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Identification and characterization of *Burkholderia* isolates obtained from bacterial rot of saffron (*Crocus sativus* L.) grown in Italy

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Summary. Twenty five isolates of *Burkholderia gladioli*, the causal agent of a bacterial disease recently reported on saffron (*Crocus sativus* L.) grown in central Sardinia (Italy), were characterized using different approaches. The characteristic symptoms of the disease on saffron plants were rot of emerging shoots and leaves and spots on leaves and corms. In the field, the disease was destructive and reduced flowering by about 80%. Two types of colonies of bacteria cultured from affected plants were selected on the basis of their characteristic morphology and pigment production on nutrient-glucose-agar. One type was round, wrinkled, and producing yellowish pigment; while the second was round, smooth and without pigment. All 25 selected isolates were pathogenic on saffron leaves and corms. Ten were pathogenic on gladiolus and lily leaves. None of the tested isolates was pathogenic on onion plants. The isolates were characterized by conventional tests, Biolog, PCR and PCR-RFLP analysis. Conventional tests and PCR identified all isolates as *B. gladioli*. PCR-RFLP analysis of 16S rDNA products digested with the three restriction enzymes *Alu* I, *Dde* I and *Bss* KI, identified ten of the isolates as *B. gladioli* pv. *gladioli*. Sequencing and comparison of the 16S rDNA PCR products confirmed that ten of the isolates were *B. gladioli* and the remaining 15 were an unidentified *Burkholderia* species. Sequencing the gene encoding for β-subunit polypeptide of DNA gyrase (*gyrB*) did not assist identification of these isolates. This study suggests that other *Burkholderia* species are involved with bacterial softrot of saffron in Sardinia, and further studies are in progress to verify this hypothesis.

Key words: *Burkholderia gladioli* pv. *gladioli*, PCR-RFLP, gene sequencing.

Introduction

Saffron (*Crocus sativus* L.) has been cultivated in Sardinia since ancient times. The plant was known during the Roman period and its cultivation and uses were later spread by the Arabs and Spanish (Picci, 1986). Since the early years of the 20th century, saffron cultivation has progressively decreased, as it is the case in most European countries, with the exception of Spain and Greece. In spite of this collapse in production, 80–90% of the world market is still controlled by European companies.

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The area currently planted with saffron in Sardinia is about 35 ha, and most is cultivated in the centre of the island (San Gavino Monreale, Turri and Villafranca). Saffron cultivation is traditional in these areas and plants are cultivated through biological systems. The dried stigmas are sold exclusively as threads. Even though this kind of farming is profitable, it is not widely used because of the out-dated techniques used and the low levels of mechanization. Saffron cultivation involves intense manual work both for the harvest and the treatment of flowers. In addition, saffron monoculture has resulted in the development of serious pathogens that infect plants and corms adversely affecting production.

Different pathogens, mainly fungi, have been

reported on saffron. These include: *Macrophomina phaseolina* (Carta *et al*., 1982; Thakur *et al*., 1992; Ionita *et al*., 1995); *Fusarium oxysporum* f. sp. *gladioli* (Yamamoto *et al*., 1956; Shah and Srivastava, 1984; Garcia-Jimenez *et al*., 1985; Garcia-Jimenez and Alfaro-Garcia, 1987; Cappelli, 1994); *Fusarium solani* (Sud *et al*., 1999); *Penicillium aurantiogriseum* Dierckx (*Penicillium cyclopium* Westl.) and *Penicillium hirsutum* Dierckx (*Penicillium corymbiferum* Westl.) (Francesconi, 1973; Picci, 1986; Cappelli *et al*., 1991; Gu and Zhi, 1997; Fiori, 2002); *Rhizoctonia violacea* (Voglino, 1905; Nannizzi, 1941); *Stromatinia gladioli* (Drayton) Whetzel (Fiori *et al*., 2007); *Phoma crocophila* (Mont.) Sacc. (Nannizzi, 1941); *Rhizoctonia violacea* var. *crocorum* (Nannizzi, 1941); *Bacillus croci* (Mizusawa, 1923) *Burkholderia* (*Pseudomonas*) *gladioli* pv. *gladioli* (Xu and Ge, 1990); *Bean yellow mosaic virus* (Russo *et al*., 1979); and *Turnip mosaic virus* (Chen and Chen, 2000).

Since 1980 researchers have found that different fungal diseases were associated with saffron diseased plants in Sardinia (Carta *et al*., 1982; Fiori, 2002; Fiori *et al*., 2007). Some of these are transmitted through the corms (Cappelli *et al*., 1991; Cappelli, 1994; Fiori, 2002). In addition, in 2003 a disease caused by bacteria was observed on saffron plants in the areas of San Gavino Monreale and Turri (Fiori *et al.,* 2005). Severe outbreaks of the disease occurred in the field, reducing flowering by about 80%.

Bacillus croci and *B*. (*Pseudomonas*) *gladioli* pv. *gladioli* are the only bacterial pathogens found in saffron. *Bacillus croci* is Gram negative, facultatively anaerobic with peritrichous flagella, pathogenic on *Crocus sativus* and the causal agent of rot lesions mainly on tubers. *Burkholderia gladioli* pv. *Gladioli* was isolated for the first time in 1921 (McCulloch, 1921), and is normally pathogenic on *Gladiolus* spp. and *Iris* spp. (Palleroni, 1984).

The objective of the present study was to characterize *Burkholderia* spp. isolates obtained from diseased saffron plants grown in Sardinia, using conventional and molecular methods.

Materials and methods

Surveys and samples collection

Surveys were mainly carried out in saffron fields in San Gavino Monreale and Turri, and some fields in Villafranca, an area where bacterial softrot was not observed. A preliminary survey was carried out in 2003; subsequently the observations were made in 2005, 2006 and 2007. Field samples were collected in November (at the beginning of blooming) and then in March. Corms and plants were sampled in fields. In total 204 samples were processed (20 were collected from flowers, 80 from leaves and 104 from corms).

Symptoms

Field symptoms were observed in autumn and spring. They were characterized by rot on plants and spots on leaves and corms. In particular in autumn, during rainy and mild periods just before blooming, the sheaths that wrapped emerging shoots showed brown lesions (Figure 1a). Later the disease spread to the leaves and flowers and they rotted (Figure 1b and c). Brown rounded marks, surrounded by widespread reddish brown halos were observed on corms (Figure 1f). At the last stage of the disease, under high humidity, the spots became rotted. Symptoms on leaves were observed in autumn and also in spring. Reddish-brown spots, surrounded by widespread chlorotic halos occurred on foliar limbs (Figure 1, d and e). Subsequently, during wet periods, the veins and leaf edges are sometimes affected by the disease and in some cases the leaves bend and distal parts wither.

Isolation and purification of bacteria

The isolations were performed as reported by Fiori *et al*. (2005). Small portions (2–3 cm long) of flowers and leaves were disinfected by immersion in 0.5% sodium hypochlorite, while corms were washed in tap water before being disinfected by immersion in 0.5% sodium hypochlorite. After washing several times with sterile distilled water, pieces of infected tissues were taken from the edges of the symptomatic areas, and each sample was ground in a mortar with sterile phosphate buffer (PBS 0.1 M, pH 7.0). The resulting suspensions were diluted 1:10, streaked onto nutrient agar (NA, OXOID CM3, Basingstoke, Hampshire, UK) supplemented with 1% of D-glucose (NDA) Petri dishes, and then incubated at 25°C. The dishes were regularly checked and two different colony types appeared within 72 h: one type was smooth and the other wrinkled. Colonies were purified

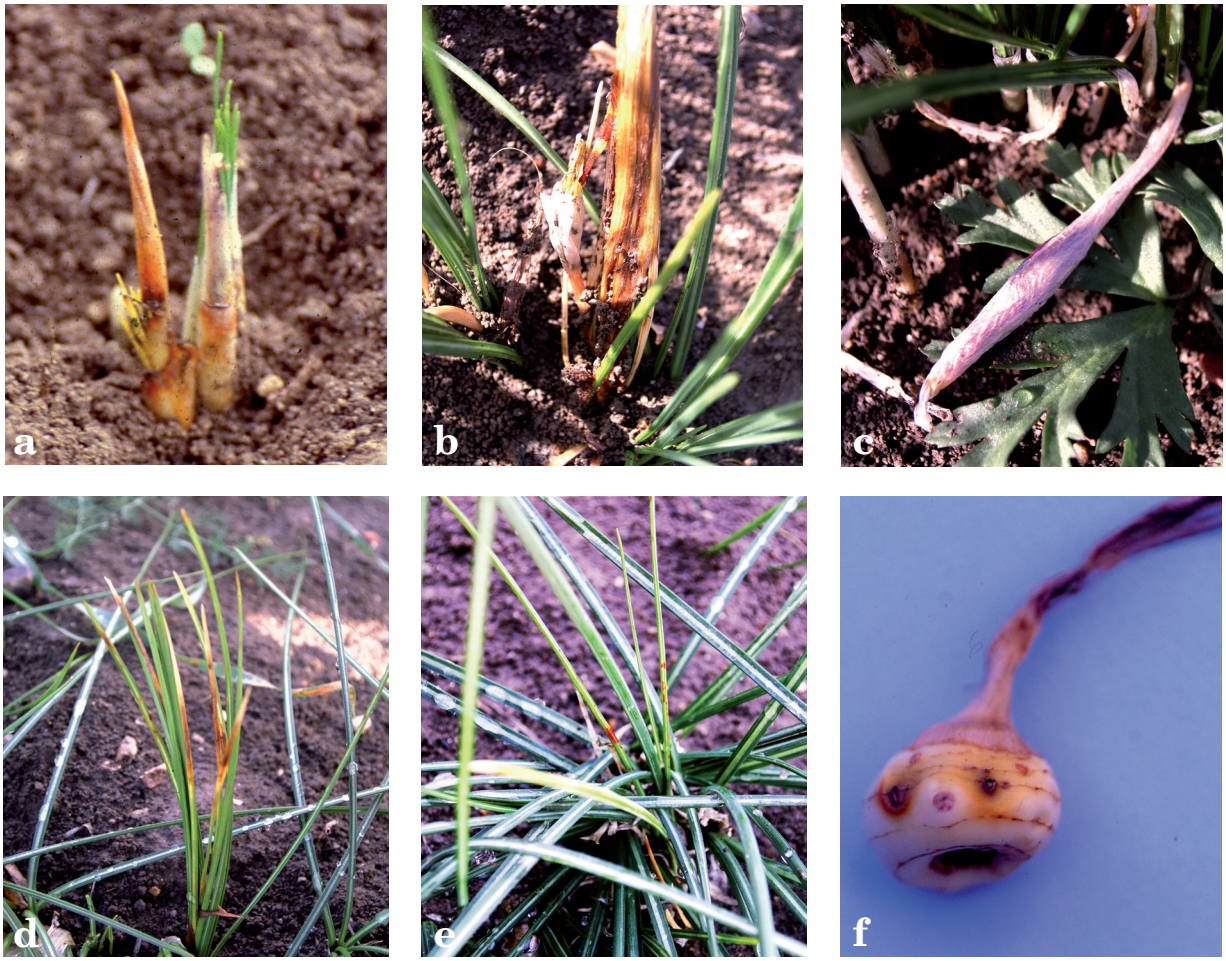

Figure 1. Symptoms of bacterial softrot in naturally infected saffron plants and corms: rot on emerging shoots, leaves and flowers (a, b and c); spots on leaves (d and e); brown marks surrounded by reddish brown halos on corm (f).

twice on NDA and streaked on NA for long-term storage at 4°C.

Pathogenicity test

The isolates were grown on NA for 3 d at 25°C and then colonies were resuspended in sterile distilled water (SDW). Bacterial suspensions were spectrophotometrically measured and adjusted to a concentration of about 10^7 cfu mL^{-1}. Each isolate was inoculated into ten saffron plants and ten corms, four gladiolus, four lily and four onion plants. Saffron plants were inoculated by nebulizing leaves each previously injured with a sterile needle. Corms, gladiolus, lily and onion plants were inoculated by pouring a drop of bacterial suspension on injured surfaces. The same number of saffron plants and corms, gladiolus, lily and onion plants were inoculated with SDW as controls. The inoculated plants were covered with plastic bags and kept in greenhouse at a minimum temperature of 20°C, and fertilised and watered as required. Saffron corms, after inoculation, were planted in pots containing a sterile commercial soil, kept in greenhouse and similarly managed. Plants and corms were checked regularly for symptom development. Re-isolations were made from diseased plants and corms, as described above.

Identification of bacteria

Bacterial isolates and their re-isolates were identified using biochemical and physiological tests (Palleroni, 1984; Chun and Jones, 2001) (Table 1), nutritional profile analysis using computerised the Biolog system (Biolog Inc., Hayward, CA, USA) and PCR.

The Biolog system is based on tests for the oxidation of 95 substrates in 96-well microtiter plates. For nutritional profile analysis the isolates were grown on Biolog universal growth medium (Biolog Inc.) and incubated at 28°C. After 18 h the cultures were collected using swabs and suspended in 0.85% saline solution (GN/GP-IF; Biolog Inc.). Each plate was inoculated with 150 µl per well of bacterial suspension, which had been turbidimetrically adjusted with saline solution to the appropriate density (optical density was determined at 590 nm). The microplates were incubated at 28°C for 20 h, analyzed and identified using the database software, version 6.01 (Biolog Inc.).

For PCR identification, total DNA was extracted by Dneasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. PCRs were performed using primers LP1 (5'-GGGGGGTCCATTGCG-3') and LP4

Table 1. Main characteristics of bacterial isolates from saffron and *B. gladioli* pv. *gladioli* CFBP2427 type strain.

Characteristic	Bacterial isolates from saffron		*B. gladioli* pv. *gladioli* CFBP2427
	Group A[a]	Group B[b]	
Gram reaction	-	-	-
Oxidase	+	+	+
Catalase	+	+	+
Poly-b-hydroxybutyrate	+	+	+
Arginine dihydrolase	-	-	-
Nitrate reduction to NO_2	-	+	-
Hydrolysis of: gelatin	-	+	+
starch	-	-	-
O-F Glucose test	O	O	O
Growth at: pH 4	-	+	+
pH 8	+	+	+
pH 9	-	-	-
Growth in 3% NaCl	-	+	+
Growth at 40° C	+	+	+
Utilization of: Arabinose	+	+	+
Maltose	-	-	-
Mannose	+	+	+
L-Arabitol	+	-	-
meso-Erythritol	-	-	-
D-Tartrate	-	+	+
L- Tartrate	-	+	+
meso-Tartrate	+	-	+
L-Threonine	+	+	+

[a] Group A = DPPZ 9, DPPZ 10, DPPZ 11, DPPZ 12, DPPZ 13, DPPZ 14, DPPZ 15, DPPZ 16, DPPZ 17, DPPZ 18, DPPZ 19, DPPZ 20, DPPZ 21, DPPZ 23 and DPPZ 27:
[b] Group B = DPPZ 22, DPPZ 24, DPPZ 25, DPPZ 26, DPPZ 28, DPPZ 29, DPPZ 30, DPPZ 31, DPPZ 32, DPPZ 33
+, Positive reaction; - , negative reaction; O, oxidative reaction.

(5'-AGAAGCTCGCGCCACG-3') designed on 23S ribosomal DNA sequence (Whitby *et al*., 2000), with a PCR express Thermal cycler (Hybaid). Each reaction contained 1 μM of each primer, 200 μM each deoxynucleoside triphosphate, 1 U of Go Taq DNA polymerase (Promega), 1× buffer containing 1.5 mM $MgCl_2$, 10 ng of genomic DNA in a total volume of 50 μl. The PCR program consisted of an initial denaturation of 95°C for 3 min and subsequent 30-cycle amplification: annealing at 60°C for 10 s, denaturation at 95°C for 10 s and extension at 72°C for 60 s. Following amplification, 10 μl of each reaction mixture was subjected to electrophoresis in a 1% agarose gel in TAE buffer (pH 8.0). The PCR products were visualized and photographed after SYBR Safe (Invitrogen, Carlsbad, CA, USA) DNA gel staining. Positive results were assessed by the amplification of a 700 bp band (Whitby *et al*., 2000).

Molecular characterization of bacteria

RFLP analysis was carried out using the restriction endonucleases *Alu* I, *Dde* I and *Bss* KI (New England Biolab, Beverly, MA, USA) on PCR products (about 1500 bp) obtained with primers fD1 (5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC-3') (Segonds *et al*., 1999) which amplify the 16S rDNA. Type strains of *B. gladioli* pv. *gladioli* CFBP 2427, *B. gladioli* pv. *agaricicola* CFBP 3580, *B. gladioli* pv. *alliicola* CFBP 2422, *B. glumae* CFBP 2430 and *B. plantarii* CFBP 3997 were used as reference controls.

PCR amplification of *gyrB* gene, encoding the β-subunit polypeptide of DNA gyrase, was carried out according to Maeda *et al*. (2006). The degenerate primers UP-1E (5'-CAGGAAACAGCTATGACCAYGSNGGNGGNAARTTYRA-3') and AprU (5'-TGTAAAACGACGGCCAGTGCNGGRTCYTTYTCYTGRCA-3') were used for the first amplification and a nested PCR was carried out on the obtained product with primers M13(-21) (5'-TGTAAAACGACGGCCAGT- 3') and M13R (5'- CAGGAAACAGCTATGACC-3') (Maeda *et al*., 2006). A fragment of 1000 bp was produced for this gene.

In order to obtain the entire sequence of *gyrB* and 16S rDNA both strands of the fragments were sequenced by BMR Genomics s.r.l., DNA sequencing service (Padua, Italy; www.bmr-genomics.it). The 16S rDNA gene of the type strains *B. gladioli* pv. *gladioli* CFBP 2427, *B. gladioli* pv. *agaricicola* CFBP 3580 and *B. gladioli* pv. *alliicola* CFBP 2422 were also sequenced as they were not present in Genbank. Sequences were aligned using the CLUSTAL W software (Chenna *et al*., 2003; www.ebi.ac.uk/clustalw) and compared with those present in Genbank using the BLAST software (Altschul *et al*., 1997; www.ncbi.nlm.nih.gov/blastn). Comparison with other *Burkholderia* sequences were made using BLASTN 2.2.22 software (Altschul *et al*., 1997).

Results

The bacterial isolates obtained from diseased saffron plants were divided in two groups according to their colonial morphology on NDA medium after 4 d incubation. The colonies of the first group were smooth, white-cream and, with backlight, pale green in colour. The colonies of the second group were wrinkled, green-yellow in colour and each produced a yellow diffusible pigment. On the basis of these characteristics, a total of 25 isolates were selected: 15 belonged to the group A (smooth colony type) and 10 to the group B (wrinkled type) (Table 1).

All the 25 isolates were pathogenic and induced specific symptoms on saffron leaves and corms (Figure 2a and b). The isolates of the group B (isolates DPPZ 22, DPPZ 24, DPPZ 25, DPPZ 26, DPPZ 28, DPPZ 29, DPPZ 30, DPPZ 31, DPPZ 32, DPPZ 33) were also pathogenic on gladiolus and lily leaves (Figure 2, c and d). None of the isolates were pathogenic on onion plants.

The results of the main biochemical and physiological tests are reported in Table 1. Although there were some differences, on the basis of these results group A and group B isolates could be ascribed to *B. gladioli*. Characterisation using the Biolog system resulted in the identification of ten isolates as *B. gladioli* (probability ranged from 82 to 99%), while the remaining fifteen isolates were not identified (Table 2).

In the PCR analysis using the primers LP1 and LP4, the 25 saffron isolates and the type strains of *B. gladioli.* pv. *gladioli* CFBP 2427 and *B. gladioli* pv. *alliicola* CFBP 2422 used as controls produced the same species-specific amplification product of 700 bp (Whitby *et al*., 2000) (Figure 3). Moreover,

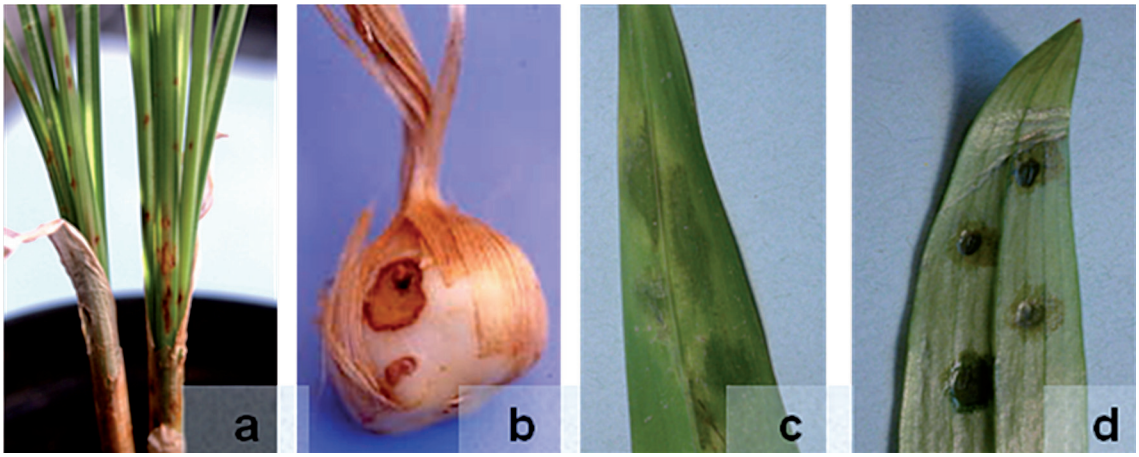

Figure 2. Symptoms of bacterial softrot in an experimentally infected saffron plant and corm (a and b), and on gladiolus (c) and lily (d) leaves.

a single 16S rDNA amplification product of about 1500 bp was produced for all the isolates using primers fD1 and rD1 (Figure 4). RFLP analysis of the 16S rDNA fragments using *Alu* I, *Dde* I and *Bss* KI restriction endonucleases gave the same patterns for the group B isolates (B, D, F) of the type strain *B. gladioli* pv. *gladioli* CFBP 2427. The patterns produced by the isolates of the group A were different from all the reference strains used in this study (Figure 4).

Both strands of PCR products were sequenced using primers fD1 and rD1. The 16S ribosomal gene sequences were aligned. Two types of sequences were obtained with about 96% similarity; they differed for 58 out of 1460 nucleotides. The first type of sequence, which was common to the group B isolates, had the most significant similarity (99%) with the 16S ribosomal RNA gene sequence of *B. gladioli* with accession number EF088208. They differed only for two nucleotides: C instead of T (nt 176) and T instead of C (nt 178). One of these identical sequences, DPPZ22, was deposited in Genbank under accession number GU479033.

The group A isolates had a second type of sequence which had the highest similarity (99%) with a *Burkholderia* sp. that degrades phenanthrene AF247494 (Friedrich *et al*., 2000). The sequence of DPPZ13, representative of this group of isolates, was deposited in Genbank under accession no. GU479034. None of the Genbank sequences that matched this type of sequence belonged to *B. gladioli*.

The two types of 16S sequences, GU479033 and GU479034 *B. gladioli*, were aligned with the sequences of *B. gladioli* EF088208, *B. cepacia* EU024184, *B. multivorans* EU024178, *B. plantarii* EU024175 and *B. glumae* EU24181, already present in Genbank, and with *B. gladioli* pv. *gladioli* CFBP 2427 (GU936677), *B. gladioli* pv. *agaricicola* CFBP 3580 (GU936678) and *B. gladioli* pv. *alliicola* CFBP 2422 (GU936679), sequenced in the present study, as they were not present in Genbank (Tables 3 and 4).

Comparison of the data obtained indicated a similarity of 99% between sequence GU479033 and the following: *B. gladioli* (EF088208), *B. gladioli* pv. *gladioli* CFBP 2427 (GU936677), *B. gladioli* pv. *agaricicola* CFBP 3580 (GU936678) and *B. gladioli* pv. *alliicola* CFBP 2422 (GU936679). The comparison with *B. plantarii* (EU024175) and *B. glumae* (EU024181) revealed 98% similarity, while 97% similarity was found with *B. cepacia* (EU024184) and *B. multivorans* (EU024178) (Table 3).

Sequence GU479034 had 96% similarity with *B. gladioli* (EF088208), *B. gladioli* pv. *gladioli* CFBP 2427 (GU936677), *B. gladioli* pv. *agaricicola* CFBP 3580 (GU936678) and *B. gladioli* pv. *alliicola* CFBP 2422 (GU936679), *B. plantarii* (EU024175), *B. glumae* (EU024181), *B. cepacia* (EU024184) and 95% with *B. multivorans* (EU024178) (Table 4). *Burkholderia* sp. GU479034 sequence has 41 nucleotides which are present only in this species.

Table 2. Results of Biolog test for 25 saffron isolates.

Isolate	Identification BIOLOG	PROB	SIM	DIST
DPP 9z	Not identified (*B. gladioli*)	-	0.355	10.85
DPP 10z	Not identified (*B. gladioli*)	-	0.121	13.31
DPP 11z	Not identified (*P. fluorescens;*	-	0.275	12.87
	B. gladioli)	-	0.00	15.31
DPP 12z	Not identified (*P. fluorescens;*	-	0.304	11.84
	B. gladioli)	-	0.011	12.91
DPP 13z	Not identified (*P. fluorescens;*	-	0.313	11.95
	B. gladioli)	-	0.00	14.15
DPP 14z	Not identified (*P. fluorescens;*	-	0.237	13.95
	B. gladioli)	-	0.002	15.59
DPP 15z	Not identified (*B. gladioli;*	-	0.122	14.28
	Acinetobacter calcoaceticus gen. 1)	-	0.060	14.51
DPP 16z	Not identified (*B. gladioli*)	-	0.351	10.97
DPP 17z	Not identified (*P. fluorescens;*	-	0.274	12.95
	B. gladioli)	-	0.00	15.04
DPP 18z	Not identified (*P. fluorescens;*	-	0.274	12.95
	B. gladioli)	-	0.00	15.04
DPP 19z	Not identified (*B. pyrrocinia*;	-	0.202	11.66
	B. gladioli)	-	0.103	11.88
DPP 20z	Not identified (*B. gladioli*;	-	0.350	10.18
	P. fluorescens)	-	0.037	10.92
DPP 21z	Not identified (*P. fluorescens;*	-	0.343	11.20
	B. gladioli)	-	0.00	14.65
DPP 23z	Not identified (*B. phenazinium*;	-	0.328	11.54
	P. fluorescens)	-	0.001	13.34
DPP 27z	Not identified (B. gladioli)	-	0.256	13.45
DPP 22z	Identified *B. gladioli*	96%	0.784	2.80
DPP 24z	Identified *B. gladioli*	91%	0.691	3.62
DPP 25z	Identified *B. gladioli*	98%	0.742	3.62
DPP 26z	Identified *B. gladioli*	96%	0.744	3.41
DPP 28z	Identified *B. gladioli*	99%	0.706	4.40
DPP 29z	Identified *B. gladioli*	96%	0.784	2.80
DPP 30z	Identified *B. gladioli*	98%	0.824	2.36
DPP 31z	Identified *B. gladioli*	99%	0.830	2.44
DPP 32z	Identified *B. gladioli*	98%	0.839	2.19
DPP 33z	Identified *B. gladioli*	82%	0.527	5.44

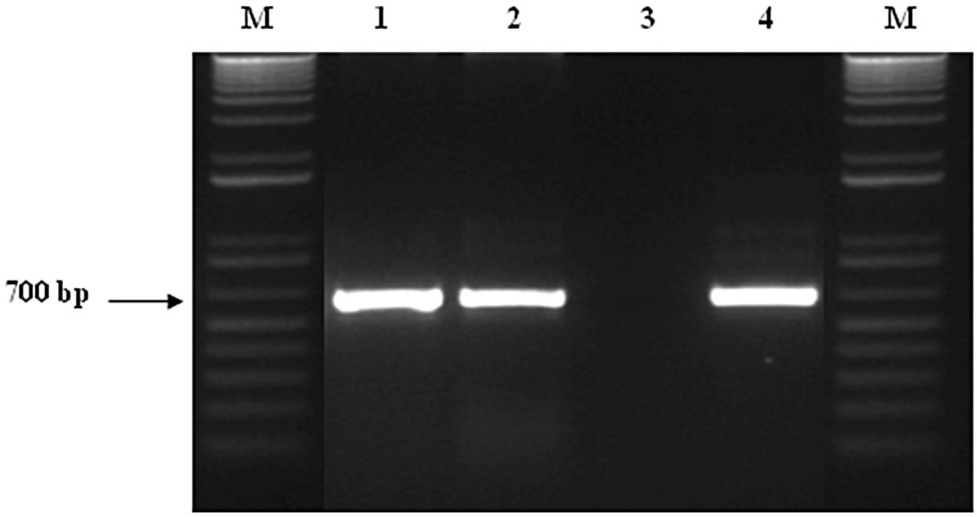

Figure 3. Amplification products from *Burkholderia* spp. obtained using LP1 and LP4 primers. Lane M, 1 Kb plus DNA ladder (Invitrogen); lane 1, type strain *B. gladioli* pv. *gladioli* CFBP 2427; lane 2, *B. g.* pv. *alliicola* CFBP 2422; lane 3, SDW; lane 4, band produced by group A and group B saffron isolates.

To obtain more precise identification of the group A saffron isolates, DPPZ 13 *gyrB* gene was sequenced (data not shown) and analysed. BLAST search gave the following results: the highest similarity of 90% was found with *B. thailandensis* (CP000086); 87% with *B. plantarii* (AB190644) and *B. cepacia* (AB190575); only 86% with *B. gladioli* (AB190622), *B. gladioli* pv. *agaricicola* (AB220902) and *B. gladioli* pv. *alliicola* (AB190640) (Table 5).

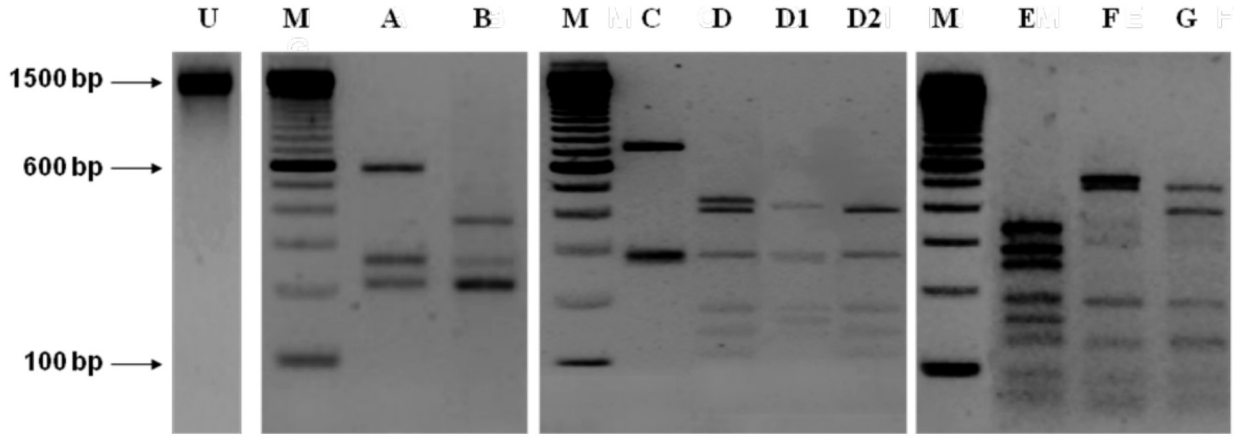

Figure 4. Restriction fragment length polymorphism patterns of *Burkholderia gladioli* and unidentified strains isolated from saffron after digestion of PCR amplification products obtained with fD1 and rD1 primers with the restriction endonucleases *Alu*I (A and B), *Dde*I (C, D, D1 and D2) and *BssK*I (E, F and G). M = 100 bp ladder (Invitrogen); U = uncut product; (B, D, F) = *B. gladioli* pv. *gladioli* CFBP 2427; (B, D, F) = Group B isolates; (B, D2, F) = *B. gladioli* pv. *alliicola* CFBP 2422; (A, D1, F) = *B. glumae* CFBP 2430; (B, D, G) = *B. gladioli* pv. *agaricicola* CFBP 3580; (B, D1, F) = *B. plantarii* CFBP 3997; (A, C, E) = Group A isolates.

Table 3. Comparison between *B. gladioli* pv. *gladioli* (strain DPPZ22, group B) GU479033 and other *Burkholderia* species.

Accession No.	Description	Max score	Total score	Query coverage (%)	E value	Max ident. (%)
EF088208	*Burkholderia gladioli* strain S10 16S ribosomal RNA gene, partial sequence	2626	2626	99	0.0	99
GU936677	*Burkholderia gladioli* pv. *gladioli* CFBP 2427	2610	2610	100	0.0	99
GU936678	*Burkholderia gladioli* pv. *agaricicola* CFBP 3580	2610	2610	100	0.0	99
GU936679	*Burkholderia gladioli* pv. *alliicola* CFBP 2422	2628	2628	100	0.0	99
EU024184	*Burkholderia cepacia* strain CIP 8272 16S ribosomal RNA gene, partial sequence	2244	2244	89	0.0	97
AF148555	*Burkholderia multivorans* 16S ribosomal RNA gene, partial sequence	2277	2277	99	0.0	97
EU024175	*Burkholderia plantarii* strain CIP 105979 16S ribosomal RNA gene, partial sequence	2298	2298	89	0.0	98
EU024181	*Burkholderia glumae* strain CIP 106418 16S ribosomal RNA gene, partial sequence	2286	2286	89	0.0	98
GU479034	*Burkholderia* sp. strain DPPZ13 16S ribosomal RNA gene, complete sequence	2372	2372	100	0.0	96

Table 4. Comparison between *Burkholderia* sp. (strain DPPZ13, group A) GU479034 and other *Burkholderia* species.

Accession No.	Description	Max score	Total score	Query coverage (%)	E value	Max ident. (%)
AF247494	*Burkholderia* sp. S2.1 16S ribosomal RNA gene, partial sequence	2607	2607	98	0.0	100
EF088208	*Burkholderia gladioli* strain S10 16S ribosomal RNA gene, partial sequence	2385	2385	99	0.0	96
GU936677	*Burkholderia gladioli* pv. *gladioli* CFBP 2427	2396	2396	99	0.0	96
GU936678	*Burkholderia gladioli* pv. *agaricicola* CFBP 3580	2405	2405	99	0.0	96
GU936679	*Burkholderia gladioli* pv. *alliicola* CFBP 2422	2385	2385	99	0.0	96
EU024184	*Burkholderia cepacia* strain CIP 8272 16S ribosomal RNA gene, partial sequence	2168	2168	89	0.0	96
EU024181	*Burkholderia glumae* strain CIP 106418 16S ribosomal RNA gene, partial sequence	2154	2154	89	0.0	96
EU024175	*Burkholderia plantarii* strain CIP 105979 16S ribosomal RNA gene, partial sequence	2145	2145	89	0.0	96
AF148555	*Burkholderia multivorans* 16S ribosomal RNA gene, partial sequence	2143	2376	90	0.0	95
GU479033	*Burkholderia gladioli* pv. *gladioli* 16S ribosomal RNA gene, partial sequence	2369	2369	99	0.0	95

Table 5. Comparison between *Burkholderia* sp. (strain DPPZ13, group A) *gyrB* gene sequence and other *Burkholderia* species.

Accession No.	Description	Max score	Total score	Query coverage (%)	E value	Max ident. (%)
CP000086	*Burkholderia thailandensis E264 chromosome I, complete sequence.*	1258	1320	95	0.0	90
AB207070	*Burkholderia glumae gyrB* gene for DNA gyrase subunit B, partial cds, isolate: H94	1218	1218	95	0.0	88
AP009385	*Burkholderia multivorans* ATCC 17616 DNA, complete genome, chromosome 1	1186	1228	97	0.0	87
AB190644	*Burkholderia plantarii* gene for DNA gyrase subunit B, partial cds, strain: MAFF 302381	1007	1053	82	0.0	87
AB190575	*Burkholderia cepacia* gene for DNA gyrase subunit B, partial cds, strain: ATCC 25416	980	1047	81	0.0	87
AB220902	*Burkholderia gladioli* pv. *agaricicola gyrB* for DNA gyrase subunit B, partial cds, strain: GTC1730	962	1011	82	0.0	86
AB190640	*Burkholderia gladioli* pv. *alliicola* gene for DNA gyrase subunit B, partial cds, strain: ATCC 19302	971	1020	82	0.0	86
AB190622	*Burkholderia gladioli* gene for DNA gyrase subunit B, partial cds, strain: MAFF 302385	944	993	82	0.0	86

Discussion

The genus *Burkholderia* includes opportunistic or obligate plant, animal and human pathogens. Also included are species and strains that are beneficial to plants (Compant *et al*., 2008). Originally, plant pathogenic strains were included in the genus *Pseudomonas* (Severini 1913; McCulloch, 1921). Currently, these bacteria are included in *Burkholderia* (Yabuuchi *et al*., 1992).

Two groups of bacterial pathogens isolated from diseased saffron plants were characterized in the present study. Preliminary identification of the ten isolates of group B (Table 1 and Table 2) was based on results of biochemical and physiological tests and Biolog analysis. On the basis of these characteristics they could be ascribed to *B. gladioli*. Further differentiation from the isolates of the group A was based on pathogenicity on gladiolus and lily; on the basis of these results they can be ascribed to the pathovar *gladioli*.

Burkholderia gladioli is an heterogeneous species with phenotypic and genetic variability. Their discrimination is based mainly on pathogenicity (Saddler, 1994). The species was divided into three pathovars (pv. *gladioli,* pv. *alliicola* and pv. *agaricicola*) based on their host ranges (Young *et al*., 1996). *Burkholderia gladioli* pv. *gladioli* causes rot of stems and corms mainly on *Gladiolus* and *Iris* spp. and other plants (e.g. orchids) including *Crocus* species, rot of rice, and leaf spots and blight of ferns (Saddler, 1994; Ura *et al*., 2006; Compant *et al*., 2008; Nandakumar *et al*., 2009). On saffron this pathogen was only reported in China as the causal agent of corm rot (Xu and Ge, 1990). This bacterium is also an opportunistic human pathogen and may be involved in various nosocomial infections observed in cystic fibrosis patients (Bauernfeind *et al*., 1998).

The identification of group B isolates was confirmed also using genomic analyses: PCR (Whitby *et al*., 2000) (Figure 3) and RFLP of the 16S rDNA fragments produced the same patterns (B, D, F) of the type strain *B. gladioli* pv. *gladioli* CFBP 2427 (Segonds *et al*., 1999) (Figure 4).

Sequencing of the 16S rDNA gene was useful for strain differentiation and to compare the saffron isolates with *Burkholderia* spp. reported in Genbank.

Sequences of the strains belonging to the group B had the most significant similarity (99%) with the 16S ribosomal RNA gene sequence of *B. gladioli* EF088208. The sequence GU479033, representative of this group, was compared with other *Burkholderia* species and a similarity of 99% was found also with the sequences of *B. gladioli* pv. *gladioli* CFBP 2427 (GU936677), *B. gladioli* pv. *agaricicola* CFBP 3580 (GU936678) and *B. gladioli* pv. *alliicola* CFBP 2422 (GU936679) (Table 3). These three sequences were very similar and not distinguishable. For example, the sequences of *B. gladioli* EF088208 and *B. gladioli* pv. *alliicola* GU936679 were identical.

Isolates of group A were pathogenic on saffron, but not pathogenic on gladiolus and lily. On the basis of the results of biochemical and physiological tests they can be ascribed to *B. gladioli*. The differences recorded in some tests (Table 1), except the lack of growth at pH 4, have also been reported elsewhere (Palleroni, 1984; Saddler, 1994; Chun and Jones, 2001).

The results of Biolog and molecular analyses did not allow us to identify the group A isolates. It was not possible either to reach species determination by 16S sequencing. BLAST analysis of *Burkholderia* sp. sequence GU479034, representative of this second group, showed the highest similarity (99%) with *Burkholderia* sp. AF247494. The comparison of GU479034 with *B. gladioli* sequences gave a similarity of 96% for all the pathovars reported above, while it was 95% with the saffron strain *B. gladioli* pv. *gladioli* GU479033 (Table 4). We also found that GU479034 had 41 nucleotides which differed from all the other *Burkholderia* species considered. These could be peculiar bases of a new *Burkholderia* species. In addition, even the analysis of the *gyrB* gene did not give complete identification, as the similarity with the sequences available in Genbank was not sufficiently high to determine the species (Table 5).

In conclusion, these strains of bacteria, which have the ability to cause softrot in saffron, are close to *B. gladioli*. Therefore, further studies are necessary to verify if the isolates of this group belong to a new species or pathovar of this bacterium.

Literature cited

Altschul S.F., T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller and D.J. Lipman, 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25, 3389–3402.

Bauernfeind A., I. Schneider, R. Jungwirth and C. Roller, 1998. Discrimination of *Burkholderia gladioli* from other *Burkholderia* species detectable in cystic fibrosis patients by PCR. *Journal of Clinical Microbiology* 36 (9), 2748–2751.

Cappelli C., R. Buonaurio and A. Polverari, 1991. Occurrence of *Penicillium corymbiferum* on saffron in Italy. *Plant Pathology* 40, 148–149.

Cappelli C., 1994. Occurrence of *Fusarium oxysporum* f. sp. *gladioli* on saffron in Italy. *Phytopathologia Mediterranea* 33, 93–94.

Carta C., M. Fiori and A. Franceschini, 1982. Il "marciume carbonioso" dei bulbi dello zafferano (*Crocus sativus* L.). *Studi Sassaresi, Annali della Facoltà di Agraria, Università di Sassari* XXIX, 193–197.

Chen J. and J.S. Chen, 2000. Occurrence and control of mosaic disease (turnip mosaic virus) in saffron (*Crocus sativus*). *Zhejiang Nongye Kexue* 3, 132–135.

Chenna R., H. Sugawara, T. Koike, R. Lopez, T.J. Gibson, D.G. Higgins and J.D. Thompson, 2003. Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Research* 31, 3497–500.

Chun W. and J.B. Jones, 2001. II. Gram-negative Bacteria. Burkholderia. In: *Laboratory Guide for Identification of Plant Pathogenic Bacteria*. Third Edition. (Schaad N.W., Jones J.B., Chun W. ed.), APS Press, St. Paul, MN, USA, 139–150.

Compant S., J. Nowak, T. Coenye, C. Clément and Ait Barka E., 2008. Diversity and occurrence of *Burkholderia* spp. in the natural environment. *FEMS Microbiology Reviews* 32, 607–626.

Fiori M., 2002. Avversità. In: *Zafferano. Storia, Cultura, Coltivazione e Impiego a San Gavino Monreale e in Sardegna*. (F. Sanna, ed.) E.R.S.A.T. Centro Zonale di Guspini. Medio Campidano, Sardinia, Italy, 68–73.

Fiori M., S. Virdis and A. Schiaffino, 2005. A bacterial disease of saffron caused by *Burkholderia gladioli* and *Pseudomonas* spp. In: *Atti del XII Congresso Nazionale S.I.Pa.V. Reggio Calabria, 29 settembre–1 Ottobre 2005, Journal of Plant Pathology* 87 (4, special issue), 294.

Fiori M., G. Falchi, M. Quaglia and C. Cappelli, 2007. Saffron (*Crocus sativus* L.) diseases in Italy. In: *Atti del XIV Congresso Nazionale SIPaV. Perugia 18–21 Settembre 2007*, *Journal of Plant Pathology* 89 (3, special issue), 55.

Francesconi A., 1973. The rotting of bulbs of *Crocus sativus* L. by *Penicillium cyclopium* Westling. *Annali di Botanica* 32, 63–70.

Friedrich M., R.J. Grosser, E.A. Kern, W.P. Inskeep and D.M. Ward, 2000. Effect of model sorptive phases on phenanthrene biodegradation: molecular analysis of enrichments and isolates suggests selection based on bioavailability. *Applied and Environmental Microbiology* 66 (7), 2703–2710.

Garcia-Jimenez J. and A. Alfaro-Garcia., 1987. *Fusarium oxysporum* Schecht. as causal agent of a seedborne disease of saffron (*Crocus sativus* L.). In: *Proceedings of*

the 7th Congress of the Mediterranean Phytopathological Union. September 1987, Granada, Spain, 156.

Garcia-Jimenez J., V.J. Piera and A. Alfaro-Garcia, 1985. Los "soldatos", una nueva enfermedad del azafaran (*Crocus sativus* L.) en Espana. In: *Proceedings IV Congreso Nacional de Fitopatologia. Sociedad Espanola de Fitopatologia*. Pamplona, Spain October 1985, 76.

Gu Z.F. and Y. Zhi, 1997. A study on biological characteristics of *Penicillium corymbiferum* on *crocus*. *Acta Agriculturae Shanghai* 13 (4), 71–74.

Ionita A., H. Iliescu, V. Jinga and E. Iordache, 1995. *Macrophomina phaseolina* a dangerous parasite of cropped plants possibilities for control. *Probleme de Protectio Plantelor* 23 (2), 179–196.

Maeda Y., H. Shinohara, A. Kiba, K. Ohnishi, N. Furuya, Y. Kawamura, T. Ezaki, P. Vandamme, S. Tsushima and Y. Hikichi, 2006. Phylogenetic study and multiplex PCR-based detection of *Burkholderia plantarii*, *Burkholderia glumae* and *Burkholderia gladioli* using *gyrB* and *rpoD* sequences. *International Journal of Systematic and Evolutionary Microbiology* 56, 1031–1038.

McCulloch L., 1921. A bacterial disease of gladiolus. *Science* 54, 115–116.

Mizusawa Y., 1923. A bacterial rot disease of saffrons. *Annals of the Phytopathological Society of Japan* 1 (5), 1–12.

Nandakumar R., A.K.M. Shahjahan, X.L. Yuan, E.R. Dickstein, D.E. Groth, C.A. Clark, R.D. Cartwright and M.C. Rush, 2009. *Burkholderia glumae* and *B. gladioli* cause bacterial panicle blight in rice in the southern United States. *Plant Disease* 93, 896–905.

Nannizzi A., 1941. I parassiti delle piante officinali. Roma, Istituto Poligrafico dello Stato. 1–544.

Palleroni N.J., 1984. Family I. Pseudomonadaceae Winslow, Broadhurst, Buchanan, Krumwiede, Rogers and Smith 1917, 555^{AL}. In: *Bergey's Manual of Systematic Bacteriology*. Volume 1. (Krig N.R., Holt J.G., ed), Baltimore/London, Williams & Wilkins, 141–219.

Picci V., 1985-86. Lo Zafferano *Crocus sativus* Linnaeus Coltivazione e preparazione della droga in Sardegna. *Annali della Facoltà di Agraria dell'Università di Sassari. Studi Sassaresi Sezione III*, 32, 15–31.

Russo M., G.P. Martelli, M. Cresti and F. Ciampolini, 1979. Bean Yellow Mosaic Virus in saffron. *Phytopathologia Mediterranea* 18, 189–191.

Saddler G.S. (ed.), 1994. *Burkholderia gladioli* pv. *gladioli*. *IMI Descriptions of Fungi and Bacteria*, 122. CAB International, Wallingford, UK, 1218.

Segonds C., T. Heulin, N. Marty and G. Chabanoni, 1999. Differentiation of *Burkholderia* Species by PCR-Restriction Fragment Length Polymorphism Analysis of the 16S rRNA Gene and Application to Cystic Fibrosis Isolates. *Journal of Clinical Microbiol*ogy 37 (7), 2201–2208.

Severini G., 1913. Una batteriosi dell'*Ixia maculata* e del *Gladiolus colvilli*. *Annali di Botanica* 11, 413–424.

Shah A. and K.K. Srivastava, 1984. Horticultural Experiments and Training Centre, Chaubattia, Almora, India. *Progressive Horticulture* 16, 141–143.

Sud A.K., Y.S. Paul and B.R. Thakur, 1999. Corm rot of saffron and its management. *Journal of Mycology and Plant Pathology* 29 (3), 380–382.

Thakur R.N., C. Singh and B.L. Kaul, 1992. First report of corm rot in *Crocus sativus*. *Indian Phytopathology* 45 (2), 278.

Ura H., N. Furuya, K. Iiyama, M. Hidaka, K. Tsuchiya and N. Matsuyama, 2006. *Burkholderia gladioli* associated with symptoms of bacterial grain rot and leaf-sheath browning of rice plants. *Journal of General Plant Pathology* 72, 98–103.

Voglino P. (ed.), 1905. Patologia vegetale. Fanerogame parassite, mixomiceti, bacterii, ifomiceti od eumiceti (funghi). *Unione Tipografico-Editrice (Già Ditta Pombe & C)* Torino, Italy, pp. 287.

Whitby P.W., L.C. Pope, K.B. Carter, J.J. LiPuma and T.L. Stull, 2000. Species-specific PCR as a tool for the identification of *Burkholderia gladioli*. *Journal of Clinical Microbiol*ogy 38 (1), 282–285.

Xu C.X. and Q.X Ge, 1990. A preliminary study on corm rot of *Crocus sativus* L. *Acta Agriculturae, Universitatis Zhejiangensis* 16 (suppl. 2), 241–246.

Yamamoto W., M. Maeda and N. Oyasu, 1956. Studies on the *Penicillium* diseases occurring on cultivated plants. *Scientific Reports of the Hyogo University of Agriculture* 2, 23–28 (*Review of Applied Mycology*, 1958, 37, 170).

Young J.M., G.S. Saddler, Y. Takikawa, S.H. De Boer, L. Vauterin, L. Gardan, R.I. Gvozdyak and D.E. Stead, 1996. Names of plant pathogenic bacteria 1864-1995. *Review of Plant Pathology* 75, 721–763.

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