (Fig. 4). Based on significant differences of rotten tissues between the inoculated and control

samples, only three out of the strains developed different degrees of soft rot in plant segments tested, i.e. *P. aeruginosa* SCUC-36 on the fruit of tomato, *P. entomophila* SCUC-Dol on the fruit of sweet pepper and *P. putida* SCUC-43 on the fruit of eggplant. The disease progress was commonly similar for all the inoculated samples and the symptoms started on plant segments as water-soaked spots (Fig. 4). After 3-5 days of incubation, these spots extended externally and internally and became softens and watery. These soft-rotting strains were recovered from the diseased segments, and re-identified as the same species. No soft-rotting bacterium was re-isolated from the controls.

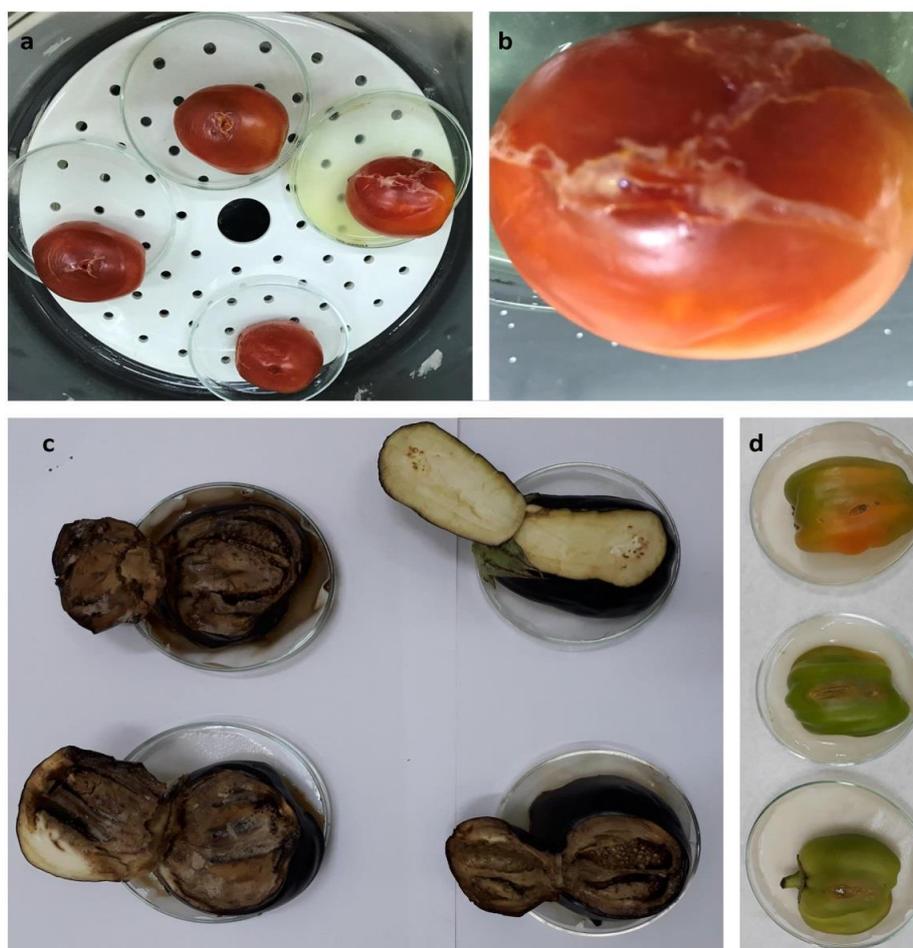


Fig. 4- Pathogenicity tests. a-b. Soft rot symptoms of tomato three days after inoculation of *Pseudomonas aeruginosa* SCUC-36, c. Fruits of sweet pepper which artificially infected by *P. entomophila* SCUC-Dol (four days after inoculation), D. Soft rot symptoms of eggplant three days after inoculation of *P. putida* SCUC-43.

Discussion and Conclusion

This study set out with the aim of identifying *Pseudomonas* species causing soft rot in some agricultural and ornamental products. In reviewing the literature, the identification of strains at the species level based on biochemical and phenotypic characteristics are not always practical and proper and in some cases may be resulted to misleading (26). Thus, these conventional methods which are based on shape, color, size, spore production, growth conditions, staining properties, motility, tolerance to salts, fluorescence, host-range, pathogenicity, utilization of carbon sources and other biochemical tests cannot provide sufficient resolution to distinguish many bacterial species (12, 13, 26). Therefore, accurate identification of the strains under study was supported by DNA analysis of *tuf* gene. Until now, some house-keeping genes, which vary slowly during evolution, have been used individually or in combination for phylogenetic and diagnosis studies, i.e. *16S rRNA*, *gyrB*, *rpoA*, *rpoB*, *rpoC*, *rpoD*, *tuf* and *GroEL* (<https://www.ncbi.nlm.nih.gov>). In this study, the *tuf* gene, encoding elongation factor Tu, were amplified and used for phylogenesis among *Pseudomonas* species. It is preferred to use *tuf* gene for diagnostic purposes because of ubiquitous distributions and the highly evolutionarily conserved part (27). The findings of the current study are consistent with those of Li et. al (28) who found that *tuf* gene provides a better separation of species than the *16S rRNA* gene for accurate identification of strains.

In pathogenicity tests, some strains of the genus *Pseudomonas* formed the water-soaked and soft rot areas on the inoculated segments of plant host, i.e. *P. aeruginosa*

(SCUC-36) *P. entomophila* (SCUC-Dol) and *P. putida* (SCUC-43). *P. aeruginosa*, the type species of the genus *Pseudomonas*, has been known to be in close association with animals and plants, where it commonly acts as opportunistic pathogen (29). This species was reported to cause a variety of plant diseases including root rot of ginseng (30), fruit rot of tinda (31), internal brown rot and soft rot of onion bulbs (32, 33), leaf disease on *Arabidopsis*, lettuce and tobacco (34-36), root disease of *Arabidopsis* and sweet basil and collar rot of calla lily plant (37). To now, the validly described species of the genus *Pseudomonas* which are commonly known as soft rot pathogen include *P. cichorii*, *P. marginalis* and *P. viridiflava* (38, 39). Pectinolytic species of *Pseudomonas* are capable of producing a wide variety of pectolytic enzymes degrading the pectic components of plant cell walls (39-42). The species *P. marginalis* was known, which cause the maceration and soft rot on broccoli (43), cauliflower (44, 45), carrot (46), faba bean (47), onion (48-50), potato (51), tomato (52) and *Zantedeschia* spp. (53). Bacterium *P. viridiflava* was reported as one of the pathogens causing soft rot on carrot (46, 54), potato (55) and tomato (56). Pectinolytic species *P. cichorii* was also known as one of the causal agent of soft rot in butter-head lettuce (57) and lettuce (58).

The strain *P. mosselii* SCUC-45 capable of synthesizing pectic enzymes were not able to soft-rot the leaf of radiator plant in pathogenicity tests. *P. mosselii* is a validly described species of the *P. putida* group (59), which has been known as a potential emerging pathogen in human (60) and plant growth promoting bacterium (61), no data on its infectiveness or pathogenicity in plant were available.

To the best of our knowledge, this is the first report of pathogenicity of *P. aeruginosa* on tomato, *P. entomophila* on sweet pepper and *P. putida* on eggplant. Furthermore, this study firstly reports the association of *P. aeruginosa* and *P. mosselii* with soft rot in maize and radiator plant, respectively.

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