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RESEARCH ARTICLE

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Virulence and indole-3-acetic acid (IAA) biosynthesis ability of Turkish *Pseudomonas savastanoi* pv. *savastanoi* isolates and susceptibility of some native olive genotypes

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Abstract

Aim of study: To evaluate the virulence and indole-3-acetic acid (IAA) biosynthesis ability of several Turkish *P. savastanoi* pv. *savastanoi* isolates and the susceptibility of some native genotypes to olive knot.

Area of study: The Aegean, Marmara, and Mediterranean Regions of Turkey.

Material and methods: 101 isolated bacteria were identified on the basis of biochemical, PCR for amplification of the bacterial *iaaL* gene, and pathogenicity tests. The virulence of the isolates was determined in a randomized experimental trial carried out by stem inoculation of pot-grown seedlings of olive (cv. 'Manzanilla') in the growing chamber. The amounts of IAA produced by the isolates were determined colorimetrically. The susceptibility of native olive genotypes was evaluated on 2-yr old plants inoculated with two distinct strains.

Main results: Tested *P. savastanoi* pv. *savastanoi* isolates showed significant differences in virulence found to be associated with their geographical origin. The isolates produced IAA amounts varied from 148.67 to 0.3 μg mL^{-1}. The geographical variation in IAA biosynthesis ability of the isolates was observed. No correlation (R=0.0225) was determined between virulence and IAA amounts of the isolates. Native olive genotypes indicated different susceptibility levels to the olive knot pathogen. No genotype tested had complete resistance. However, low susceptible genotypes ('Memecik', 'Ayvalık' and 'Uslu') were identified. Some genotypes had variable reactions depending on the isolate used.

Research highlights: The results undergird the differences in the virulence and IAA production of the isolates within the area and also between geographical locations. Genotypes with low susceptibility can be used as genitors in further breeding studies.

Additional key words: olive knot; pathogenicity; IAA production; cultivar resistance

Abbreviations used: IAA (indole-3-acetic acid); *iaaL* (indole-3-acetyl-L-lysine synthetase gene); FAME (fatty acid methyl ester); KB (King's medium B); LOPAT (levan production, oxidase, potato soft rot, arginine dehydrolase, and tobacco hypersensitivity reaction); NA (nutrient agar); PCR (polymerase chain reaction); PFGE (pulsed-field gel electrophoresis); rep-PCR (repetitive element palindromic PCR); SDW (sterile distilled water)

Authors' contributions: Conceived and performed the studies; coordinated the research project: NU. Biochemical and molecular analysis; inoculation assays: NU, NG. Both authors read and approved the final manuscript.

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Introduction

The olive (*Olea europaea* L.) is an economically important crop cultivated traditionally in Near East and Mediterranean basin. Turkey is the fourth biggest producer of olives in the world after Spain, Italy and Greece, with total harvested area 864,428 ha and production 1,500,467 tons (FAO, 2018; http://www.fao.org/faostat).

Main olive growing areas in Turkey are concentrated in the Aegean, Marmara, and Mediterranean Regions. However, olive cultivation has increased also in the Black Sea and South Anatolia Regions recently. More than 80

olive cultivars have been authorized for commercial plantations in the country. Native cultivars such as 'Ayvalık', 'Gemlik', 'Memeli', 'Memecik', 'Domat' etc. are still representing a substantial part of olive production.

Olive knot, having significant negative impact on olive yield and oil quality (Schroth *et al.*, 1968, 1973), is widespread in Turkey, with incidence ranging from 15 to 100% in some production sites of the Aegean, Mediterranean and Marmara Regions (Basım *et al.*, 2000; Tatlı & Benlioğlu, 2004; Mirik *et al.*, 2006, 2007; Mirik & Aysan, 2011, Ustun & Arslan, 2013; Doksöz & Bozkurt, 2020). The tumorous woody outgrowths on young stems as well as on branches, twigs and rarely on leaves and fruits can lead to severe damage, resulting in reduced yield and fruit quality (Young, 2004; Godena *et al.*, 2012). The causal agent is the gammaproteobacterium *Pseudomonas savastanoi* pv. *savastanoi*, which belongs to the *Pseudomonas syringae* complex, including also tumorigenic lineages causing overgrowths (knots) in economically relevant woody plants like *P. savastanoi* pv. *nerii* on oleander (and other Oleaceae), and *P. savastanoi* pv. *fraxini* on *Fraxinus* sp. and olive (artificial inoculation) (Janse, 1982; Gardan *et al.*, 1992; Young, 2004; Caballo *et al.*, 2017).

P. savastanoi pv. *savastanoi* isolates are heterogeneous in their phenotypic and genetic features (Alvarez *et al.*, 1998; Bella *et al.*, 2003; Scortichini *et al.*, 2004; Sisto *et al.*, 2007; Quesada *et al.*, 2008; Ramos *et al.*, 2012; Moretti *et al.*, 2017). Rep-PCR and cluster analyses divided the isolates from different countries into two clusters and four sub-clusters (Moretti *et al.*, 2017). Fatty acid methyl ester (FAME) and molecular (rep-PCR, BOX-PCR, PFGE (pulsed-field gel electrophoresis) analysis of several Turkish olive strain indicated the presence of two heterogenic groups by FAME, and BOX-PCR and three groups by PFGE (Basım & Basım, 2001; Mirik *et al.*, 2011; Basım *et al.*, 2019).

The pathogen colonizes the apoplast and produces phytohormones playing an active role in disease development (Surico *et al.*, 1985). The virulence of the bacterium is closely related to the bacterial secretion of indole-3-acetic acid (IAA) and cytokinins at the site of infection stimulating olive cells to divide and develop new tissue and overgrowths (Surico *et al.*, 1985; Powell & Morris, 1986; Glass & Kosuge, 1988; Temsah *et al.*, 2008; Rodríguez-Moreno *et al.*, 2009; Quesada *et al.*, 2012; Ramos *et al.*, 2012). Presence of the hrp/hrc gene cluster in the *P. savastanoi* pv. *savastanoi* bacterial genome encoding a functional type III secretion system triggers the pathogenicity of the bacterium or hypersensitive response (HR) in susceptible/resistant plants (Sisto *et al.*, 2004; Perez-Martinez *et al.*, 2010). Matas *et al.* (2012) identified novel virulence mechanisms in the pathogen such as the type IV secretion system, a battery of genes involved in tolerance and detoxification of ROS (reactive oxygen species), a set of genes required for the biosynthesis of the cell wall, and a gene that regulates c-di-GMP (3′,5′-cyclic diguanylic acid) levels. Furthermore, Ca^{2+} influx in bacterial cells was found to be mediated by a Na^{+}/Ca^{2+} exchanger which is crucial for the virulence of the bacterium (Moretti *et al.*, 2019). Other virulence determinants are linked to different metabolisms of phenolic compounds necessary for knot induction in woody plants; phytotoxins, cell wall-degrading hydrolytic enzymes, extracellular polysaccharides, etc. (Ramos *et al.*, 2012). Hosni *et al.* (2011) also indicated that the ability of *P. savastanoi* pv. *savastanoi* to induce olive knot formation is affected by its interaction with the resident bacterial flora by sharing quorum sensing intercellular communication signals belonging to the N-acyl-homoserine lactone family.

The virulence of the bacterium strongly depends on the cultivars and various climatic conditions. The strains generate various size of tumors on distinct olive cultivars in artificial inoculations (Penyalver *et al.*, 2006; Pérez-Martínez *et al.*, 2007). Moreover, the different strains may induce variable knot dimensions on the same cultivar. Moretti *et al.* (2017) also demonstrated an association of the virulence with the geographical origin of the strains.

The use of resistant cultivars is considered one of the most suitable approaches for disease control, but unfortunately, little information is available. Limited studies on cultivar resistance have showed large differences in the susceptibility of genotypes to olive knot (Quesada *et al.*, 2012), and only a few tolerant varieties with no complete resistance have been documented up to now (Osman *et al.*, 1980; Sisto & Iacobellıs, 1999; Hassani *et al.*, 2003; Young, 2004). Native olive germplasms, particularly in Mediterranean countries like Spain, Italy, Greece and Turkey, where cultivated varieties believed to be derived from wild-type olive (Sakar *et al.*, 2016), can provide resistance sources. In addition in Anatolia as a motherland of olive native genotypes, are likely to bear resistance to overcome the pathogen virulence in the process of co-evolution. So evaluation of such genotypes may provide valuable information for disease management, particularly in most affected areas, and also resistance breeding.

In this study we determined the virulence of Turkish *P. savastanoi* pv. *savastanoi* isolates from different regions and their IAA biosynthesis ability and evaluated the susceptibility of some native genotypes to olive knot.

Material and methods

Sample collection and isolation

Knot samples were collected from infected olive trees in Marmara regions of Turkey between the years of 2010-2015: Balıkesir (Bandırma, Burhaniye, Edremit,

Erdek), Çanakkale (Ayvacık, Geyikli, Ezine, Küçükkuyu, Gökçeada), Aegean (İzmir (Karaburun), Manisa (Akhisar) and Mediterranean (Hatay (Iskenderun). Symptoms observed were recorded.

Greenish-grey young knots from shoots, leaf or fruit stalks and fruit spots were surface disinfected, cut aseptically to small pieces (1-2 mm) and macerated in 1 mL sterile distilled water. An aliquot (50 µL) of suspension was streaked on King's medium B (KB; King *et al.*, 1954) and PVF-1 agar (Surico & Lavermicocca, 1989). Plates were incubated at 26°C for 3 to 5 days. Presumptive weak fluorescent, creamy colonies were selected and purified on KB medium. Isolates were stored in 20% glycerol at –80°C for further identification.

Identification of bacterial isolates

Biochemical tests. LOPAT (levan production, oxidase, potato soft rot, arginine dihydrolase and hypersensitivity reaction on ''White Burley'' tobacco leaves) and some additional biochemical tests growth at 37°C; hydrolysis of gelatin and aesculin; acid from carbohydrates (melezitose, inositol, arabinose, dulcit, raffinose, sucrose, xylose, ethanol); growth at 2% NaCl were performed as recommended by Lelliott & Stead (1987).

PCR. Genomic DNAs were extracted from pure cultures of selected isolates. A cell suspension from each putative strain was prepared from 24 hr-old bacteria grown on KB medium. Bacterial cells were lysed by heating 15 min at 95°C, and subsequently cool on ice for 10 min. Then suspensions were centrifuged for 1 min at 10,000 rpm and supernatants were used as target DNA in the PCR. DNAs were stored at -20°C until usage. Two specific primers designed by Penyalver *et al.* (2000) — iaaF (5'-GGCACCAGCGGCAACATCAA-3'); iaaLR (5'-CGCCCTCGGAACTGCCATAC-3') of the bacterial gene *iaaL* responsible for indole-3-acetyl-L-lysine synthetase ın IAA biosynthesis — were used for DNA amplification. PCR reaction mixture in a final volume of 20 µL included 10× Taq buffer (Thermo), 1.5 mM $MgCl_2$, 5% formamide, 0.2 mM concentrations of each deoxynucleoside triphosphate (Thermo), 0.6 µM concentrations of each primer, and 1.5 U of Taq DNA polymerase (Thermo) per reaction. Then, 5 µL of the DNA extract from pure culture was added to each reaction. The PCR was performed in a thermal cycler (Eppendorf Master cycler Gradient) with the following program: 1 cycle of 94°C (5 min), followed by 35 cycles of 94°C (30 s), 62°C (30 s), and 72°C (30 s) and then 1 cycle of 72°C for 5 min. PCR-products were separated on electrophoresis gel (1.5% agarose). The gel was stained with 5 mg mL^{-1} ethidium bromide and DNA bands were visualized under an ultraviolet (UV) transilluminator. The expected fragment length of amplicons was 454 bp.

Pathogenicity tests

The isolates were inoculated on the 6-month-old olive plant (cv. 'Manzanilla') to verify their pathogenicity. The inoculum was prepared from 24-48 hr old bacterial growth on nutrient agar (NA) medium at 27 ± 2°C, suspended in sterile distilled water (SDW) and spectrophotometrically adjusted to 10^6 CFU mL^{-1}. The young plants were stem-inoculated by putting 10 µL aliquots of bacterial suspension into wounds open in stem bark with a sterile scalpel. Three plants per isolate and 3 wounded sites per plant were inoculated. SDW was used for inoculation of control plants. Inoculated plants were kept at 60-80% relative humidity, 22-26°C, and 16 hr daylight until knot formation. Approximately 4 weeks post-inoculation the disease symptoms were recorded.

Immature healthy fruits 'Ayvalık' were inoculated by injecting 5 µL aliquots of bacterial suspensions prepared as described above, in order to confirm the ability of the pathogen to cause fruit spots on olive fruits. Three isolates from spots on fruit 'Ayvalık' (Ayvacık, Aegean region) were used. For each isolate 5 fruits were injected at 4 points per fruit. Control fruits were inoculated with SDW. Inoculated fruits were kept in a humid chamber at 27 ± 2°C. Ten days later the symptoms were recorded.

Virulence tests

Virulence tests were performed with selected pathogenic isolates in order to determine high virulent ones for further cultivar susceptibility experiments. The isolates representing different geographical areas with various environmental conditions or distinct locations in the same area and/or isolated from different plant parts were included in these tests. In general, for the same orchard only one isolate from the knots was chosen. A second isolate was included only if they were isolated from fruit spots which were not common for all orchards.

All selected isolates were tested in the same trial organized as randomized parcel design with 3 plants per plot and 3 replications in the growing chamber. The inoculum was prepared as described in the Pathogenicity part. One year old pot-grown seedlings of olive 'Manzanilla' were stem inoculated following Penyalver *et al.* (2006). The young plants were wounded (10 wounds per plant) on the main stem and lateral branches by using a sterile scalpel. Each wound site was inoculated with 10 µL aliquots of bacterial suspension containing approximately 10^6 CFU/mL of *P. savastanoi*. pv. *savastanoi*. In control plants SDW was used for inoculation. For each isolate, 3 plants (30 wounded sites) with 3 replications were inoculated.

Inoculated plants were kept at 60-80% relative humidity, 22-26°C, and 16 hr daylight / 8 hr darkness conditions for three months. At the end of the period, the inoculated

wound sites developing knots at each inoculation site were cut and weighed separately and the average knot weight (g) per plant was estimated. In statistical analyses, Tukey's HST tests were used to compare the isolates.

IAA biosynthesis of the isolates

IAA production by different isolates was determined using Salkowski's reagent (Gordon & Weber, 1951; Mayer, 1958). The bacterial suspensions containing 10^8 CFU mL^{-1} from 24 hr old freshly grown cultures were prepared, and 0.1 mL from each suspension was transferred to 500 mL Erlenmeyer flasks including liquid 200 mL nutrient broth medium supplemented with 0.5 M L-tryptophan. Bacterial suspensions were shaken at 120 rpm at 28°C for 24 hr. The broth was then centrifuged for 5 min at 10,000 rpm and in the supernatant equal volume of Salkowski's reagent (1.0 mL 0.5 M $FeCl_2$ solution + 50 mL of 35% of perchloric acid) was added. The contents were mixed and allowed to stand at room temperature for 30 min to develop color. The OD was then recorded at 535 nm. Uninoculated broth served as control. Standard curve was prepared with 0-1000 (0, 10, 20, 30, 40, 50, 60, 70, 100, 200, 1000) µg mL^{-1} of IAA (Sigma Chemicals). The linear regression equation y=bx+a of the curve was used for quantification.

Absorbance values for 45 isolates − 43 olive isolates; *P. savastanoi* pv. *nerii* (Kavak & Ustun, 2009) as positive control; and *P. syringae* pv *lacrymans* (Plac) as negative control, because it does not produce IAA− were compared with the IAA standard curve and the IAA quantity of each strain was determined by using the formula y=bx+a of the linear regression equation.

Cultivar susceptibility

The ten following native common grown olive genotypes were evaluated in two separate experiments: 'Ayvalık' (olive oil, Aegean Region), 'Domat' (table consumption, Aegean Region), 'Edincik' (table consumption, Marmara Region), 'Gemlik' (olive oil and table consumption, Marmara and others regions), 'Memecik', 'Memeli' and 'Uslu' (olive oil and table consumption, Aegean Region), 'Kilis' and 'Saurani' (olive oil, Mediterranean Region), 'Sari Ulak' (table consumption, Mediterranean Region). The cultivar 'Manzanilla' (introduced) was used as control.

In the first experiment 'Manzanilla', 'Ayvalık', 'Gemlik', 'Sarı Ulak', 'Memecik', 'Domat' and 'Memeli' were inoculated separately with two isolates (Akh3 and Hat 3b) defined as the most virulent in virulence tests. In the second experiment 'Edincik', 'Saurani', 'Kilis', and 'Uslu' and the same isolates (Akh3 and Hat 8) were used. For inoculum preparation, bacterial isolates were grown on NA for 24 h and suspended in SDW. The suspensions were spectrophotometrically adjusted to 10^6 CFU mL^{-1}. Stems and shoots of 2-yr-old plants of each genotype were wounded (10 wounds per plant) with a sterile scalpel and 0.1 mL of bacterial suspensions were put into wounds. For control plants, SDW was used instead of bacterial suspensions. Ten plants (2 plants × 5 replications) per genotype were inoculated by each isolate. Three months post-inoculation knots were cut and weighed separately and the average knot weight (g) per plant was estimated.

The genotypes were evaluated, as proposed by Penyalver *et al.* (2006), as highly susceptible (H; cultivars with significantly higher weight of knots per diseased plant than the average value of the trial), medium susceptible (M; cultivars with no significant differences with the average value), and low susceptible (L; cultivars with a significantly lower weight than the average value).

Statistical analysis

Analysis of variance (ANOVA) was performed by using XLSTAT software (by Addinsoft). Tukey's HSD (Tukey's honestly significant difference test) tests ($p \leq 0.05$) were used to define the differences between the isolates in virulence tests (knot weights) and their IAA biosynthesis. The test was used also to compare the genotypes for their susceptibility to two different isolates. The linear regression equation y=bx+a was used to quantify the IAA amount produced by each isolate.

Pearson correlation coefficient (Pearson, 1895) was calculated to determine the correlation between IAA biosynthesis and knot weight reproduced by the isolates by using the formula $r = \sum((X\text{-}M_y)(Y\text{-}M_x))/\sqrt{(SSx)(SSy)}$, (r= correlation coefficient; x = X values; y = Y values; M_x = mean of X values; M_y = mean of Y values; $X\text{-}M_x$ & $Y\text{-}M_y$ = deviation scores; $(X\text{-}M_x)^2$ & $(Y\text{-}M_y)^2$ = deviation squared; (X-Mx)(Y-My)= product of deviation scores). Scores near zero mean no (or weak) correlation; scores near 1 positive correlation; and -1 negative correlation.

Results

Isolation

Olive knot symptoms recorded in olive growing areas between 2010 and 2015 in the Marmara, Aegean and Mediterranean Regions of Turkey were usually overgrowths (knots) on young shoots, branches, stems. No knot symptoms were observed on the leaves, fruits, and roots. However, small knots were frequently seen on fruit stalks at the point of joining with fruit. Also, rarely only in some

areas (Çanakkale province-Ezine and Kucukkuyu; Balıkesir-Burhaniye, Erdek) small, circular, sunken, brown spots 2.5-4.0 mm in diameter were noticed on fruits sometimes at the site of fruit stalk joining (Fig. S1 [suppl]).Totally 90 samples were collected (mainly from young knots on shoots, branches, fruit stalks; 10 samples from fruit lesions included) and 101 bacteria were isolated.

Biochemical and pathogenic characters of the isolates

In LOPAT tests isolated bacteria were found identical with fluorescent pseudomonads I b group (Lelliot *et al.*, 1966), where *Pseudomonas savastanoi* pv. *savastanoi* is located. All isolates were Gram, oxidase, pectinolytic activity, levan and arginine dihydrolase negative; but positive for hypersensitive reaction on tobacco leaves (except two isolates) (Table 1). Also, they grew at 37ºC and in the presence of 2% NaCl, assimilated mannitol, sorbitol, citrate (except three isolates), *m*-inositol (except four isolates), and sucrose, but were not able to hydrolyze gelatin and aesculin and assimilate melezitose, L-arabinose, dulcit, raffinose, trehalose and ethanol. The isolates showed similar biochemical properties with a few exceptions for tobacco hypersensitivity reaction (THR) and assimilation of citrate and m-inositol.

Table 1. Biochemical and pathogenic features of *Pseudomonas savastanoi* pv. *savastanoi* isolates from the Aegean, Marmara and Mediterranean Region of Turkey. In parenthesis, the number of isolates tested

Tests	Pv[a]	Psav[b]	Isolates[c]										
			GA (7)	Ayv (10)	Akh (15)	Ba (7)	Bur (6)	Gy (8)	Edr (12)	Erd (12)	Ezi (6)	Kar (10)	Hat (8)
Fluorescent pigment	+	+	+	+	+	+	+	+	+	+	+	+	+
Levan	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-
Pectinolytic activity	+	-	-	-	-	-	-	-	-	-	-	-	-
Arginine dihydrolase	-	-	-	-	-	-	-	-	-	-	-	-	-
THR [d]	+	+	+	+	+	+	+	+	+[e]	+	+	+[e]	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate	ND	+	+	+[e]	+	+	+	+	+[e]	+	+	+	+
Growth at 37ºC	-	-	-	-	-	-	-	-	-	-	-	-	-
Aesculin hydrolysis	+	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin hydrolysis	+	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 2% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+
Melezitose	-	-	-	-	-	-	-	-	-	-	-	-	-
m-Inositol	+	+	+	+	+	+	+	+	+	+	+	+	V
L-arabinose	+	-	-	-	-	-	-	-	-	-	-	-	-
Dulcit	-	-	-	-	-	-	-	-	-	-	-	-	-
Raffinose	+	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	+	+	+	+	+	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+	+	+	+	+	+
Trehalose	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethanol	-	-	-	-	-	-	-	-	-	-	-	-	-
Olive knot	-	+	+	+	+	+	+	+	+	+	+	+	+
Fruit spots	ND	ND	ND	+	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] Pv: *Pseudomonas viridiflava* CFBP 1466. [b] Psav: *Pseudomonas savastanoi* pv. *nerii* (Kavak & Ustun, 2009). [c] GA: Gökçeada; Ayv: Ayvacık; Akh: Akhisar; Ba: Bandırma; Bur: Burhaniye; Gy: Geyikli; Edr: Edremit; Erd: Erdek; Ezi: Ezine; Kar: Karaburun; Hat: Hatay. [d] THR: tobacco hypersensitivity reaction. [e] Only isolate was negative in these cases, *e.g.* one out of 10 isolates (Ayv) and 1 out of 12 isolates (Edr) were negative for citrate production, the rest were positive. +: 80% or more strains were positive. V: between 21-79% of isolates were positive. -: 80% or more strains were negative. ND: not determined.

Pathogenicity of all isolated bacteria was confirmed on cv 'Manzanilla' and knots were produced on inoculated sites two-three weeks post-inoculation (Table 1; Fig. S2 [suppl], up). Tested isolates caused brownish sunken spots surrounded by a halo on immature fruits. No symptoms were observed on control fruits (Fig. S2 [suppl], down).

Molecular identification

The iaaLF/iaaLR primers yielded the expected 454 bp DNA sequence from all pathogenic isolates.

Virulence tests

The virulence of tested isolates (40) varied significantly (p<0.05). The weight of generated knots changed from 1.35 to 0.075 g. Hat 8, Akh 3, Ayv 4a, and Edrmt 6 showed the highest virulence. In opposite, Krbrn10 and Erdk12 had the lowest virulence. The isolates from the same locations differed in their virulence, high and less virulent ones were defined in the same location (Fig. 1).

Comparison of the means of the average knot weights (g) of all tested isolates from diverse locations indicated significant differences in the ANOVA test (Tukey's HSD test, $p \leq 0.05$). Akhisar, Edremit, Burhaniye and Hatay isolates were in the same statistical group, but they differed from Ayvacık, Erdek and Karaburun isolates in the weight of produced knots (Fig. 2).

IAA biosynthesis ability

The absorbance values for different concentrations (0-1000 µg mL^{-1}) of IAA (OD 535 nm) increased from 0 to 1.161 µg mL^{-1}. The linear regression equation of the standard IAA concentration-response curve used for quantification of IAA production of each isolate was determined as Y = 0.00122X + 0.08263, where Y means experimentally measured absorbance value (at OD 535 nm) and X the calculated amount of IAA. Based on the absorbance values, the calculated IAA amounts of isolates changed from 148.67 to 0.3 µg mL^{-1} (Fig. 3).

The average IAA values of *P. savastanoi* pv. *savastanoi* isolates belonging to different locations of Turkey differed statistically from each other (Fig. 4). The isolates belonging to Karaburun (Izmir, Aegean region) produced the

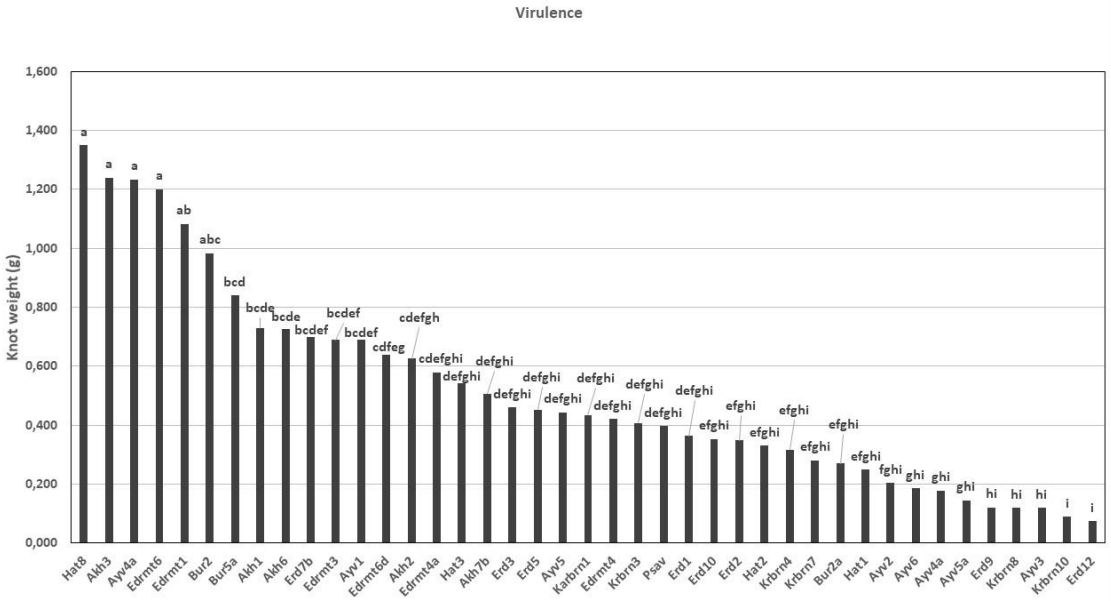

Figure 1. Knot weights generated by selected *Pseudomonas savastanoi* pv. *savastanoi* isolates in virulence tests on cv. 'Manzanilla'. Isolates: Akh1, Akh2, Akh3, Akh6, Akh7b from Akhisar (Manisa, the Aegean region); Ayv1, Ayv2, Ayv3, Ayv4a, Ayv5a, Ayv6 from Ayvacık (Çanakkale, Marmara region); Bur 2, Bur 2a, Bur 5 from Burhaniye (Balıkesir, the Marmara region); Edrmt 1, Edrmt 3, Edrmt 4, Edrmt 4a, Edrmt 6, Edrmt 6d from Edremit (Balıkesir, the Marmara region); Erd 1, Erd 2, Erd 3, Erd 5, Erd 7b, Erd 9, Erd 10, Erd 12 from Erdek (Balıkesir, the Marmara region); Hat1, Hat2; Hat3; Hat8 from Hatay (the Mediterranean region); Kar1, Kar3, Kar4, Kar7, Ka8, Kar10 from Karaburun (İzmir, the Aegean region). Isolates names marked only with number indicate they were isolated from knots, and those marked with number and letter were isolated from fruit spots. Psav: *P. savastanoi* pv *nerii* (Kavak & Ustun, 2009) positive control. Negative control: SDW (not included in the graph). Different letters above the histogram bars indicate differences between groups at $p \leq 0.05$ (Tukey's HSD test).

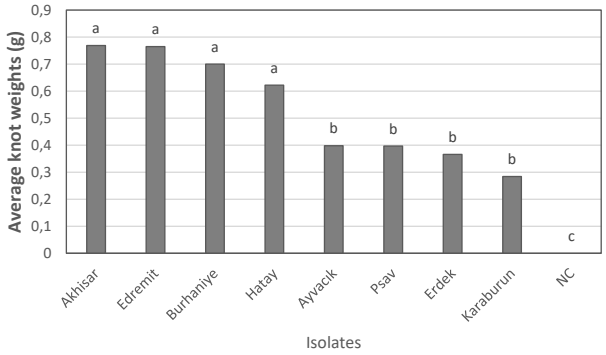

Figure 2. Average knot weights (g) of *Pseudomonas savastanoi* pv. *savastanoi* isolates from different locations of Turkey. Average knot weight values (g) were calculated as a mean for all selected isolates from each location. Number of tested isolates for each location are: Akhisar (5), from Manisa, Aegean region; Burhaniye (3), Edremit (6) and Erdek (8) from Balıkesir, Marmara region; Ayvacık (6) from Çanakkale, Marmara region; Karaburun (6) from Izmir, Aegean region; Hatay (4) from Mediterranean region. Psav: *P. savastanoi* pv *nerii*, positive control. Negative control: water. Different letters above the histogram bars indicate differences between groups at $p \leq 0.05$ (Tukey's HSD test).

highest amounts of IAA, in opposite those from Ayvacık (Çanakkale province, Marmara region) and Gökçeada (Çanakkale province, Marmara region) the lowest amounts.

Pearson coefficient R calculated in order to determine the correlation between knot weight (g) and IAA amount (μg mL^{-1}) was 0.0225, meaning that no correlation was determined.

Cultivar susceptibility

In the first experiment, the response to Akh 3 isolate was highly susceptible for 'Manzanilla', medium susceptible for 'Memeli', 'Domat', and 'Gemlik', and low susceptible for 'Ayvalık' and 'Sarı Ulak'. The reaction to Hat 8 also varied, with high susceptibility for 'Manzanilla', medium for 'Memeli', 'Domat', and 'Sari Ulak', and low for 'Memecik' and 'Gemlik'. Most of the genotypes tested had the same reactions to the two different isolates. Only 'Gemlik' and 'Sari Ulak' cultivars showed differences in susceptibility to each isolate (Table 2).

In the second experiment, the genotypes had a different response to Akh3, with high susceptibility for 'Saurani', medium for 'Manzanilla' and 'Kilis', and low for 'Edincik' and 'Uslu'. For Hat 8 'Edincik' was highly susceptible, 'Manzanilla' and 'Kilis' were medium and 'Saurani' and 'Uslu' low. 'Edincik' and 'Saurani' revealed different susceptibility to the isolates used (Table 2).

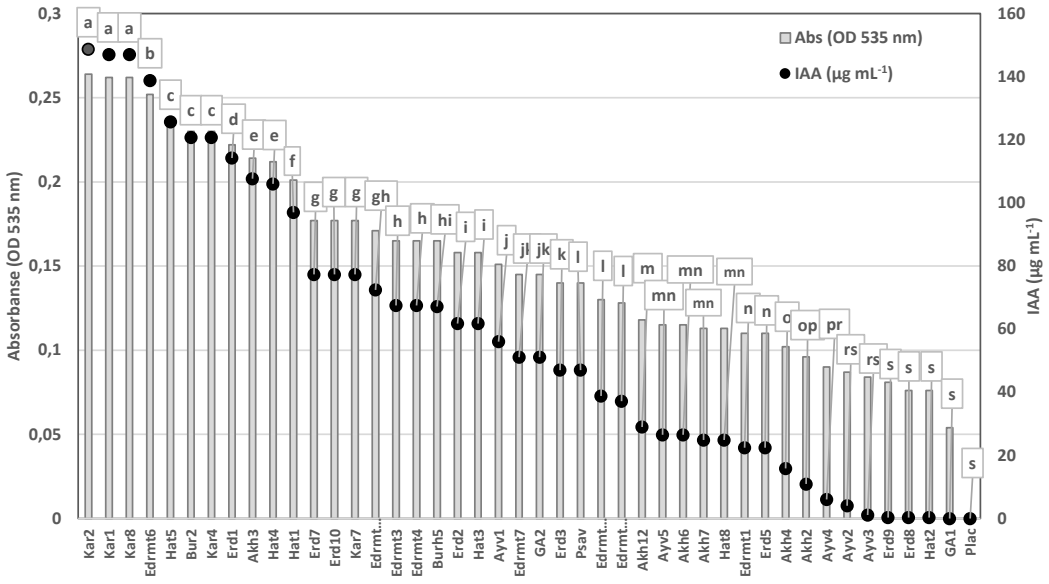

Figure 3. Absorbance values and IAA amount of *Pseudomonas savastanoi* pv. *savastanoi* isolates from the Aegean, Marmara and Mediterranean regions of Turkey. Isolates: Akh2, Akh3, Akh 4, Akh6, Akh7, Akh 12 from Akhisar (Manisa, Aegean region); Ayv1, Ayv2, Ayv3, Ayv4, Ayv5 from Ayvacık and GA1 and GA 2 from Gökçeada (Çanakkale, Marmara region); Bur 2, Bur 5 from Burhaniye (Balıkesir, Marmara region); Edrmt 1, Edrmt 3, Edrmt 4, Edrmt 4a, Edrmt 6, Edrmt 6d from Edremit (Balıkesir, Marmara region); Erd 1, Erd 2, Erd 3, Erd 5, Erd 6, Erd 7, Erd 8, Erd 9, Erd 10 from Erdek (Balıkesir, Marmara region); Hat1, Hat2; Hat3; Hat 4, Hat 5, Hat 8 from Hatay (Mediterranean region); Kar1, Kar2, Kar4, Kar7, Ka8 from Karaburun (Izmir, Aegean region). Absorbance and IAA values of each isolates are means of three replications. Different letters above the histogram bars indicate differences between groups at $p \leq 0.05$ (Tukey's HSD test). Psav: positive control. Plac: negative control.

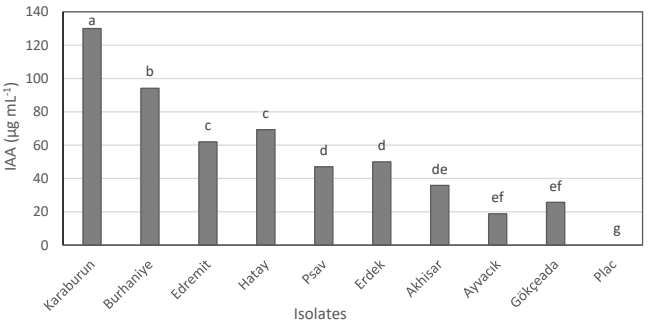

Figure 4. Average IAA values of *Pseudomonas savastanoi* pv. *savastanoi* isolates from different locations of Turkey. IAA values were calculated as a mean for all isolates from each location. The numbers of isolates tested for each location are: Karaburun (5), Burhaniye (2), Edremit (7), Erdek (9), Hatay (6), Ayvacık (5), Gökçeada (2), Akhisar (5). Psav: positive control. Plac: negative control. Different letters above the histogram bars indicate differences between groups at $p \leq 0.05$ (Tukey's HSD test).

Discussion

One hundred and one bacteria were isolated from samples collected from olive growing areas in the Aegean, Marmara, and Mediterranean Regions of Turkey between the years 2010-2015. The symptoms recorded were knots on young shoots, branches, stems, and also fruit stalks. The pathogen prefers woody tissue to herbaceous (Varvaro & Surico, 1978) and the appearance of these overgrowths on leaf and fruit stalks is sporadic. Fruit spotting firstly recorded by Zachos (1958) in Greece was another disease form observed only in some areas primarily located near the sea (Canakkale province-Ezine and Kucukkuyu; Balikesir province-Burhaniye, Erdek etc.). Panagoupoulos (1993) explained that their occurrence can be dependent on wet summer and high relative humidity in these areas. In Greece, the fruit spots were reported to reduce the marketable value of the fruits, particularly those grown for canning. However, in our study fruit spots were very sporadically observed in limited areas in Turkey.

The isolates were highly homogeneous in the biochemical tests performed. Bella *et al.* (2003) reported homogeneity of olive and oleander strains in the assimilation of carbon sources clustered in one phenon. However, heterogeneity in phenotypic and genetic features was determined in further studies (*e.g.*, Sisto *et al.*, 2007; Quesada *et al.*, 2008; Ramos *et al.*, 2012; Moretti *et al.*, 2017). Although existing two clusters within the species, Moretti *et al.* (2017) suggested that today's *P. savastanoi* pv. *savastanoi* population is the result of clonal expansion of a single strain that moderates migration of the pathogen occurred between the countries and that changes in the virulence arose during its evolution. This hypothesis supports the differences in the virulence of the strains (Young, 2004) shown to be associated by Moretti *et al.* (2017) with the geographical origin.

Our study undergirds the differences in the virulence of the isolates within the area and also between geographical

Table 2. Susceptibility of native olive genotypes to *Pseudomonas savastanoi* pv. *savastanoi*. (isolates Akh 3 and Hat 8)

Genotype	Akh 3		Hat 8	
	Average knot weight (g) [a]	Average knot weight (g) [a]	Average knot weight (g) [a]	Susceptibility level [b]
		Experiment I		
'Manzanilla'	1.644 a*	H	1.749 a	H
'Memecik'	0.206 e	L	0.397 d	L
'Memeli'	0.612 b	M	0.642 bc	M
'Ayvalık'	0.342 de	L	0.312 de	L
'Domat'	0.608 b	M	0.576 c	M
'Gemlik'	0.428 cd	M	0.218 e	L
'Sarı Ulak'	0.318 de	L	0.759 b	M
General mean	0.595 bc		0.664 bc	
		Experiment II		
'Manzanilla'	0.558 b*	M	0.633 b	M
'Edincik'	0.225 c	L	0.782 a	H
'Saurani'	0.919 a	H	0.415 c	L
'Kilis'	0.51 b	M	0.557 b	M
'Uslu'	0.343 c	L	0.395 c	L
General mean	0.509 b		0.556 b	

[a] Values followed by the same letter are not statistically different at $p \leq 0.05$ (Tukey's HSD test). [b] H: highly susceptible. M: medium susceptible. L: low susceptible.

locations. Virulence tests results indicated that from highly virulent to less virulent isolates can be present in the same area (Fig. 1). On the other hand, locations differed among each other based on the average knot weight of tested isolates (Fig. 2). They were statistically divided into two groups (I: Akhisar, Edremit, Burhaniye and Hatay isolates; II: Ayvacık, Erdek and Karaburun isolates). Differences in environmental conditions, ecological niches, ecosystem composition, and dynamics cultivars, general cultivation practices, etc. between the areas may be considered to affect host-pathogen interactions and also the evolutionary potential and virulence of the pathogen as it was revealed previously (Sacristán & García-Arenal, 2008; Abdullah *et al.*, 2017; Velásquez *et al.*, 2019).

Besides type III and IV secretion systems, quorum sensing (QS) regulation, and some other metabolic adaptation mechanisms, phytohormones are among the outstanding virulence determinants of *P. savastanoi* pv. *savastanoi* (Surico *et al.*, 1985; Powell & Morris, 1986; Glass & Kosuge, 1988; Sisto *et al.*, 2004; Temsah *et al.*, 2008; Rodríguez-Moreno *et al.*, 2009; Perez-Martinez *et al.*, 2010; Matas *et al.*, 2012; Quesada *et al.*, 2012; Ramos *et al.*, 2012). In this study, the isolates produced significantly different amounts of IAA (Fig. 3). The biosynthesis of this plant hormone known also for many endophytic bacteria occurs through multiple pathways (like indole-3-pyruvate (IPyA) pathway, indole3-acetamide (IAM) pathway, indole-3-acetonitrile (IAN) pathway, tryptamine pathway and tryptophan side-chain oxidase pathway), and even a single organism can have more than one pathway (Spaepen *et al.*, 2007; Jasim *et al.*, 2014) which can explicate the differences between the strains. The average IAA production of the isolates for the locations studied was also variable (Fig. 4). The isolates belonging to Karaburun (Izmir, Aegean Region) produced the highest amounts of IAA, in opposite these from Ayvacık and Gökçeada (Çanakkale province - Marmara Region) the lowest IAA amounts. However further researches are needed to confirm geographical variation between the isolates in IAA biosynthesis and to explain its genetic basis.

Although all tested isolates produced IAA, a no correlation (Pearson coefficient of correlation R = 0.0225) was determined between virulence (knot weights) and IAA of the isolates. Not only IAA biosynthesis but also other virulence mechanisms (Ramos *et al.*, 2012) of *P. savastanoi* pv. *savastanoi* switched on in different isolates can be the reason for this lack of correlation.

Identifying resistant olive genotypes is an important point for successful disease management and improvement of quantity/quality of fruit and oil yield (Young, 2004). Unfortunately, no completely resistant varieties are yet available, but there are several reports of useful tolerance (Varvaro & Surico, 1978; Benjama *et al.*, 1987; Benjama *et al.*, 1992; Benjama, 1994; Marcelo *et al.*, 1999; Young *et al.*, 2005). The data for cultivar reactions were gathered from comparative studies with artificial inoculations or field trials based on the visual evaluation of disease symptoms (Godena *et al.*, 2012; Salman *et al.*, 2020, Valverde *et al.*, 2020). Comparative studies carried out under controlled conditions with artificial inoculations seem to give more adequate information for cultivar resistance/susceptibility. Field observations do not always allow getting universally valid knowledge on the intrinsic susceptibility of each cultivar because the initial quantity of bacterial inoculum differs between plants and factors favoring infection can vary in different areas (Quesada *et al.*, 2012). However, the disease response of the cultivar is closely related to plant age, strain virulence and inoculum dose in inoculation assays (Penyalver *et al.*, 2006).

Turkey has a large number of olive genotypes but information regarding their susceptibility has not been found. The present study evaluated experimentally the susceptibility of native olive genotypes grown widely in Turkey. Two experiments' results indicated that no genotype tested has complete resistance to the pathogen. However, low susceptible genotypes (‘Memecik’, ‘Ayvalık’ and ‘Uslu’) were identified (Table 2). Some of the genotypes (‘Memeli’, ‘Domat’, ‘Kilis’) were moderately susceptible and others (‘Gemlik’ and ‘Sari Ulak’; ‘Edincik’ and ‘Saurani’) had variable responses depending on the isolate (Table 2). In addition, the susceptibility of the same genotype (‘Manzanilla’) varied in different experiments considered to be linked with distinct genotypes tested in each experiment (Table 2). Different response of some olive cultivars (‘Frantoio Oblonga’, ‘Lectin de Sevilla’, ‘Morisca’, etc) to olive knot pathogen in different experiments was previously reported by Penyalver *et al.* (2006). ‘Manzanilla’ was evaluated as intermediate susceptible in Italy, but highly susceptible in Australia priory (Sisto *et al.*, 2001, Young, 2004; Young *et al.*, 2005). Our study supported strain-depending variation in the reactions of olive genotypes (Benjama, 1994; Penyalver *et al.*, 2006).

In the present study, the differences in the virulence and IAA production of Turkish olive knot isolates within the area and between geographical locations were found, IAA secretion of the isolates was firstly estimated quantitatively and associated with geographical locations, also the susceptibility of common Turkish native olive genotypes to the pathogen was determined for the first time. The useful information for the behavior of main native Turkish cultivars to one of the most relevant diseases affecting olive cultivation was provided; however, only a small part of Turkish olive germplasm was evaluated. Further researches are necessary to assess the other Turkish olive genotypes registered in the ‘’National cultivar list’’ or locally grown. Genotypes with low susceptibility can be used as genitors in further breeding studies.

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