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Prevalence and characterization of *Burkholderia gladioli* in Iran, from bacterial dry rot of saffron corms (*Crocus sativus* L.)

Mahmoud-Reza KARIMI-SHAHRI[1], Mohammad ZAKIAGHL[2]

[1] *Department of Plant Protection, Razavi-Khorasan Agricultural and Natural Resources Research Center, (AREEO), Mashhad, Iran*
[2] *Department of Plant Protection, College of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran*
The authors contributed equally to this research.
Corresponding authors. E-mail: zakiaghl@um.ac.ir; karimi_in@yahoo.com

Summary. Iran is the main world producer of saffron (*Crocus sativus* L.), but a bacterial disease continues to threaten saffron production, causing severe flower failure, rot on flowering tubes, delayed vegetative growth, premature yellowing of leaves, bare patches in saffron farms, reddish-brown lesions in the germination zones of roots, and rot of saffron corms. Field surveys in Razavi-Khorasan and Southern-Khorasan provinces revealed high incidence of Burkholderia gladioli dry rot symptoms in saffron farms, with symptoms observed during flowering on leaves and corms. Twenty-four bacterial isolates from symptomatic saffron corms from different parts of Iran were characterized. These bacteria were identified as *Burkholderia gladioli*, based using phenotypic characteristics, species-specific PCR, and sequencing analyses of the 16S rRNA and 16S-23S intergenic transcribed spacer regions. All 24 isolates triggered hypersensitive reactions in tobacco and pelargonium leaves, although pathogenicity tests showed that only 21 isolates were capable of causing rots on saffron corms.

Keywords. Saffron dry rot, pathogenicity, phenotypic tests, molecular identification.

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ORCID:
M-RK-S: 0000-0003-3183-457X
MZ: 0000-0001-5032-8344

INTRODUCTION

Burkholderia (Yabuuchi *et al.*, 1992) comprises aerobic, Gram-negative and rod-shaped bacteria, and includes more than 60 species, some of which are plant growth-promoting, endophytic and antifungal biocontrol agents (Compant *et al.,* 2008). Other species are important pathogens for humans, animals and plants (Coenye and Vandamme, 2003; Suárez-Moreno *et al.,* 2012). Important plant pathogens include *B. andropogonis* (bacterial leaf stripe of sorghum and maize; Li and De Boer 2005), *B. caryophylli* (bacterial wilt of carnation; EPPO, 2006), *B. gladioli* (bacterial blight of gladiolus; McCulloch, 1921), *B. glumae* (bacterial panicle blight of rice; Nandakumar *et al.,* 2009), and *B. plantarii* (seedling blight of rice; Wang *et al.,* 2016). Although *B. gladioli* was originally described as the causal agent of gladiolus

blight, this species has been found to be pathogenic on other plants, and four pathovars of *B. gladioli* have been identified based on their host ranges. These pathovars are: *B. gladioli* pv. *alliicola* (Bga) (formerly *Pseudomonas alliicola*), causing onion rot; *B. gladioli* pv. *gladioli* (Bgg) (formerly *Pseudomonas marginata*), responsible for leaf and corm rot of gladiolus and iris, and also affecting other plants (Saddler, 1994); *B. gladioli* pv. *agaricicola*, causing soft rot of mushrooms (*Agaricus bitorquis*) (Lincoln *et al*., 1991; Yabuuchi *et al.,* 1992; Ura *et al.,* 2006; Nandakumar *et al.,* 2009; Kowalska *et al.,* 2015; Moon *et al.,* 2017); and *B. gladioli* pv. *cocovenenans* (formerly *Pseudomonas cocovenenans*), which causes rot of coconut rot and producing the human toxin bongkrekic acid (Jiao *et al.,* 2003).

Burkholderia gladioli pv. *gladioli* was first identified in China (Xu and Ge, 1990), and later in Sardinia (Italy) (Fiori *et al.,* 2011), as a destructive pathogen of saffron. In addition, the bacterium has been considered a quarantine pathogen that causes soft rot during growth and storage of many vegetables, leading to significant economic losses in China (Lee *et al.,* 2012; 2021). This pathogen has been detected in onion growing areas in the United States, Bulgaria, Korea, and elsewhere (Lee *et al.,* 2005).

Several methods have been developed for detection and identification of *Burkholderia* species, including culture in a semi-selective medium (Castro-González *et al.,* 2011), pathogenicity assays (Nandakumar *et al.,* 2009), multiplex PCR (Maeda *et al.,* 2006), and real-time PCR (Thibault *et al.,* 2004). Sequence analysis of the 16S ribosomal RNA gene (rRNA) is a powerful tool for understanding phylogenetic and evolutionary relationships in bacteria (Woo *et al.,* 2003). Nevertheless, *Burkholderia* species show a high degree of similarity in their 16S rRNA gene sequences (Vermis *et al.,* 2002; Chiarini *et al.,* 2006), so the 23S rRNA or the internal transcribed spacer (ITS) region of the 16-23S rRNA has been used in addition to the 16S rRNA, for improved species (Liguori *et al.,* 2011).

Saffron (*Crocus sativus* L., *Iridaceae*) is a reliable industrial and medicinal plant. Propagation of saffron is vegetative from corms, as the plant is a sterile triploid (3n = 24) that does not produce seeds (Koocheki and Khajeh-Hosseini, 2020). Iran is the world leader in saffron production, growing approx. 408 tons of saffron from 112,000 ha in 2021. Approximately 60% of cultivated saffron area is in the three provinces of Khorasan (UNIDO, 2022). However, due to the lack of packaging, marketing and production of saffron-based edible products, a significant portion of Iranian saffron is distributed by other countries. In recent years, saffron production in Iran has faced challenges. In many farms, saffron bloom, a measure of potential yield, is not reached. In addition, in some farms vegetative growth of saffron is delayed, leading to formation of small daughter corms that do not flower in the following season. Saffron corms may also fail to germinate, and bare patches occur in affected fields.

The present paper describes isolation, identification, and prevalence of the bacterial pathogen *B. gladioli* from diseased saffron corms in Iran. This research used pathogenicity assessments, and physiological, biochemical, and molecular characteristics to characterize this pathgen.

MATERIALS AND METHODS

Field survey and sample collection

Saffron fields in Razavi-Khorasan and South-Khorasan provinces of Iran were surveyed for Burkholderia gladioli dry rot (BGR) in saffron corms from 2016 to 2022. Field symptoms on affected plants were early leaf yellowing followed by drying in the autumn and winter seasons. Corm samples were collected from October (before saffron blooming) until June. A total of 455 corm samples were collected from 108 saffron fields in different regions (Table 1). The samples were kept at 4°C until analyses.

Isolation and purification of bacteria

Isolations were carried out as reported by Fiori *et al.* (2011). Corms were washed in tap water and then disinfected by immersion in 0.5% sodium hypochlorite for 5 min. Diseased scale tissues were then cut into 10-15 mm cubes from the edges of symptomatic areas using a sterilized scalpel. Fragments of these tissues were then disinfected in ethanol for 30 s, and washed several times in sterile water. Each sample was then ground in a sterile mortar with Tris-HCl buffer (50 mM, pH 7.0). The resulting suspensions were streaked onto Petri plates containing nutrient agar (NA). The plates were then incubated at 28°C for 48 h. Resulting colonies were subcultured twice onto NA, and then stored at 4°C in sterile 0.1 M $MgSO_4$ for short-term use. The isolates were also maintained at -80°C in nutrient broth medium containing 50 % (v/v) glycerol for long-term storage.

Pathogenicity assays

The isolates were grown in Luria Bertani (LB) broth at 28°C until OD_{600} = 2. Resulting cells were pelleted by

Table 1. Farm locations in Iran from which saffron samples were harvested, including numbers of farms sampled, incidence of Burkholderia gladioli dry rot, and numbers of *Burkholderia gladioli* isolates obtained.

District	Years of sampling	Location	Number of farms	Number of infected samples	Number of isolates
Razavi-Khorasan Province					
Sabzevar	2018-2019	35°56'N/57°30'E	22	0	0
Torbat-e Heydariyeh	2016-2017	35°25'N/ 59°09'E	55	23	13
Zaveh	2016-2019	35°15'N/ 59°43'E	168	62	42
Bajestan	2019-2020	34°34'N/58°12'E	15	15	15
Rashtkhar/ Khaf	2018-2019	34°26'N/60°09'E	3	3	3
Mahvelat	2018-2019	35°02'N/58°40'E	24	0	0
Torbat-e-Jam	2018-2019	35°20'N/60°37'E	10	10	2
Chenaran	2019-2020	36°43'N/ 59°00'E	9	9	9
Gonabad	2019-2020	34°26'N/58°52'E	15	15	15
Fariman	2018-2019	35°43'N/60°01'E	3	3	3
Neyshabur	2020-2021	36°25'N/58°37'E	5	5	5
Quchan	2021-2022	37°09'N/58°35'E	8	8	8
Average of incidence in Razavi-Khorasan Province				45.4	
South-Khorasan Province					
Boshruyeh	2018-2020	34°06'N/57°23'E	19	15	5
Tabas	2018-2019	33°26'N/56°48'E	4	0	0
Ferdows	2018-2019	33°51'N/58°01'E	7	0	0
Sarayan	2020-2021	33°28'N/58°19'E	13	5	5
Qaen	2018-2019	33°39'N/59°15'E	23	4	0
Zirkuh	2018-2019	33°32'N/60°10'E	8	3	0
Birjand	2018-2019	33°05'N/59°10'E	11	0	0
Sarbisheh	2018-2019	32°29'N/60°03'E	18	2	0
Khosf	2018-2019	32°19'N/58°38'E	13	8	0
Nehbandan	2018-2019	31°26'N/59°43'E	2	2	0
Average of incidence in South-Khorasan province				33.1	
Overall average of incidence				42.2	

centrifugation at 7,000 rpm for 2 min, and then resuspended in sterile distilled water.

Hypersensivity reaction (HR) tests of selected isolates were conducted using tobacco (*Nicotiana tabacum* 'Samsun') and pelargoniums leaves. A bacterial suspension of each isolate was prepared in 1×PBS buffer using a 24 h culture in LB broth, and was adjusted to 5×10^8 cfu mL^{-1}. The bacterial suspension was then injected into the intercellular spaces of leaves, and positive pathogenicity was recorded where complete collapse of the tissues occurred after 24 h. The test was repeated at least twice with each isolate. Healthy corms of saffron were peeled, washed with running water, disinfected by dipping in 0.5% sodium hypochlorite for 2 min, disinfected in 70% ethanol for 30 s and then washed with sterile water. For each isolate, four corms were inoculated at 1 cm depth on two opposite sides of each corm with a 20 µL aliquot of the bacterial suspension, using a syringe. Control corms were mock inoculated with sterile distilled water. Each isolate was also inoculated into onion and carrot disks, by adding a drop of the bacterial suspension onto injured surfaces. Controls were mock inoculated with sterile distilled water. The inoculated material was maintained in a high humidity chamber at 28°C for symptom development. Re-isolations were made from diseased material, as described above. Some saffron corms were also wounded with a laboratory needle to make a 5 mm long scratch on each corm, and these were each inoculated with 20 µL of suspension (5×108 cfu mL^{-1}) from a 24 h culture, and were then planted into pots containing a sterile commercial soil, and these were maintained in a greenhouse at 28°C for 7 d. Plants and corms were checked regularly for symptom development. Re-isolations were made from the inoculated corms, and from shoots and leaves that developed from the inoculated corms.

Phenotypic tests of bacterial isolates

Physiological and biochemical characteristics of the bacterial isolates were determined, as described by Holt (1994). Culture tubes each containing saline solution, were each inoculated with 150 µL of bacterial suspension (5×10^8 cfu mL^{-1}). The tubes were then incubated at 28°C for 24 h. Twenty-four selected bacterial isolates were characterized by Gram staining, colony morphology, catalase and oxidase production, oxidative/fermentative metabolism of glucose, starch and gelatin hydrolysis, and H_2S production. Utilization of glucose, lactose, maltose, galactose, arabinose, raffinose, dextrose, sucrose, citrate, adonitol, mannitol, sorbitol, and urease and arginine decarboxylase activity, were also assessed. Abilities of the isolates to grow at pHs of 4 to 9, in 3% NaCl 3%, and at 41°C were also assayed (Table 2). The phenotypic data were analyzed by cluster analyses using Pearson correlation coefficient similarity indices (http://genomes.urv.cat/UPGMA/).

Molecular characterization of bacteria

For PCR identifications, total DNA was extracted using the DNA extraction kit (DNP™) (Sinaclon), according to the manufacturer's instructions for Gram-negative bacteria. PCRs were carried out using universal primers 16S-27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 16S-1492R (5'-TACGGYTACCTTGTTACGACTT-3'); CMG16-1 (5'-AGAGTTTGATCMTGGCTCAG-3') and CMG16-2 (5'-CGAAGGATATTAGCCCTC-3'); GLA-f (5'-CGAGCTAATACCGCGAAA-3') and GLA-r (5'-AGACTCGAGTCAACTGA-3'); and LP1 (5'-GGGGGGTCCATTGCG-3') and LP4 (5'-AGAAGCTCGCGCCACG-3') designed on 16S and 23S rRNA sequences (Whitby *et al.,* 2000; Furuya *et al.,* 2002; Fiori *et al.,* 2011; Stoyanova *et al.,* 2011b; Li *et al.,* 2019). A 869-bp fragment of the *Burkholderia recA* gene was also amplified using a specific primer pair BUR1 (5'-GATCGA(AG)AAGCAGTTCGGCAA-3') and BUR2 (5'-TTGTCCTTGCCCTG(AG)CCGAT-3') (Payne *et al.,* 2005). Each reaction contained 10 µL of Amplicon 2× ready to use PCR master mix, 1 µM of each primer, 1 µL of genomic DNA in a total volume of 20 µL. The PCR program consisted of an initial denaturation of 97°C for 7 min followed by 30 cycles of denaturation each at 95°C for 30 s, annealing at 57°C for 60 s, and extension at 72°C for 80 s. The PCR products were subjected to electrophoresis in a 1% agarose gel containing DNA Safe Stain (Sinaclon) in TBE buffer (pH 8.0), and photographed. The PCR products were cloned into the pGT19 vector (Vivantis), according to the manufacturer's recommendations, and were Sanger sequenced by Pishgam Co., Iran. Phylogenetic trees were constructed using the neighbor-joining method, with the General Time Reversible nucleotides substitution model with 500 bootstrap replicates, using MegaX software (Tamura *et al.,* 2011).

RESULTS

Prevalence of BGR in saffron farms of Iran

Of the 455 samples taken, 192 showed BGR symptoms. BGR incidence in Razavi-Khorasan was 45.4% (153 of 337 samples), and the greatest incidence was observed in Zaveh and Torbat-e-Heydarieh districts. In South Khorasan province, incidence was 33.1% (39 of 118 samples), and the greatest incidence was observed in Beshrouye (79% of samples infected. In some farms, no Bgg was detected (Table 1).

A total of 125 isolates were obtained from the 192 saffron corms. From these 125 isolates, 21 isolates from saffron farms in Razavi-Khorasan, which had the greatest BGR incidence, were selected for subsequent for phenotypic and pathogenicity tests, based on the type and severity of host plant symptoms and colony morphology. In addition, two isolates from diseased saffron corms from Kermanshah, one isolate from a diseased saffron corm from Afghanistan, and a Bgg isolate from an onion, were used for comparisons.

Symptoms of BGR in the field

Symptoms of BGR on saffron plants could be categorized into three distinct types.

1) Symptoms during flowering. Symptoms during the flowering period in November were absence of flowering or decreased numbers of flowers, and decreased quantity and quality of flower stigmas. BGR caused rot and collapse in underground shoots. Infested saffron plants had decayed sheaths, while newly formed sprouts exhibited tissue burning and browning. These shoots were incapable of emergence from soil, saffron shoot rot led to reduction in flower production and appearance of bare patches in affected areas on the affected farms (Figure 1).
2) Symptoms on leaves. These symptoms became evident after the leaves had fully grown. Affected leaves showed symptoms of yellowing and necrosis, and corms were shorter than those of the healthy corms. BGR manifested as irregular bare patches in saffron farms, and patches were observed throughout different sections of individual farms. BGR triggered premature yellowing of saffron leaves. During midwin-

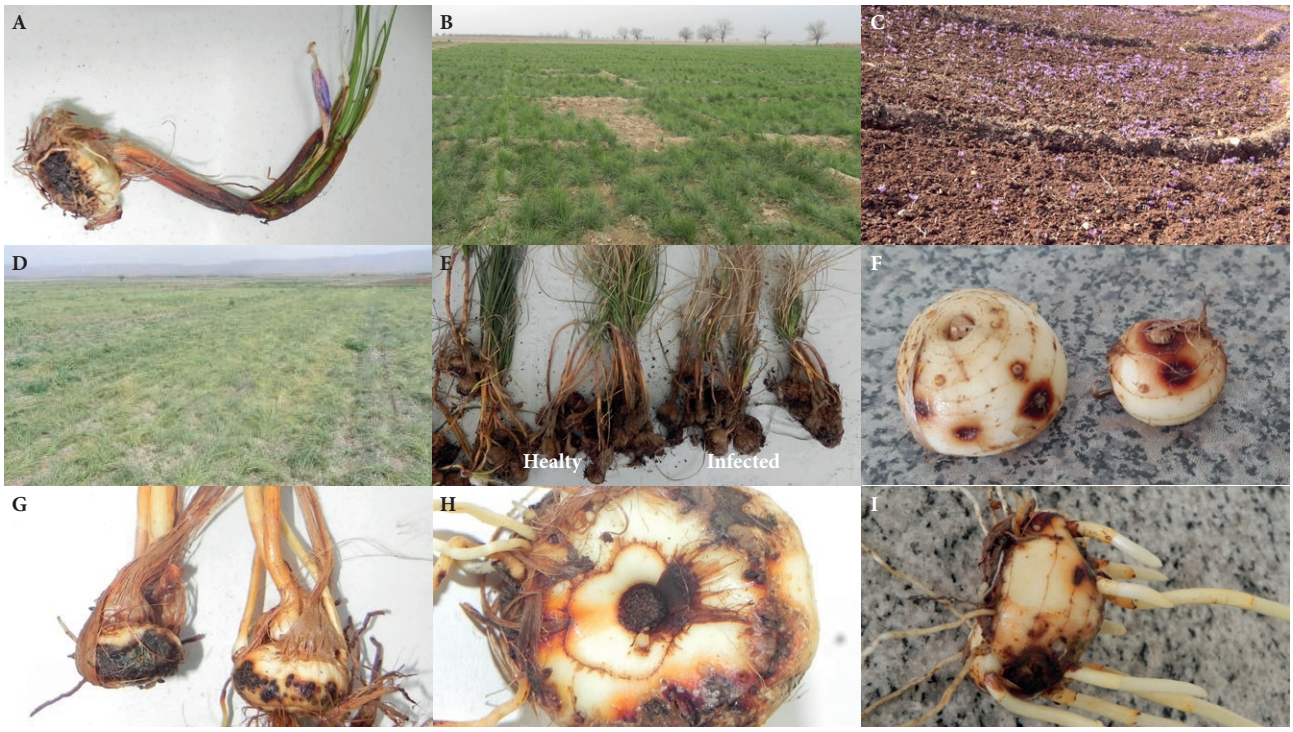

Figure 1. Symptoms of BGR in naturally infected saffron plants and corms. (A) burning and browning in newly formed sprout tissues; (B) bare patches in saffron farms; (C) flowering failure; (D) premature yellowing of saffron leaves; (E) yellowing and necrosis at the ends of leaves; (F) ring-shaped red to brown lesions on saffron corms; (G) progressive spread of lesions to larger areas of necrosis; (H) ring-shaped red to brown lesions within the root germination zone in o saffron corm; (I) a large number of weak flowering tubes from the lateral buds of a corm with emerging leaves.

ter, most infected saffron leaves turned yellow and fell (Figure 1).

3) Symptoms on corms. These symptoms were predominantly manifested on the mother corms, and were characterized by a distinctive ring-shaped red-brown discolouration precisely within the root germination zones. These zones progressively decayed and extended into the deeper layers of each corm. BGR was initially manifested as burnt spots, and gradually extended to cover an increasing area. Affected tissue surfaces acquired glistening appearance, occasionally covered by a thin gray layer. Lateral buds of infected corms began to grow and produced large numbers of weak cataphylls that exhibited abnormal growth, and had necrosis and red discolouration, flowering failure and also produced very small daughter corms (Figure 1).

Pathogenicity tests

The results of the pathogenicity tests with BGR-associated bacteria are shown in Figure 2. A necrotic zone or rot lesion at least 2 mm beyond the inoculation site was considered as evidence of infection in all the pathogenicity tests. All 24 selected isolates induced HR after inoculations of tobacco and pelargonium leaves. Inoculation of saffron corms with BGR isolates resulted in development of lesions with black necrosis around the inoculation site. Water-soaked lesions appeared 4 to 8 d post inoculation (dpi), and these lesions rapidly enlarged and turned dark-brown to black lesions within 7 to 10 dpi. Negative controls displayed small wounding sites without further development in 10 d. Symptoms were similar to those observed in the greenhouse, as shown in Figure 2.

Most of the isolates were infectious on saffron corms, except isolates 110, 126 and 160. Isolate 126 was obtained from onion, and isolates 110 and 160 were obtained from saffron corms with BGR symptoms. The most severe corm symptoms were caused by isolates 169 and 255. Symptoms included necrosis and rotting, which could be superficial or deep. Based on symptom severity, a 0 to 5 rating scale was established, in which isolates inducing complete rot and tissue decay were scored as 5, and non-pathogenic isolates scored 0. The isolates were assigned into six groups (Figure 3).

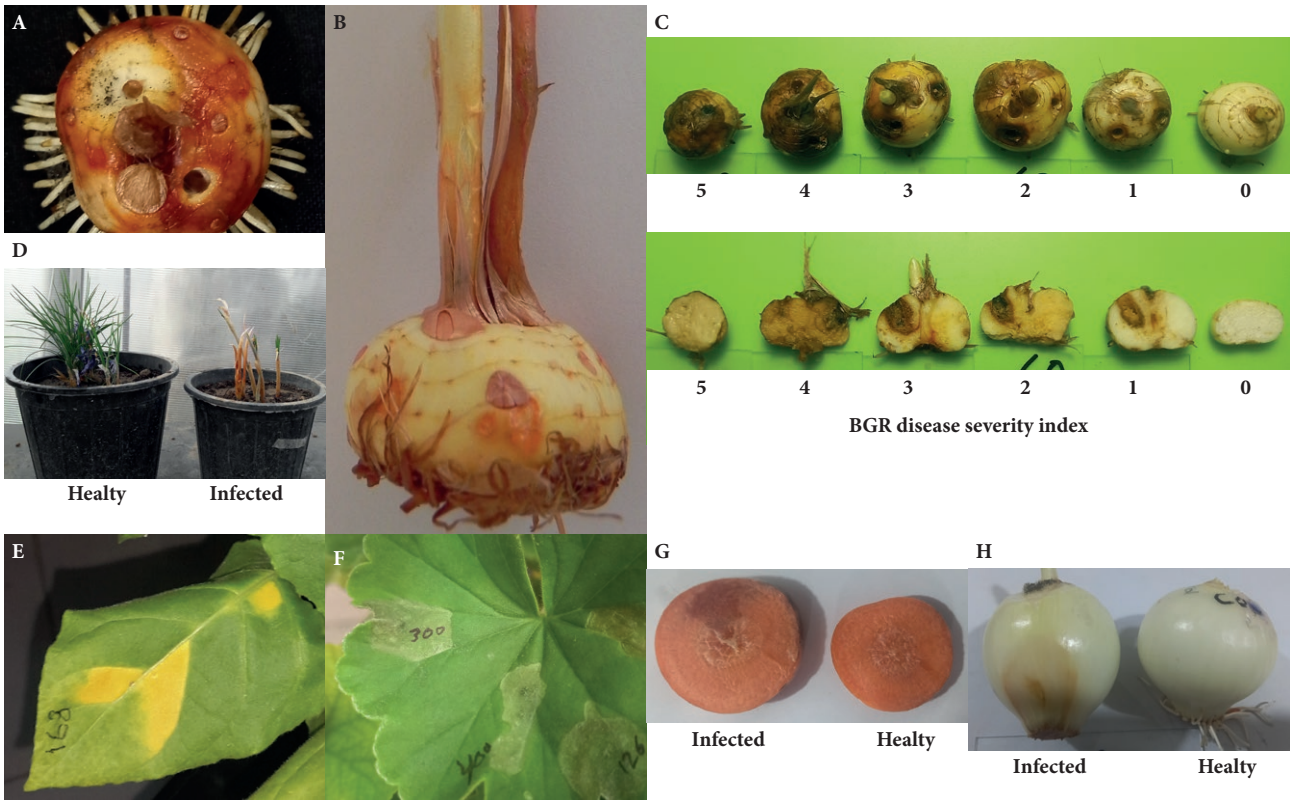

Figure 2. Results of pathogenicity tests of *B. gladioli* isolates on different plants. (A) Severe red-brown discolouration on an inoculated saffron corm; (B) rotted, twisted and truncated flowering tubes turned orange in inoculated corms; (C) disease severity rating scales for BGR on entire (upper) and cross sections (lower) of corms; (D) yellowing, tip necrosis and growth delay of leaves from inoculated corms; (E) HR in a tobacco leaf; (F) HR response in a pelargonium leaf; (G) watery rot on carrot discs; (H) severe watery decay on onions.

Cataphylls of inoculated saffron corms turned orange, and became twisted and truncated, and the flowers did not emerge from the tubes. The leaves of the inoculated corms showed yellowing and tip necroses (Figure 2). The pathogen was re-isolated from inoculated and symptomatic corms, their 16s RNA fragments were amplified with PCR, and the amplified fragments were identical to those of the inoculated bacterial strains, thus fulfilling Koch's postulates.

With the exception of isolates 110, 148, 149, 150 and 160, the remaining isolates caused watery rot on carrot discs, and in onions, and with the exception of isolates 149, 150, 160, 168, 169, 250, 251, 262, 300 and 400, the remaining isolates caused watery rot (Figure 2).

Based on the pathogenicity test data for saffron, the BGR-associated isolates were categorized into six groups (correlation coefficient 94%) (Figure 3).

Physiological and biochemical tests of BGR-associated isolates

The results of the most relevant phenotypic tests are listed in Table 2. Most of the assessed islotes were oxidase and catalase positive and hydrolyzed gelatin, and most were positive for arginine decarboxylase activity, and grew in 3% NaCl. Some of the isolates grew at 41°C, but none produced indole or acidified glucose. There were differences among the isolates for their profiles of utilization of carbohydrate sources, and urease activity. Results of the morphological, physiological and biochemical characteristics showed that all 24 BGR-associated isolates were identified as *B. gladioli* (with 82 to 98% probability). However, some differences were observed, and according to the phenotype tests, the isolates clustered into four groups. Nine isolates (102, 155, 161, 169, 252, 255, 262, SA4 and SA14), nine isolates (106, 110, 126, 147, 148, 151, 160, 168 and 300), five isolates (149, 150, 250, 251 and 400) and one isolate (176) were categorized into four phenotypic groups of BGR-associated bacteria (Figure 3, Table 2).

Molecular identifications

Molecular identification of the BGR-associated isolates was achieved by PCR amplification of the rRNA and *recA* gene. Expected fragments of 1500, 470 and 300 bp belonging to the 16S rRNA and 16S-23S rRNA

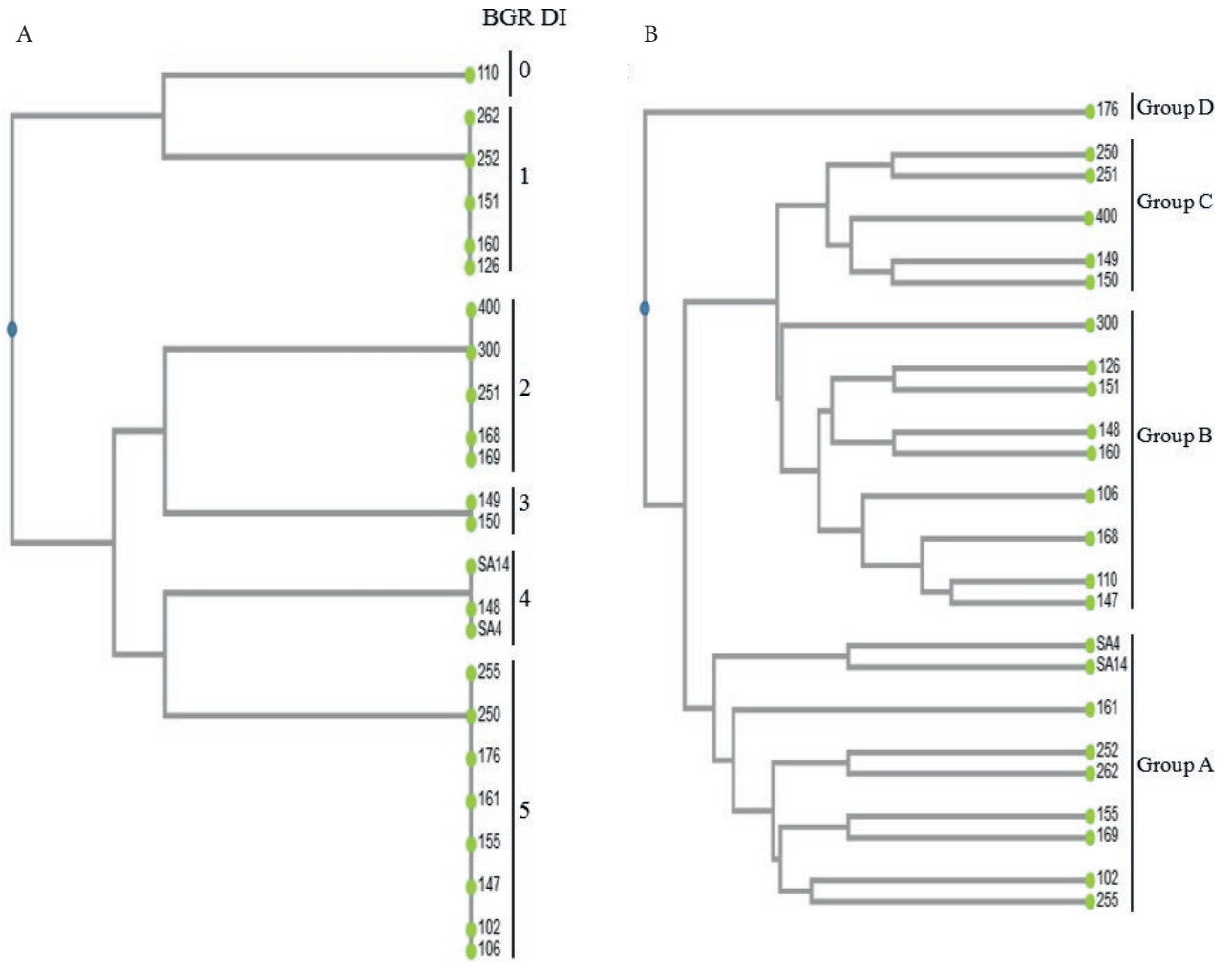

Figure 3. Representative dendrograms of clustering of 24 BGR-associated isolates, based on disease severity scale on saffron corms (A); and diversity in physiological and biochemical characteristics (B). DI = disease index.

ITS region were amplified from all isolates, using the primer pairs 27F/1492R (1500 bp), CMG16-1/CMG16-2 (470 bp) and GLA-f/GLA-r (300 bp). An approx.i700 bp fragment of the 23S rRNA was amplified from most of the isolates using the LP1/LP4 primer pair, but amplification was unsuccessful from isolates 14 of 24. In most isolates (18 of 24), the expected *Burkholderia recA*-related fragment of 869 bp was amplified with the BUR1/BUR2 primer pair. A 470 bp fragment of the 16S rRNA from two isolates (110 from phenotype group A, and 155 from group B) was cloned and sequenced. The sequences were deposited in GenBank with accession numbers PQ120996 and PQ120997. These sequenced fragments of the BGR-associated isolates showed 97 to 99.5% similarity with other *Burkholderia gladioli* isolates deposited in GenBank, and were put in *B. gladioli* group in the phylogentic tree (Figure 4 A). In a phylogenetic tree, *B. gladioli* pv. *gladioli* isolates from saffron isolates from Italy, India and Iran clustered in a separate clade from other *B. gladioli* pathovars (Figure 4 B). *Burkholderia gladioli* isolates from saffron had, in the 16S rRNA gene, 99.5 to 99.9% similarity to *B. gladioli* pv. *gladioli* 99.5 to 100% similarity to *B. gladioli* pv. *alliicola*, and 99.1 to 99.7% similarity to *B. gladioli* pv. *agaricicola*. The 16S rRNA fragments from two isolates of *B. gladioli* had 97.3 to 98.5% similarity to homologous sequences from *B. cepacia* and showed 92.5 to 96.4% similarity to those of other *Burkholderia* species.

DISCUSSION

In this study, a polyphasic approach was used, by combining pathogenicity, biochemical and molecular

Table 2. Main characteristics of bacterial isolates from saffron.

Characteristic	Group A	Group B	Group C	Group D	*B. gladioli* pv. *gladioli* CFBP2427[a] (Fiori *et al.,* 2011)
Gram reaction	-	-	-	-	-
Colony colour	Yellowish/ orange-milky	Milky	Milky	Milky	
Oxidase	+/-	+/-	+	+	+
Catalase	+	+/-	+	-	+
Arginine decarboxylase	-/+	+	+	-	
Hydrolysis of: gelatin	+	+	+	-	+
Hydrolysis of starch	+/-	+/-	+	+	-
O-F Glucose test	+/-	+/-	+/-	+/-	+/-
Growth at: pH 4	+	+	+	+	+
pH 8	+	+	+	+	+
pH 9	+	+	+	+	-
Growth in 3% NaCl	+	+	+	-	+
Growth at 41° C	-/+	-/+	-/+	-	-/+
Utilization of					
Raphinose	+/-	-/+	+/-	+	
Arabinose	-/+	-/+	+	+	
Adonitol	+	-	-	-	+
Glucose	$+^{w}$	$+^{w}$	$+^{w}$	$+^{w}$	+
Dextrose	+	-/+	+	-	
Maltose	++	+/-	+	++	-
Sucrose	-	-	-	-	
Lactose	+	-	-	+	
Galactose	-/+	-/+	-	+	
Urease	+	++	+	+	

[a] CFBP 2427 is the pathotype strain of *Burkholderia gladioli* pv. *gladioli*
+ = positive; - = negative; -/+ = mostly negative, but positive in some isolates; +/- = mostly positive, but negative in some isolates; ++ = strongly positive; $+^{w:}$ weakly positive.

test to identify and characterize a collection of bacterial isolates causing dry rot of saffron corms in Iran. The main symptom of the disease was reddish-brown lesions on saffron corms, which spread to the inner corm tissues. In many cases, the corms were completely rotted. The colonies of isolated bacteria were milky-yellowish and round in 2 day cultures, and secreted yellowish pigments into the culture media. Based on results of biochemical and physiological tests, 24 isolates were initially identified as *B. gladioli*. These isolates were also categorized into six groups based on disease severity indices on saffron corms, and four groups determined from results of physiological and biochemical tests. No correlation was found between the BGR disease indices and the phenotypic grouping of the isolates or their geographical distributions. *B. gladioli* is a heterogeneous species with variations in phenotypic and genetic characteristics; its differentiation is mainly based on host range (Sadler, 1994; Coenye *et al.,* 1999; Nandakumar *et al.,* 2009; Castro-González *et al.,* 2011; Fiori *et al.,* 2011).

Due to the high degree of phenotypic similarity between *B. gladioli*, *B. glumae* (Coenye and Vandamme 2003; Coenye *et al.,* 1999), and *B. cepacia* (Baxter *et al.,* 1997), biochemical characterization if these bacteria is not accurate for precise identification of *B. gladioli*. PCR with *B. gladioli*-specific primers was used to increase detection sensitivity, specificity, and simplicity, and for more rapid identifications than from phenotypic methods. Specific fragments of *B. gladioli* fragments were amplified from selected Iranian isolates. Sequences of 16S and 23S rRNA have been used for rapid identification and differentiation of *B. gladioli* from *B. cepacia*, *B. multivorans*, *B. vietnamiensis*, *B. mallei*, *B. pseudomallei* and *Ralstonia pickettii* (Bauernfeind *et al.,* 1998). BLAST analysis showed that the BGR-associated isolates were most similar to *Burkholderia* species. Comparison of the 16S rRNA sequence of the Iranian BGR-associated saffron isolates revealed 99.5 to 99.9% nucleotide similar-

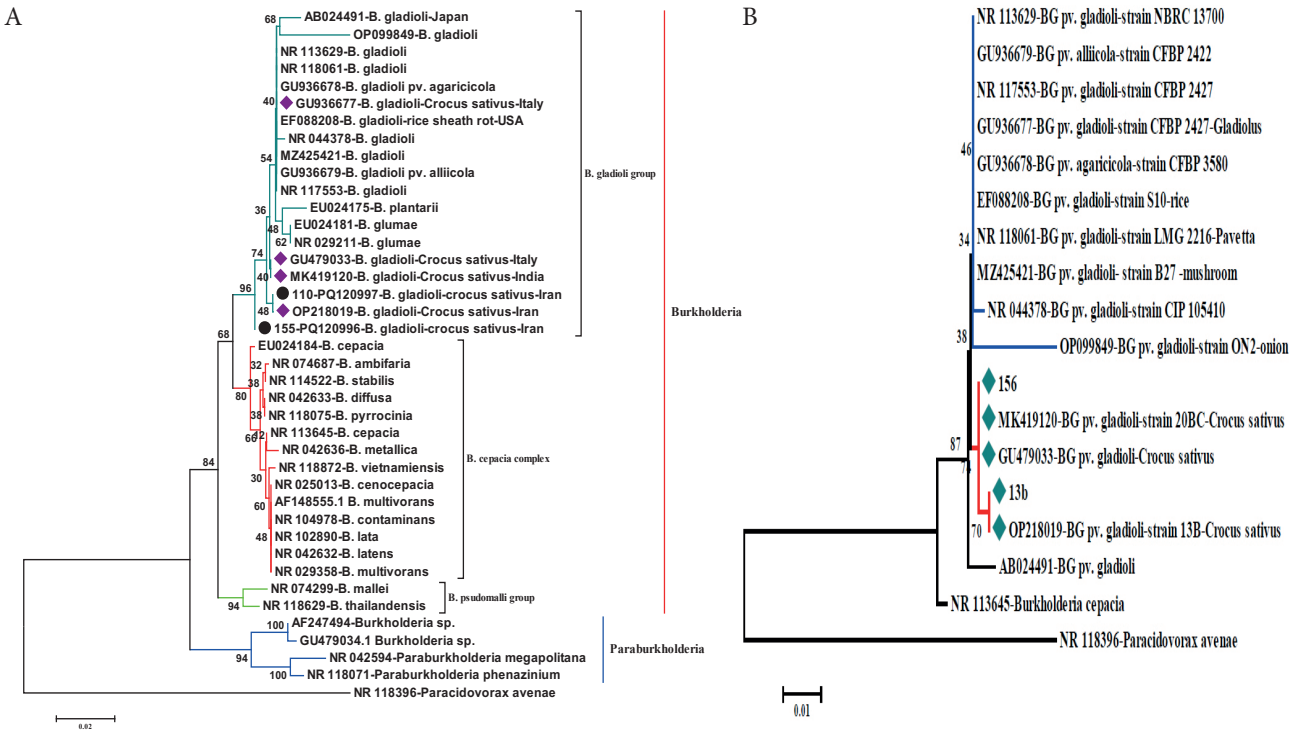

Figure 4. Neighbor-joining phylogenetic tree based on partial sequences of the 16S rRNA gene of *Burkholderia* species (A) and 16S-23S rRNA ITS region of *Burkholderia gladioli* pathovars (B). Numbers on branch nodes are percentage bootstrap values based on 1,000 replicates. *Paracidovorax avenae* was used as an outgroup. Bar shows substitutions per nucleotide position. • indicates represented *Burkholderia gladioli* isolates from saffron (*Crocus sativus*) from Iran, and ♦ indicates isolates from owther countries.

ity with other *B. gladioli* isolates (Gee *et al.,* 2003; Kim *et al.,* 2009). Similarity of this fragment with sequences from other *Burkholderia* species was in the range 92.5 to 96.4%. Phylogenetic analysis of the 16S rRNA gene placed the BGR-associated isolates in the *Burkholderia* group, close to the *B. gladioli* group. Due to the similarity of the 16S rRNA sequence from *B. gladioli* and *B. glumae* (Nandakumar *et al.*, 2009), the 16S-23S rRNA ITS region was used to distinguish *B. gladioli* from *B. glumae* (Furuya *et al.,* 2002). In the phylogenetic tree, the Iranian saffron BGR-associated isolates were placed next to *B. gladioli* isolates.

Bgg is pathogenic to gladiolus, orchids, *Crocus* spp., rice, and fern, and other plant species (Sadler, 1994; Ura *et al.,* 2006; Compant *et al.,* 2008; Nandakumar *et al.,* 2009). Sequence analysis of the 16S rRNA of the Iranian saffron BGR-associated isolates showed similarity of 99.1 to 100% with the pathovars Bgg and Bga of *B. gladioli*.

To determine the pathovars of the Iranian BGR-associated isolates, pathogenicity tests were carried in onion as a possible alternative host. Presumptive Bgg isolates caused dry rot in onion, but possible Bga gave soft rot and internal rot in onion (Lee *et al.,* 2005). Most of the BGR-associated isolates caused soft rot in onion, making it possible that BGR-associated saffron isolates from Iran are Bga. However, phylogenetic analyses of some BGR-associated isolates using the 16S-23S rRNA ITS region showed that they were very similar to pathovar *gladioli*. These results indicate that the causal agent of saffron dry rot in Iran could be *B. gladioli* pv. *gladioli*. Recently, *B. gladioli* has been reported from saffron, garlic and wild mushrooms in Iran (Abachi *et al.,* 2024; Hamidizade *et al.* 2024; Khezri *et al.,* 2023), but the present report is the first from Iran, where biochemical and molecular characterization have been achieved for the pathogen causing bacterial dry rot of saffron Iranian saffron *B. gladioli* strains possess pathogenicity characteristics that overlap with Bgg and Bga pathovars of *B. gladioli*. This shows that using phenotypic pathovar-oriented assays to classify *B. gladioli* strains should be replaced by phylogenetic or phylogenomic analyses for identification of this pathogen (Abachi *et al.*, 2024).

Inoculation by injection of saffron corms with the BGR-associated isolates, or through wounds in the corm skins, caused lesions in the corms. These results agree with the observations of Fiori *et al.* (2011). Nevertheless, the severity of symptoms depended on virulence of the different isolated bacteria.

It has been reported that *Burkholderia* is more frequently isolated from rhizospheres of plants than from bulk soil of the same field (Marques *et al.,* 2014). *Burkholderia gladioli* has been identified as an endophyte in coffee (Vega *et al.,* 2005) and soybean (Kuklinsky-Sobral *et al.,* 2004), but not as an endophyte in saffron (Marques *et al.,* 2015; Sharma *et al.,* 2015; Xuan *et al.,* 2016). However, Ahmad *et al.* (2022) reported *B. gladioli* as an endophyte in saffron, so it is still unclear whether *B. gladioli* is in the rhizosphere community of *Crocus* species. Studies have shown that toxoflavin produced by *B. gladioli* and *B. glumae* is an important pathogenic factor causing grain wilt in rice and rot in many crops (Jeong *et al.,* 2003; Jung *et al.,* 2011). Inoculation with toxoflavin led to infections in crops such as tomato, sesame, eggplant and bell pepper (Jeong *et al.,* 2003; Nandakumar *et al.,* 2009). The reddish-brown lesions on saffron corms could be due to the presence of toxoflavin. Therefore, further investigation is required on the role of toxoflavin production in regulating pathogenicity of *B. gladioli* in saffron.

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