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# SEEDBORNE BACTERIA OF ORANGE AND BLACK COLOUR CARROTS IN TURKEY

**Kubilay K. Bastas, Haris Butt, Aysegul Gur**

*Department of Plant Protection, Faculty of Agriculture, Selcuk University, Campus, Konya, Turkey.*

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**ABSTRACT**

Carrot (*Daucus carota* L.) is among the most economically important vegetable crops worldwide. Seedborne bacterial pathogens of carrot cause important damages to seed quality and yield of plants. In this study, seedborne bacteria were determined on some carrot seeds sown in Turkey. Seeds of different orange and black color varieties of carrot were collected from Eregli and Kasınhanı districts of Konya province, where the highest carrot production is reported. Subsamples of 10,000 seeds were soaked in 100 ml sterile saline (0.85% NaCl) with 0.02% Tween 20 overnight at 5 °C, YDCA, KB, MKM, MD5A and mTBM media were used for bacterial isolation and bacterial morphological characterization. Biochemical, physiological and molecular methods were used for the identification of the bacterial isolates. Pathogenicity tests of strains were performed on orange color carrots, and pathogenic strains induced a hypersensitive reaction in tobacco plants. The 60 pathogenic and saprophytic bacterial strains were obtained belong to Pseudomonaceae, Bacillaceae, and Xanthomonadaceae families. There were twenty-three seed samples on 5 different orange carrot cultivars Maestro, Bolero, Sireco, Natuna and Romans, and 11 black carrot genotype of traditional cultivar 'Eregli'. Two pathogenic bacteria were defined as *Xanthomonas hortorum* pv. *carotae* and *Pseudomonas viridiflava* at different percent infestation ratios (17.39-18.18%) and (9.09-13.04%) on orange and black carrot seed samples. To the best of our knowledge, it is the first report of *P. viridiflava* on carrot seeds in Turkey.

*Corresponding Author: Kubilay K. Bastas*

*Email: kbastas@selcuk.edu.tr*



## INTRODUCTION

Carrot (*Daucus carota* L.) is consumed as edible roots; usually, orange in color but red, white, purple, and yellow varieties also exist. Carrot is native to Europe and southwestern Asia and is a domesticated form of the wild carrot *Daucus carota*. Selective breeding has been practised for domestic carrot to obtain large, more delectable, and edible taproot with a less woody texture. Most carrot cultivars contain water (88%), sugar (7%), fibre (1%), ash (1%), protein (1%), and fat (0.2%) with almost no starch content. The fiber comprises mostly of cellulose, with smaller proportions of hemicellulose and lignin. Fructose, sucrose, glucose and xylose are free sugars found in carrots. Glutamic acid and other free amino acids are responsible for the taste while β-carotene mainly, and α-carotene and γ-carotene to lesser extent, gives carrot the bright orange colour. In humans, α and β-carotenes are partly metabolized into vitamin A. Among carotenoid, β-carotene is the predominant while α-carotene and γ-carotene are present in lesser amounts. Typically, there are between 6000 and 54,000µg of carotenoids in 100 grams of carrot root. There are abundant amounts of antioxidants and minerals in carrots having anti-aging, anti-inflammatory, antiproliferative, and anti-carcinogenic properties. Additionally, carrot

consumption helps in maintenance of normal blood glucose and cholesterol levels, minimizes the risk of cardiovascular diseases, and protects against diabetes and Alzheimer's disease (Ahmad *et al.*, 2019).

According to the Food and Agriculture Organization of the United Nations (FAO), 44.76 million tonnes of carrots and turnips were produced worldwide in the calendar year 2019 (FAOSTAT, 2019). China led the chart with 21.48 million tonnes that accounted for 47.99% of the global output, followed by Uzbekistan, the USA and Russia, with almost 2.77, 2.26, and 1.56 million tonnes, respectively. Asia produced about 64.8% of the world's carrot followed by Europe (19%) and Americas (10%). Turkey is among the world's important carrot producers, with its annual production of 663.882 tonnes (TUIK, 2019).

Bacteria, fungi and viruses can be transmitted through seeds in plants (Baker and Smith, 1966; Mundt and Hinkle, 1976). Thus one would expect natural selection to favor host plants that tightly control the kind and number of microbes that migrate into the developing seeds. Carrots are affected by several bacterial diseases; leaf blight (*Xanthomonas hortorum* pv. *carotae*), soft rot (*Dickeya dadantii*, *Pectobacterium carotovorum* subsp. *carotovorum* and *Pectobacterium atrosepticum*), crown gall (*Rhizobium radiobacter*), hairy root (*R. rhizogenes*), milky disease (*Bacillus popilliae* var. *rhopaea*) and scab (*Streptomyces scabies*) (Strandberg, 2000). Almeida *et al.* (2013) reported that *Pseudomonas viridiflava* was determined on carrot seeds, and this could explain how post-harvest damage might originate from contaminated seeds.

The world's carrot seed industry is concerned directly with a seed-borne disease i.e., bacterial blight, caused by *X. hortorum* pv. *carotae*. Under optimum conditions, the quality of *X. hortorum* pv. *carotae* infested carrot seeds may reduce noticeably, which leads to a significant yield loss. Brown stem, blighted umbels, petiole lesions, necrotic leaf spots with irregular yellow halos, and gummy bacterial exudates on stems and umbels are typical symptoms of this disease. Use of *X. hortorum* pv. *carotae* free seed is important management practice as contaminated carrot seeds can be a potential primary source of inoculum. Therefore, inspection services, seed industry and commercial seed testing laboratories needed sensitive detection methods suitable for routine application (Meijerink and Van Breukelen, 1995). Despite being able to detect the seed-borne infection, development of seed contamination thresholds for specific regions of carrot production (Umesh *et al.*, 1998), and the availability of seed treatments to eliminate seed-borne inoculum (Howard *et al.*, 1994; Pscheidt and Ocamb, 2001), the carrot industry is still facing losses due to this disease (Kuan *et al.*, 1985; Umesh *et al.*, 1996). Studies regarding the prevalence of *X. hortorum* pv. *carotae* in carrot seeds in Turkey are very scarce and the pathogen was reported only once by Demir and Ustun (2001) in one carrot seed sample in Aegean Region. So it is important to study further the presence of this pathogen in carrot seeds in Turkey.

The pectinolytic species *Pseudomonas viridiflava* is a multi-host and seed-borne pathogen causing severe damages to tomato (Alivizatos, 1986; Goumas *et al.*, 1999), melon (Aysan *et al.*, 2003) bean, birdsfoot, cabbage, cauliflower, kiwifruit, fennel, grape, lettuce, lupine, parsnip, passion fruit, pea, pepper, poinsettia, poppy, pumpkin, rape, soybean, and zinia, *Amaranthus* sp., *Chrysanthemum* sp., eggplant (Goumas *et al.*, 1999), and *Arabidopsis thaliana* (Goss *et al.*, 2005). Symptoms of *P. viridiflava* infection include yellowing of the plant and inner part of the stem, wilting, brown discoloration of vascular tissses and pith often developing soft rot. Particularly in Aegean islands, this pathogen is significant in the eastern Mediterranean region, representing 50% and 15%, respectively, of the Pseudomonas species causing stem necrosis (Aysan, 2001; Ustun and Saygili, 2001).

Seventy percent of Turkey's orange carrots are produced in Kasınhanı town of Konya Province and exports them to the Middle East and Balkans countries. According to the provincial agricultural directorate, 300.000 tons of orange carrot is grown in the region. There are 110 carrot processing factories of varying sizes in Kasınhanı. In addition, an estimated 10,000 workers go to Kasınhanı from neighborhood towns or provinces every year as seasonal laborers. Black carrot is produced about 140,000-160,000 tons in only Eregli district of Konya Province, Turkey.

This study aimed to determine the seed-borne bacterial pathogens on seeds of black and orange carrots in the areas with highest production, i.e., Kasınhanı town and Eregli district of Konya.

## MATERIAL AND METHODS

### Plant material

Seed samples were obtained from carrot producers in

2 districts (Kaşınhanı and Ereğli) of Konya province of Central Anatolia Region, Turkey. Twenty three seed samples from five different orange carrot cultivars; Maestro, Bolero, Sireco, Natuna and Romans, and 11 black carrot genotypes of traditional cultivar 'Ereğli' were used in the experiments.

For bacterial identification, carrot cv. Maestro and *Nicotiana tobaccum* cv. White Burley were used for pathogenicity tests and hypersensitivity reaction (HR) assays.

**Reference cultures**

Reference cultures used in all the tests are given in Table 1. Stock cultures were preserved at -80 °C in 30% glycerol solution. Colonies grown on NA at 23-25 °C were used to prepare the bacterial suspensions, adjusted to a density of $10^8$ CFU $ml^{-1}$ ($OD_{600}$~0.15) using biophotometer (Eppendorph, Germany). Sterile distilled water (SDW) was used for dilutions, and the inoculum was kept on ice and plants were inoculated within 2hrs of inoculum preparation.

Table 1. Reference cultures and their sources used in the experiments.

| Bacterium | Code No | Source |
|---|---|---|
| *Xanthomonas hortorum* pv. *carotae* | NCPPB3651 | Nat. Collec. of Plant Pathogenic Bacteria, England |
| *Xanthomonas translucens* pv*. translucens* | XttKk521 | Prof. K. K. Bastas, Selcuk University, Turkey |
| *Xanthomonas axonopodis* pv. *phaseoli* | NCPPB2665 | Nat. Collec. of Plant Pathogenic Bacteria, England |
| *Pseudomonas viridiflava* | YA12 | Prof. Y. Aysan, Cukurova University, Turkey |
| *Pseudomonas syringae* pv. *syringae* | Pss74 | Prof. K. K. Bastas, Selcuk University, Turkey |
| *Pectobacterium caratovorum subsp. caratovorum* | EccLk7 | Prof. K. K. Bastas, Selcuk University, Turkey |
| *Pectobacterium atrosepticum* | EcaKp37 | Prof. K. K. Bastas, Selcuk University, Turkey |

**Isolation of bacteria from the seeds**

Seed sample was taken from the bottom of the bag (100g each) and was divided into 5 subsamples of 20g seeds each according to the standards of International Seed Testing Association (ISTA) (Asma, 2010).

Twenty-gram seed subsamples (approx. 10.000 seeds) were added to 100 ml of sterile cold (5 °C) aqueous saline (0.85% NaCl) in 250 ml Erlenmeyer flask. After incubation in dark for 16-18 hours at 5 °C, 0.05 ml of sterile Tween 20 was added to the flasks, and the samples were shaken vigorously for one minute. Afterwards, the suspensions were filtered through sterile cheesecloth. The retained seeds were rinsed with 25 ml of cold sterile saline. The pooled washings were collected in a clean, sterile flask and centrifuged at 4000 rpm for 15 min. Pellets were resuspended in 10 ml of sterile cold saline and the suspensions were diluted to $10^3$ - $10^6$, and plated onto the following media for 4-8 days at 28 °C in triplicate (King *et al.*, 1954; Kim *et al.*, 1982; Williford and Schaad, 1984; Cubeta and Kuan, 1986; Meguıre *et al.*, 1986; Lelliott and Stead, 1987; Schaad, 1988; Schaad *et al.*, 2001; Asma, 2010); Nutrient Agar (20 g/L nutrient agar (Merck), 40 mg/L cycloheximide), King's B medium (KB; proteose peptone 20 g/L, glycerol 15.0 ml/L, $K_2HPO_4$ 1.5 g/L, $MgSO_4.7H_2O$ 1.5 g/L, agar 15 g/L), MD5A medium (0.3 g/L $MgSO_4.7H_2O$, 1 g/L $NaH_2PO_4$, 1 g/L $NH_4Cl$, 3.0 g/L $K_2HPO_4$ with 17 g/L, pH adjusted to 6.4, after autoclaving: 10 mg/L cephalexin monohydrate, 20 mg/L nystatin, 1 mg/L L-methionine, 10 mg/L bacitracin, 10 g/L filter sterilised cellobiose, and 5 mg/L L-glutamic acid), mTBM medium (10 g/L peptone, 0.3 g/L $H_3BO_3$, 10 g/L KBr, 17 g/L agar, pH adjusted to 7.4, after autoclaving: 12 mg/L 5-fluorouracil, 20 mg/L nystatin, 65 mg/L cephalexin monohydrate, 10 g/L skimmed milk, and 10 ml/L Tween 80), YDCA medium (20 g/L $CaCO_3$, 10 g/L yeast extract, 20 g/L dextrose, 15 g/L agar), MKM medium (1.2 g/L $KH_2PO_4$, 4 g/L D(+) trehalose dihydrate, 1 g/L $NH_4Cl$, 10 g/L lactose monohydrate, 0.2 g/L 2-thiobarbituric acid with 17 g/L, 0.5 g/L yeast extract, 1.2 g/L $K_2HPO_4$, pH adjusted to 6.6, after autoclaving: 2 mg/L tobramycin sulphate, 20 mg/L nystatin, 50 mg/L bacitracin, and 10 mg/L cephalexin monohydrate).

Pure culture suspensions of known strains of *X. h.* pv. *carotae* and *P. viridiflava* were spread on above-mentioned media to prepare the positive control plates. Colonies were considered if they appeared similar to colonies of the reference strains.

**Identification of the bacteria**

**Biochemical and physiological tests**

Different biochemical tests conducted to characterize the presumptive pathogens are given in Table 2 (Kovacs,

1956; Thornley, 1960; Klement, 1963; Kuan *et al.*, 1985; Komagata, 1986; Bradbury, 1986; Lelliott and Stead, 1987; Klement *et al.*, 1990).

**Pathogenicity and hypersensitivity tests**

Individual plastic pots containing 3 kg sterile soil were fertilized once with 9.5 g ammonium sulfate, 9.5 g diammonium phosphate, 9.5 g potassium sulfate, and 50 mL of a liquid humic acid (Kaçar and Katkat, 1999), and they were grown at 25 ± 5°C, 60-75% RH, and under 12.000-14.000 Lux from tungsten-filament lamps for a 16-h photoperiod. Five seeds were planted in each pot of each carrot variety 'Maestro' at a depth of 8-10mm. Four week-old plants were sprayed with distilled water to create a fine humidity by a hand-held laboratory sprayer for bacterial development.

All suspect strains of *X. h.* pv. *carotae* were tested by atomizing the leaves to run off with a bacterial suspension containing $10^8$ CFU $ml^{-1}$. Inoculated seedlings (five plants per strains) were incubated for 48 h to enhance infection. After this incubation, plants were set in a cool condition (at 20°C, 16 h) before placing the plants in greenhouse conditions at 25- 30 °C with 90% relative humidity (Kuan, 1989; Asma *et al.*, 2002). After the 15-day incubation period, treated seedlings were observed for pathogenicity based on typical leaves symptoms and intensity of stem necrosis (Taylor, 1970; Lelliott and Stead, 1987). Each strain was inoculated on two carrot plant leaves under same conditions. Finally, SDW and reference strains were sprayed on the control plants.

The inoculation of 26 carrot plants with tuberous roots was done by pricking the crown area of the root with a sterile needle after placing a drop of *P. viridiflava* suspension ($10^8$ CFU $ml^{-1}$) to aid the bacterial entry into the tissues. SDW and reference strains were used to inoculate the control plants by the same method. Afterward, inoculated plants were transferred to a humid chamber for 72hrs followed by the transfer to the greenhouse. Daily examinations were conducted for 15 days to observe the symptom development. Carrot slices treated with SDW were used as controls.

Eight-week-old tobacco plants (*Nicotiana tobaccum* var. White Burley) were used to perform HR tests. The bacterial suspension ($10^8$ CFU $ml^{-1}$) or water (control) was injected into the mesophyll cells using a 0.46-mm-diam (26-gauge) hypodermic syringe. Treated leaves were marked with numbers of the strains using a permanent pencil for evaluations. Three leaves of each tobacco plant were inoculated with each strain.

**Polymerase chain reaction (PCR) assays**

All strains were plated in triplicate onto KB and YPGA agar plates and incubated for 48 hr, washed individually three times, each time with 1 ml of SDW. All bacterial strains were streaked on YPGA and KB agar plates in triplicate followed by 48hrs incubation and suspended in 1ml of SDW afterward. The suspensions were centrifuged at 10000g for 5 minutes to obtain bacterial pellets suspended in 800ml of extraction buffer (0.5 M NaCl, 100 mM Tris-HCl pH 8.0, 1% SDS, 50 mM EDTA) followed by a 30min incubation at 65℃. Then 5M potassium acetate (400ml) was added to each reaction and incubated for 20min on ice. The reactions were centrifuged at 10000g for 10min, RNase (40mg/ml) was added at 37℃, followed by phenol/chloroform/isoamyl alcohol (25:24:1) extraction, and finally ethanol precipitation. The pellets obtained were suspended in Tris-EDTA (TE) buffer (Goncalves and Rosato, 2002; Nunes et al., 2008). DNA concentrations were measured using Biophotometer Plus (Eppendorf, Germany). Amplifications were carried out in 0.5 ml PCR tubes with a final volume of 25µl in an Eppendorf thermocycler. Reaction mixtures contained: 2 µl target DNA, 12.5 µl PCR Master Mix (4 mM $MgCl_2$, 0.05 units / µl Taq DNA, 0.4 mM dGTP, 0.4 mM dATP, 0.4 mM dTTP, and 0.4 mM dCTP), 2 µl each of Forward and Reverse primers, 6.5 µl SDW). After amplification, the PCR products were electrophoresed in 1.5% agarose gel in 1×TBE buffer with 1000 bp marker (Fermantas). After staining with ethidium bromide, the gel was visualized using Quantum Gel Visualization System (PrizmaLab, Turkey). (Sambrook et al., 1989: Sambrook and Russell, 2001).

Primers used, 3SF 5' CATTCCAAGAAGCAGCCA3', and 3SR 5' TCGCTCTTAACACCGTCA 3', were specific for *X. h.* pv. *carotae* identification (Meng et al., 2004) amplifies a 350-bp DNA fragment. Reaction conditions applied were: 95 °C for 5 min (initial denaturation, single-step reaction), 94 °C for 15 sec (35 cycles), 58 °C for 15 sec, 72 °C for 30 sec. The final extension time was 5 min at 72 °C and 20 min at 20 °C as a single-step reaction, respectively.

The amplification of *P. viridiflava* was done using specific primers obtained from a 16SrRNA conserved region i.e., PsV-F (5'GTAGGTGGTTTGTTAAGTTGAA'3), and PsV-R

(5'ACCTCAGTGTCAGTATGAGC'3. Reaction conditions were: 95 °C for 4 min (initial denaturation), 95 °C for 1 min (35 cycles), 52 °C for 1 min, 72 °C for 90sec followed by 72 °C for 10 min (final extension) (Alimi et al., 2011). For efficient and reliable results, the amplifications were repeated twice in separate assays.

**Determination of infestation ratios (%) of the pathogens on seed samples**

The percent infestation for each carrot cultivar [PIP (%)] was calculated by commensurating the total infested seed samples (ΣISP) of relevant cultivar with the total collected seed (ΣSP) samples for each individual cultivar using following formula;

$$IC\ (\%) = \frac{\Sigma\ ISP}{\Sigma\ SP} \times 100$$

The percent infestation of all cultivars in same group carrot (orange or black) [TI (%)] was determined by commensurating the total infested seed samples (ΣISR) of relevant all cultivars in the same group carrot (orange or black) with the total collected seed samples (ΣSR) for that region using the following formula;

$$TI\ (\%) = \frac{\Sigma\ ISR}{\Sigma\ SR} \times 100$$

**RESULTS**

Total 60 bacterial isolates were obtained from twenty three seed lots on 5 different orange carrot cultivars, Maestro, Bolero, Sireco, Natuna and Romans, and 11 black carrot genotype of traditional cultivar 'Ereğli' in Konya province. Identification of bacteria was done by biochemical, morphological, physiological (Table 2) and molecular methods. According to our findings, mainly two pathogenic bacteria were identified as *X. h.* pv. *carotae* (16 strains) and *P. viridiflava* (13 strains) on orange and black carrot seeds at different percent infestation ratios (Table 3).

Table 2. Biochemical and physiological characterization tests used for *Pseudomonas viridiflava* and *Xanthomonas hortorum* pv. *carotae* strains isolated from carrot seeds in comparison with reference and negative strains.

| TEST | Reference strains | | | | PV strains obtained from seeds | Reference strains | | | XHC strains obtained from seeds |
|---|---|---|---|---|---|---|---|---|---|
| | PV | PCC | PA | PSS | | XHC | XAP | XTT | |
| Gram reaction | - | - | - | - | - | - | - | - | - |
| Fluorescent pigment on KB | + | - | - | + | + | - | - | - | - |
| Facultative growth | - | + | + | - | - | - | - | - | - |
| Levan production | - | - | - | + | - | + | + | - | + |
| Oxidase | - | - | - | - | - | + | - | - | - |
| Pectolytic activity | + | + | + | - | + | - | - | - | - |
| Arginine dihydrolase | - | - | - | - | - | - | - | - | - |
| Gelatin hydrolysis | + | + | + | + | + | + | - | + | + |
| Esculin hydrolysis | + | + | - | + | + | + | + | + | + |
| Starch hydrolysis | - | - | - | - | - | + | + | - | + |
| $H_2S$ production from cysteine | - | - | - | - | - | + | + | + | + |
| Nitrate reduction | - | + | + | - | - | - | - | - | - |
| Catalase | + | + | + | + | + | + | + | + | + |
| Urease production | - | - | - | + | - | - | - | - | - |
| Acid production from | | | | | | | | | |
| D(-) mannitol | + | + | + | + | + | V | - | + | - |
| inositol | + | - | - | + | + | ND | ND | ND | ND |
| erythritol | + | V | ND | + | + | - | - | | - |
| maltose | - | - | - | + | - | + | - | + | + |
| D(-) arabinose | - | - | - | - | - | - | - | + | - |
| D-sorbitol | + | - | + | + | + | - | - | - | - |
| Sucrose | - | - | + | + | - | + | + | + | + |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Growth at 36 ℃ | - | + | - | - | - | + | + | + | + |
| Growth at 40 ℃ | - | - | - | - | - | - | + | + | - |
| Ice nucleation | - | - | - | + | - | - | - | + | - |
| HR on tobacco | + | + | + | + | + | + | + | + | + |

PV: *Pseudomonas viridiflava*, PCC: *Pectobacterium caratovorum* subsp. *caratovorum*, PA: *Pectobacterium atrosepticum*, PSS: *Pseudomonas syringae* pv. *syringae*, XHC: *Xanthomonas hortorum* pv. *carotae*, XAP: *Xanthomonas axonopodis* pv. *phaseoli*, XTT: *Xanthomonas translucens* pv*. translucens*

(+): 80% or more strains positive; (-): %80 or more strains negative; ND: not determined, V: variable

Table 3. Infestation Ratios (%) of Pathogenic and Saprophytic Bacterial Strains on Seed Samples of Orange and Black Color Carrot Cultivars / Genotypes.

| | | Pathogenic bacteria | | | | Saprophytic bacteria | |
|---|---|---|---|---|---|---|---|
| Cultivar / Genotype | Sample Number | Infested Seed Sample with *X. h.* pv. *carotae* | Infestation Ratio (%) | Infested Seed Sample with *P. viridiflava* | Infestation Ratio (%) | Infested seed Sample with *Pseudomonas* spp. | Infested seed Sample with *Bacillus* spp. |
| Orange carrot | | | | | | | |
| Maestro | 10 | 2 | 20 | 1 | 10 | 9 | 3 |
| Bolero | 4 | 1 | 25 | - | - | 6 | 2 |
| Sireco | 3 | - | - | 1 | 33 | 1 | - |
| Natuna | 3 | 1 | 33 | - | - | 3 | - |
| Romans | 3 | - | - | 1 | 33 | 1 | 1 |
| Total | 23 | 4 | - | 3 | - | 20 | 6 |
| Infestation Ratio (%) | | 17.39 | | 13.04 | | 86.95 | 26.08 |
| Black carrot | | | | | | | |
| Ereğli | 11 | 2 | 18 | 1 | 9 | 3 | 2 |
| Total | 11 | 2 | - | 1 | - | 3 | 2 |
| Infestation Ratio (%) | | 18.18 | | 9.09 | | 27.27 | 18.18 |

Ten samples out of total 34 samples were found to be infested with *X. hortorum* pv. *carotae* and *P. viridiflava*. Population of the bacterial pathogens in infested seed samples ranged from $3.2\times10^5$ to $5.6\times10^8$ CFU/20g seed.

The present study suggested considerable seed sample infestations (%) with *X. hortorum* pv. *carotae* (on orange carrot seeds; 17.39% and on black carrot seeds; 18.18%) and with *P. viridiflava* on orange and black carrot seeds as 13.04 and 9.09%, respectively (Table 3).

Twenty three Pseudomonads and 8 *Bacillus* spp. as saprophytic bacteria were defined in orange and black carrot seeds. Antagonistic effects of these bacteria on *X. hortorum* pv. *carotae* and *P. viridiflava* were investigated on petri dishes, but remarkable results were not obtained.

**Colony morphology of bacteria**

*X. hortorum* pv*. carotae* on mTBM medium was observed after 7 days for colony characteristics. White, yellow, whitish-yellow, smooth, glistening, convex, round 1-2 mm diameter colonies with entire margins surrounded by a casein hydrolysis zone were observed. After 3-4 days of incubation at 28 ℃, typical mucoid and pale yellow growth of *X. hortorum* pv*. carotae* strains was observed on the YDCA medium. Glistening, smooth, round, straw yellow, 2-3 mm in diameter, and convex with entire margined colonies of *X. hortorum* pv*. carotae* were observed on the MD5A medium after 7-8 days. On MKM medium, 2-4 mm in diameter, light brown to peach yellow, light yellow-cream, and round colonies of *X. hortorum* pv*. carotae* were seen after 6 days.

On King's medium B, thirteen *P. viridiflava* strains formed mucoid colonies, and produced fluorescent pigment when observed under UV light. The KOH reaction test confirmed that the strains were Gram-

negative. Levan-type colonies were not observed for test bacterial strains on sucrose nutrient agar. On nutrient agar plus 40 mg/L of cycloheximide plates, convex, circular, and creamy colored colonies were observed after 3-5 days of incubation at 27 ℃.

**Biochemical and physiological characteristics**

Total twenty-nine *X. hortorum* pv. *carotae* and *P. viridiflava* strains isolated from orange and black carrot seeds were identified by biochemical and physiological tests (Table 2).

Sixteen *X. hortorum* pv. *carotae* strains gave negative results from fluorescent pigment production on KB medium, facultative growth and pectolytic activity test. However, they were positive for starch and gelatin hydrolisis, and $H_2S$ production from cysteine. The strains did not produce acid from adonitol, arabinose and sorbitol. Almost all strains gave identical results in these tests and the biochemical profiling to the *X. hortorum* pv. *carotae* reference strain. Obtaining data, only two (Xhcbc14 and Xhcoc3) out of twenty-one *X. h.* pv. *carotae* strains gave different results for gelatin hydrolysis test compare to reference strains (Table 2).

All strains were positive for catalase test and negative for oxidase and arginine dihydrolase activity tests. The strains were able to produce acid from sorbitol but not from sucrose. The strains were positive for pectolytic activity based on the ability to cause soft rot on potato slices. Results of these tests along with biochemical profiling of the test isolates were identical to the reference strain of *P. viridiflava* and were differentiated from negative references (Table 2).

**Pathogenicity and HR reaction of *X. hortorum* pv. *carotae* and *P. viridiflava* strains**

At first, the symptoms of *X. hortorum* pv. *carotae* on inoculated leaves appeared as small irregular yellowish water-soaked lesions with a tiny light brown spot in the center, which, in case of leaf spots enlarged at a later stage and became necrotic surrounded by a yellow halo. Eleven out of sixteen *X. hortorum* pv. *carotae* strains were pathogenic on leaves of cv. Maestro. Re-isolation of bacteria from inoculated plants confirmed the same as the inoculated ones.

Areas observed around the points of inoculation with *P. viridiflava* were light to dark brown. The roots' degradation was seen progressing from crown to the root tip when longitudinal sections were examined. Nine *P. viridiflava* strains out of thirteen were pathogenic on the roots on cv. Maestro. Re-isolations made from the artificially infected plants yielded pure cultures.

In HR tests, all bacterial strains that caused necrosis on tobacco leaves and brown, collapsed areas of tissues were observed at the injection sites after 48 h of incubation at 28 ℃ and 80% RH.

**PCR Assays**

Characterization of *X. hortorum* pv. *carotae* and *P. viridiflava* strains by PCR showed that all *X. hortorum* pv. *carotae* strains amplified with the primers 3SF and 3SR and almost all *P. viridiflava* strains (except str. PvBc6) reacted with the primers PVF/ PVR. *X. hortorum* pv. *carotae* strains gave a product size of 350 bp with the specific primers, 3SF and 3SR. An 860 bp PCR amplified fragment of *P. viridiflava* strains using PVF1/PVR1 primers designed for 16S rRNA sequence.

**DISCUSSION**

Carrot is among the top-ten economically important crops worldwide. According to agricultural statics, Turkey has an important place in the world carrot production (TUIK, 2019). Seventy percent of Turkey's carrots are produced in Konya province and are exported to the Balkans countries and Middle East (Livaneli, 2011).

Bacterial blight of carrot, caused by seedborne *X. hortorum* pv. *carotae* bacterial pathogen (Kendrick, 1934), concerns carrot growers in many regions of the world (Gilbertson, 2002; Cubeta and Kuan, 1986; Watson, 1948; Saad and Wade, 1972; Nishiyama *et al.*, 1979; Meng *et al.*, 2004). The pathogen can infect the seed internally or contaminate the seed surface, affecting the yield (Ark and Gardner, 1944; Umesh *et al.*, 1998). Reduction of seed germination due to the infection by *X. hortorum* pv. *carotae* can result in significant losses to the farmers (Crowe and Bafus, 2004). Unfortunately, there is insufficient knowledge about the epidemiology of *X. hortorum* pv. *carotae* about carrot seeds. It is thought that the surface infestations probably take place during seed development in umbel and harvesting operations. True infection of inner seed tissues would start during early seed development, perhaps during the living connection of seed with mother plant or via pollen or nectar (Bashan and Okon, 1986). Since the Central Anatolia region has hot and dry conditions, bacterial

diseases are seen at very low rates, and therefore, the symptoms of the disease are often not noticed in field conditions which are mistaken for the absence of the pathogen. In this regard, it should be emphasized that the disease can reach serious dimensions, especially in high irrigation conditions, humid and cool regions, and regions with different climatic conditions.

Melon and tomato crops foliage can be reduced up to 50% as a result of infection by *P. viridiflava* that causes extensive tissue necrosis (Goumans and Chatzaki, 1998). The opportunistic behavior of *P. viridiflava* allows it to survive on surfaces of its host or other weeds that can act as an inoculum source. *P. viridiflava* was responsible for severe economic losses to refrigerated carrot export from New Zealand by causing soft rot (Godfrey and Marshall, 2002). In Brazil, symptoms of *P. viridiflava* during post-harvest storage on potatoes were described by Macagnan *et al.* (2007) and observed the infection symptoms on chayote, sweet potato, yam, and pumpkin when inoculated the pathogen artificially. Association of *P. viridiflava* with carrot seeds may explain that post-harvest losses can originate from contaminated seeds (Almeida *et al.*, 2013).

Demir and Ustun (2001) tested a total of two thousand-four hundred-sixty-nine plant propagative units for the presence of some plant pathogenic bacteria. They detected *Pseudomonas viridiflava* in 3 samples (cauliflower and cabbage seeds), *X. hortorum* pv. *carotae* in one carrot seed sample in Aegean Region. Kurt *et al.* (2004) obtained *Erwinia* spp. and fluorescent and nonfluorescent plant pathogenic *Pseudomonas* spp. strains from diseased carrot roots, in the Eastern Mediterranean region.

In present study, to the best of our knowledge, this is the first report of *P. viridiflava* associated with carrot seeds in Turkey. As like *X. h.* pv. *carotae*, a high level of infestation was determined on orange and black carrot seeds by *P. viridiflava* as 9.09% and 13.04%, respectively. Lack of such studies increases the significance of the present work in Central Anatolia.

The production of commercial carrot crops depends greatly on planting seeds with zero or low contamination of *X. hortorum* pv. *carotae*. In this study, high infestation ratios of *X. hortorum* pv. *carotae* on orange and black carrot seed samples were determined, between 17.39% and 18.18% respectively (Table3). In that region, most cultivated orange carrot cultivar is Maestro, and according to findings, this cultivar was infested with both *X. hortorum* pv. *carotae* and *P. viridiflava*. In order to control these diseases, necessary measures should be considered timely.

Twenty three orange and black carrot seed samples were infested with saprophytic bacteria, Pseudomonads and *Bacillus* spp. They did not show any antagonistic effect on pathogenic bacteria under *in vitro* conditions but they can cause decay on seeds at non-suitable storage conditions because of their high infestation (%) in the seeds (18.18% - 86.95%) respectively (Table 3).

The standard procedure for detection currently in use includes seed-wash dilution plating assay, together with pathogenicity tests of representative *X. hortorum* pv. *carotae* colonies. Although effective in general, this procedure is laborious and can take as long as one month to complete (Meng *et al.*, 2004). In the present study, King's B, Nutrient Agar, MD5A, MKM, mTBM, and YDCA media were used for isolation and characterization of bacterial colony morphology. The strains were identified by biochemical, physiological pathological and HR tests for all *X. hortorum* pv. *carotae, P. viridiflava* and saprophytic bacteria.

Thus, development of more rapid PCR-based methods for detection and identification of seed contaminated by the pathogens should improve the capacity to detect and manage carrot bacterial diseases. PCR with the 3S primer pair could greatly reduce the time and facilities required for routine detection and identification of *X. hortorum* pv. *carotae* and could be used in ecological and epidemiological studies (Meng et al., 2004). In this study, the 3S primer pair were highly specific, and the about 350-bp target fragment amplified from all strains. Likewise PVF/PVR primers set confirmed *P. viridiflava* strains. The observations of present study suggested that PCR experiments were same to those reported by Goss *et al.* (2005). The PCR-based assays have the potential to improve routine detection of important carrot pathogens.

Epiphytic growth of *X. hortorum* pv. *carotae* is promoted under semi-arid areas where carrot seeds are produced (du Toit *et al.*, 2005). The bacterial association with the seeds acts as an inoculum to cause bacterial blight when carrots are grown in humid and warm areas. Carrot seed produced in Turkey harbors *X. hortorum* pv. *carotae* so there is a dire need to identify the primary inoculum source to avoid future infections when grown under semi-arid regions of Turkey. Identifying these sources of infection and the primary periods of infection through

the biennial season will assist in the development of more efficacious, regional integrated pest management programs for carrot seed crops.

Black carrots are a source for natural food coloring from Turkey and other regions of the Middle East and Asia. In Turkey, black colour carrot is produced in only Ereğli district of Konya Province and the growers use the genotypes of cv. Ereğli as seed. This situation contains two important problems; first, genetic expansions and susceptibilities to plant diseases as the same seeds are being used for many years. Second, transmission of fungal, bacterial and viral pathogens, both plant and human pathogens, from year to year and field to field. Further studies will be conducted to determine pathogens related to plant and human health, carrot yield, and orange carrots.

Plantation of healthy seed or treated seed can be an important strategy to manage bacterial blight (Umesh *et al.*, 1998; Gilbertson, 2002; Meng *et al.*, 2004). Other management recommendations include 2 to 3 year crop rotations, applications of copper bactericides, avoiding overhead irrigation that promotes dispersal of the bacterium and creates favorable conditions for infection and incorporating residues into the soil promptly after harvest (Parks and Crowe, 1999; Gilbertson, 2002; Weber *et al.*, 2004). Seed can be disinfected by hot water treatment (52 °C for up to 25 min) (Ark and Gardner, 1944; Gilbertson, 2002; Pscheidt and Ocamb, 2001). Limited resistance to bacterial leaf blight is available in some commercial cultivars. There is no study to determine susceptible or resistant carrot cultivars to fungal, bacterial or viral diseases in Turkey. This will become very important for future.

In subsequent experiments, authors plan to focus on the use of resistant cultivars for carrot bacterial diseases. Cultivar resistance is the most desirable for its cost-effectiveness and long-term stability. The development of resistant cultivars requires advanced planning in breeding programs to incorporate and maintain a diverse range of resistance genes in parental lines, but little is known about carrot resistance to bacterial diseases. Therefore, further studies should be conducted primarily between carrot varieties and *X. hortorum* pv. *carotae* and *P. viridiflava* interactions.

## CONCLUSION

Bacterial pathogens of carrot cause important damages on plant yield and seed quality. This study was conducted for detection and identification of seed borne bacterial pathogens on orange and black carrot seeds sown in Ereğli and Kaşınhanı districts of Konya province where the highest carrot production is obtained. Based on morphological, physiological, biochemical, pathological and molecular tests, the pathogenic agents were identified as *Xanthomonas hortorum* pv. *carotae* and *Pseudomonas viridiflava* with high percent infestation ratios. This to our knowledge is the first report of the occurrence of *P. viridiflava* on carrot seeds in Turkey. The information will be useful about using carrot seeds and required precautions to growers and authorities to get more healthy products and seeds.

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**CONFLICT OF INTEREST**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

**AUTHORS CONTRIBUTIONS**

Kubilay Kurtulus Bastas: Funding acquisition, Resources, Project administration, Writing – review & editing; Haris Butt: Data curation, Formal analysis, Writing – review & editing; Aysegul Gur: Data curation, Formal analysis.