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Occurrence of atypical *Pseudomonas viridiflava* strains on different host plants in southern Italy

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Abstract

Since 1997, severe bacterial disease symptoms were observed during surveys carried out in farms with field-grown Molfettese Catalogna chicory (*Cichorium intybus* var. *sylvestre*, *Catalogna* group), sugarloaf chicory (*C. intybus* var. *porphyreum*), curly type lettuce (*Lactuca sativa* var. *crispa*) cvs Langero and Aleppo, and safflower (*Carthamus tinctorius*) located in the Apulia and Basilicata regions of southern Italy. Convex colonies with yellowish mucoid material on hypersucrose medium (5% sucrose nutrient agar), producing fluorescent pigments on King's medium B, were isolated from all symptomatic plants. Based on conventional biochemical and nutritional tests, Biolog assays, and analysis of partial 16S rDNA and *rpoB* sequences, these atypical fluorescent *Pseudomonas* isolates were identified as *Pseudomonas viridiflava*. The pathogenicity of the isolates was ascertained by artificial inoculation on their respective original hosts. The analysis of *rpoB* gene sequences and RAPD-PCR profiles showed a high genetic variability among the *P. viridiflava* isolates. To the best of our knowledge, this is the first report of bacterial diseases on horticultural crops caused by atypical strains of *P. viridiflava* in Italy and the first report of the bacterium on safflower. In addition, *P. viridiflava* is reported for the first time as a causal agent of leaf spot on potato (*Solanum tuberosum*). The economic importance of chicory and curly type lettuce in southern Italy and recent spread of atypical and highly virulent *P. viridiflava* strains require more extensive and careful phytosanitary surveillance, as well as an adjustment of sustainable crop protection management to cope with this new challenge.

KEYWORDS
16S rDNA, LOPAT, *Pseudomonas syringae* species complex, RAPD-PCR, RFLP, *rpoB*

1 | INTRODUCTION

Since 1997, severe bacterial disease symptoms have been frequently observed during surveys carried out in farms with field-grown Molfettese Catalogna chicory (*Cichorium intybus* var. *sylvestre*, *Catalogna* group), sugarloaf chicory (*C. intybus* var. *porphyreum*), curly type lettuce (*Lactuca sativa* var. *crispa*) cvs Langero and Aleppo, and safflower (*Carthamus tinctorius*) in the Apulia and Basilicata regions of southern Italy. Symptoms differed depending on the host plant. Curly type lettuce and sugarloaf chicory showed

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water-soaked, dark-brown angular leaf spots located mainly along leaf margins. Necrotic streaks and rotting on the midrib were observed on Catalogna chicory. Infected safflower plants showed necrotic angular leaf spots, necrotic streaks on the midrib surrounded by a chlorotic halo, brown discolouration on the stem, stem soft rot, and finally dead plants (Figure 1a–h). Similar symptoms previously observed on curly type lettuce and common chicory in Turkey and Greece were caused by *Pseudomonas viridiflava* (Aksoy et al., 2018; Trantas et al., 2022).

Pseudomonas species are ubiquitous bacteria including important pathogens to animals, insects and plants (Lelliott et al., 1966). The pectinolytic species *P. viridiflava* was originally isolated in Switzerland from dwarf or runner bean, causing necrotic lesions on leaves and stems, as well as rots on basal stems and roots (Burkholder, 1930). This species causes diseases in many plants of agronomic and economic importance, as those belonging to the families *Asteraceae* (Nuebling et al., 2016; Trantas et al., 2022), *Brassicaceae* (Aksoy et al., 2017; Myung et al., 2010; Stravato & Buonaurio, 1997), *Cucurbitaceae* (Al-Karablieh et al., 2017; Aysan et al., 2003; Mirik et al., 2004), *Solanaceae* (Goumas et al., 1999; Goumas & Chatzaki, 1998; Jones et al., 1984; Popović et al., 2015; Sarris et al., 2012) and other herbaceous and arboreal plant species (Alippi et al., 1999; Almeida et al., 2013; Balestra et al., 2008; Beltrán et al., 2023; González et al., 2012; Odasso et al., 2012; Patel et al., 2023; Ramírez-Razo et al., 2022; Suslow & McCain, 1981; Varvaro et al., 1990).

The LOPAT determinative assay (where L is levan production; O is oxidase production; P is pectinolytic activity; A is arginine dihydrolase production; T is tobacco hypersensitive response) is the most widely used protocol for the differentiation of plant-pathogenic *Pseudomonas* (Lelliott et al., 1966). Since 1999, *Pseudomonas* isolates with an atypical LOPAT profile (L: convex colonies with uncharacteristic yellowish mucoid material on hypersucrose medium; O:

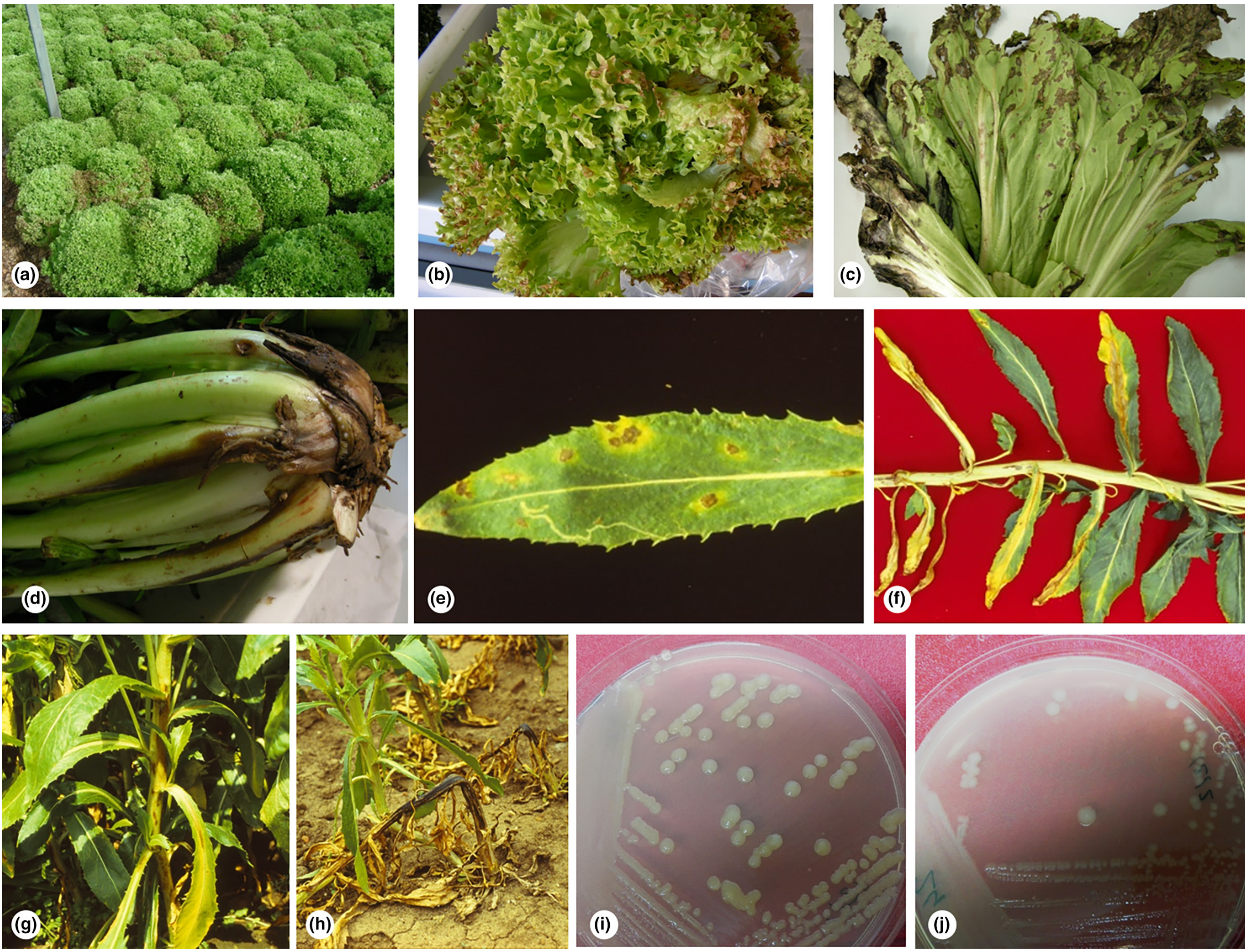

FIGURE 1 Symptoms caused by *Pseudomonas viridiflava* on different host plants: Distribution of infected plants in a curly type lettuce field (a); water-soaked, dark-brown angular leaf spot mainly along the leaf margins of curly type lettuce (b) and sugarloaf chicory (c); necrotic streaks and rotting on the midrib of Catalogna chicory (d); necrotic angular leaf spots (e) on safflower plants with necrotic streaks on the midrib surrounded by chlorotic halo (f), brown discolouration on the stem (g) stem soft rot and death (h). *P. viridiflava* colony morphology on hypersucrose medium showing convex colonies of an atypical isolate with yellowish mucoid material (i); white flat colonies of the typical *P. viridiflava* strain NCPPB 635 (j).

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negative; P: variable; A: negative; and T: positive) were detected in Spain, where they caused severe losses on common bean (*Phaseolus vulgaris*), kiwifruit (*Actinidia deliciosa*) and lettuce (*L. sativa*). The isolates were identified as *P. viridiflava* based on 16S rRNA sequences (González et al., 2003). More recently, *P. viridiflava* isolates with an atypical LOPAT profile were reported to cause bacterial leaf spot on rapeseed in South Korea (Myung et al., 2010).

Pseudomonas viridiflava is phylogenetically placed within the *Pseudomonas syringae* species complex (Lipps & Samac, 2022). Initially, it was treated as *P. syringae* and later separated by 16S rDNA analysis (Anzai et al., 2000). Although the analysis of 16S rDNA sequences distinguishes interspecies differences, it is not sufficient for studying intraspecies genetic variation (Yamamoto & Harayama, 1998). Phylogenetic analysis using nucleotide sequences of housekeeping genes, such as *gyrB*, *rpoB* and *rpoD*, providing a higher resolution, can reveal intraspecies variability (Sarris et al., 2012). Moreover, DNA–DNA hybridization, molecular fingerprinting and comparisons of whole-genome sequences were used to differentiate *P. viridiflava* from other species within the *P. syringae* species complex (Baltrus et al., 2017; Berge et al., 2014; Bull & Koike, 2015; Dillon et al., 2019; Heydari et al., 2014; Lipps & Samac, 2022).

In this study, atypical *P. viridiflava* isolates collected from different host plants over several years in the Apulia and Basilicata regions (southern Italy) were identified and characterized based on morphological, biochemical, and molecular analyses. Additionally, the genetic variability among the isolates was investigated through the analysis of the *rpoB* gene sequences and random amplified polymorphic DNA (RAPD)-PCR.

2 | MATERIALS AND METHODS

2.1 | Plant material and bacterial isolation

Symptomatic leaves and/or stems were collected from plants of Catalogna chicory, curly type lettuce, sugarloaf chicory and safflower (Figure 1a–h). Samples were rinsed with tap water, surface-decontaminated with sodium hypochlorite (1% available chloride) for 2 min and washed four times with sterile distilled water. The edges of symptomatic tissues were aseptically cut into small pieces (about 5 mm), finely ground in a drop of sterile distilled water, streaked on 5% sucrose nutrient agar medium (SNA; hypersucrose medium), and incubated in the dark at 25 ± 1°C up to 48 h. Only single convex, yellowish mucoid colonies were isolated from symptomatic tissues. The colonies were subcultured onto SNA to obtain pure cultures. These were streaked on King's medium B plates (King et al., 1954), incubated for 48 h at 25 ± 1°C in the dark, and observed for fluorescence under UV light at 365 nm. Pure colonies were stored in nutrient agar (NA) slant tubes at 4°C for short-term storage and in 20% glycerol at −80°C for long-term conservation. One representative isolate for each surveyed field was selected and subjected to subsequent analyses.

2.2 | Biochemical and nutritional characterization

The LOPAT assay, according to Lelliott and Stead (1987), was performed on seven representative atypical isolates obtained from naturally infected hosts, two reisolates from pathogenicity tests from this study, two typical strains of *P. viridiflava* (the local strain DiSSPA Pv37 obtained from potato leaf spots and the NCPPB 635 type strain) as well as the NCPPB 281 type strain of *P. syringae* pv. *syringae*, two local strains of *Pseudomonas cichorii* and one of *Pseudomonas marginalis* (Table 1). These selected isolates and strains were also characterized for their ability to perform gelatine hydrolysis, 2-ketogluconate and nitrate reduction and to utilize L(−) lactate, D(−)tartrate, L(−)tartrate, sorbitol, erythritol, inositol, mannitol, cellobiose, trehalose, rhamnose, D(−)arabinose, adonitol, betaine and malonate as carbon sources (Schaad et al., 2001). Furthermore, the same isolates and strains were assayed with the Biolog Microbial Identification System (Biolog Inc.) using 96 well-GEN III microplates. After 48 h at 25°C, the results were analysed by GEN III MicroLog M system software.

2.3 | Molecular identification

Genomic DNA was extracted from 24-h Luria Bertani broth bacterial cultures according to the protocol described by Marmur (1961) and used in PCRs to amplify the partial sequences of 16S rDNA and the *rpoB* gene using the two primer pairs fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′)/rD1 (5′-AAGGAGGTGATCCAGCC-3′), and LAPS (5′-TGGCCGAGAACCAGTTCCGCGT-3′)/LAPS27 (5′-CGGCTTCGTCCAGCTTGTTCAG-3′) (Weisburg et al., 1991; Whitelaw-Weckert et al., 2011).

PCRs contained 5 μL of 10× LA Taq buffer, 2.5 mM $MgCl_2$, 1.6 mM dNTPs mixture, 200 nM of each primer, 2.5 U of Takara LA Taq DNA polymerase, 150 ng of template DNA and ultrapure water up to 50 μL. Amplifications were carried out in a MyCycler thermocycler (Bio-Rad) using the following PCR conditions: 94°C for 3 min; followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; with a final step at 72°C for 10 min.

PCR products were purified using PCR Kleen Purification Spin Columns (Bio-Rad) and custom-sequenced (Genewiz, Takeley, UK). For molecular identification, the 16S rDNA and *rpoB* gene sequences were compared using BLASTn with those present in GenBank (https://www.ncbi.nlm.nih.gov/).

Restriction fragment length polymorphism (RFLP) analyses were performed by digesting an aliquot of the 16S rDNA amplicons with the restriction enzymes SacI and HinfI (New England Biolabs) according to the manufacturer's instructions. The size of the digested amplicons, separated by electrophoresis in 1.5% agarose gel with added GelRed (Biotium) in TAE buffer, was estimated by comparing with the molecular standard 50–2000 bp DNA ladder (Bio-Rad). Gel images were recorded and analysed by using Gel Doc 1000 and Quantity One software v. 4 (Bio-Rad).

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TABLE 1 *Pseudomonas* isolates and strains used in this study.

No.	Species	Code	Host plant	Location (year of isolation)	*rpoB* gene sequence accession no.
1	*Pseudomonas viridiflava*	DiSSPA Pv37[a,b,d,f]	*Solanum tuberosum*	Conversano, Apulia, Italy (2004)	OR340628
2		DiSSPA Pv1[a,b,c,d,g]	*Carthamus tinctorius*	Gaudiano, Basilicata, Italy (1997)	OR340621
3		DiSSPA Pv28[a,b,c,d,g]	*Cichorium intybus* var. *porphyreum*	Metaponto, Basilicata, Italy (2009)	OR340622
4		DiSSPA Pv29[a,b,c,d,g]	*Lactuca sativa* var. *crispa*	Polignano a Mare, Apulia, Italy (2015)	OR340623
5		DiSSPA Pv30[a,b,c,d,g]	*L. sativa* var. *crispa*	Polignano a Mare, Apulia, Italy (2016)	OR340624
6		DiSSPA Pv31[a,b,c,d,g]	*L. sativa* var. *crispa*	Polignano a Mare, Apulia, Italy (2017)	OR340625
7		DiSSPA Pv33[a,b,c,d,g]	*L. sativa* var. *crispa*	Polignano a Mare, Apulia, Italy (2017)	OR340626
8		DiSSPA Pv35[a,b,c,d,g]	*C. intybus* var. *sylvestre*	Molfetta, Apulia, Italy (2016)	OR340627
9		NCPPB 635[a,b,c,d,e,f]	*Phaseolus* sp.	Switzerland (1930)	AJ717483
10		PV570[g]	*Acanthus mollis*	Heraklion, Crete, Greece	JQ267558.1
11		PV574a[g]	*A. mollis*	Heraklion, Crete, Greece	JQ267554.1
12		PV527[g]	*Amaranthus blitum*	St. Pelagia, Crete, Greece	JQ267560.1
13		PV271[a,g]	*Apium graveolens*	Heraklion, Crete, Greece	JQ267553.1
14		PV272[g]	*A. graveolens*	Heraklion, Crete, Greece	JQ267548.1
15		PV272a[g]	*A. graveolens*	Heraklion, Crete, Greece	JQ267555.1
16		PV273[a,g]	*A. graveolens*	Heraklion, Crete, Greece	JQ267550.1
17		PV274[g]	*A. graveolens*	Heraklion, Crete, Greece	JQ267557.1
18		LMG2354[g]	*Chrysanthemum* sp.	United Kingdom	AJ748184.1
19		PV612[a,g]	*Cucumis melo*	Tympaki, Crete, Greece	JQ267551.1
20		PV608[g]	*Cynara cardunculus* var. *scolymus*	Heraklion, Crete, Greece	JQ267546.1
21		PV609[g]	*C. cardunculus* var. *scolymus*	Heraklion, Crete, Greece	JQ267547.1
22		KB13[g]	*Prunus armeniaca*	Iran	MW316888.1
23		KB20[g]	*Prunus persica*	Iran	MW316889.1
24		PV441[g]	*Solanum lycopersicum*	Tympaki, Crete, Greece	JQ267544.1
25		PV442[g]	*S. lycopersicum*	Tympaki, Crete, Greece	JQ267545.1
26		TKK615[g]	*S. lycopersicum*	Antiskari, Crete, Greece	JQ267549.1
27		UYT52018[g]	*S. lycopersicum*	Uruguay	OL862524.1
28		UYT62017[g]	*S. lycopersicum*	Uruguay	OL862523.1
29		UYT42017[g]	*S. lycopersicum*	Uruguay	OL862522.1
30		PV3005[g]	*Solanum melongena*	Ierapetra, Crete, Greece	JQ267559.1
31		PV3006[g]	*S. melongena*	Ierapetra, Crete, Greece	JQ267561.1
32	*Pseudomonas cichorii*	DiSSPA Pci5[a,b,d]	*L. sativa* var. *capitata*	Metaponto, Basilicata, Italy (1999)	OR340629
33		DiSSPA Pci6[a,b,d]	*L. sativa* var. *capitata*	Metaponto, Basilicata, Italy (1999)	OR340630
34	*Pseudomonas marginalis*	DiSSPA Pm8[a,b,d]	*C. cardunculus* var. *scolymus*	Polignano a Mare, Apulia, Italy	OR340631
35	*Pseudomonas syringae* pv. *syringae*	NCPPB 281[a,b,c,d,e]	*Syringa vulgaris*	United Kingdom	FN554759.1

Note: Isolates and strains used in [a]bacterium identification; [b]biochemical and nutritional tests; [c]pathogenicity test; [d]molecular genetic analyses; [e]type strain; [f]typical *P. viridiflava* isolates; [g]atypical *P. viridiflava* isolates.

Abbreviations: DiSSPA, Department of Soil, Plant and Food Sciences, University of Bari Aldo Moro, Bari, Italy; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, United Kingdom; strains labelled with PV, LMG, KB, TKKK and UYT from GenBank (https://www.ncbi.nlm.nih.gov/).

2.4 | Pathogenicity test

The pathogenicity of seven *P. viridiflava* isolates showing atypical LOPAT patterns was assessed by artificial inoculation on their respective original hosts. Two-day-old bacterial cells grown on nutrient agar (NA) at 25±1°C in the dark were suspended in sterile distilled water to obtain about 10^6 cfu/mL (optical density at 550 nm = 0.1). For curly type lettuce and sugarloaf chicory, 1-month-old plants were sprayed with the bacterial suspension and covered with a transparent plastic bag for the initial 48 h to ensure 100% relative humidity. Leaf midribs of 6-week-old Catalogna chicory plants and stems of safflower plants about 20 cm in height were inoculated with 10 μL of the bacterial

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suspension using a hypodermic needle. To compare the severity of symptoms induced by the seven atypical isolates with two typical strains of *P. viridiflava* (Table 1), these were also prick-inoculated in the midrib of common chicory as described above. Ten replicated plants were used for each combination. Ten plants of each host were mock inoculated with sterile distilled water and used as controls. All plants were maintained in a glasshouse at 25±3°C with a light period of 16h per day. Symptom development was assessed up to 20days post-inoculation (dpi). All experiments were repeated twice.

2.5 | Analysis of genetic variability

Intraspecific variation was evaluated using seven atypical *P. viridiflava* isolates (Table 1), the typical local isolate, and the type strain NCPPB 635. *P. cichorii*, *P. marginalis* and *P. syringae* pv. *syringae* strains were used as outgroups in the phylogenetic analysis.

Partial sequences of the *rpoB* gene from atypical isolates were compared with all the *rpoB* sequences of *P. viridiflava* available in GenBank. After the alignment, carried out in MEGA 6 using MUSCLE (Tamura et al., 2013), the sequences were trimmed, and phylogenetic analysis was performed using maximum parsimony (MP) with the subtree-pruning-regrafting (SPR) algorithm and maximum likelihood with Tamura–Nei model (ML) methods.

Moreover, the same isolates were submitted to RAPD-PCR analysis using 15 random primers: Opa-06, Opa-9, Opa-12, Opa-13, Opa-14, Opa-16, Opa-17, Opa-19, Opa-20, Opb-05, Opb-08, Opb-15, Opb-18, Opd-02 and Opd-04 (Operon Technologies, Inc.). The PCR mixture contained 5µL of Go Taq Flexi buffer (Promega), 2.5mM $MgCl_2$, 0.8mM dNTPs mixture, 200nM of primer, 0.15µL of Go Taq DNA polymerase (Promega), 20ng of template DNA and ultrapure water up to 25µL. Amplifications were carried out in a MyCycler thermocycler (Bio-Rad) using the following PCR conditions: 94°C for 3min; followed by 40cycles of 94°C for 1min, 37°C for 1min, and 72°C for 2min; with a final extension at 72°C for 10min. Amplified PCR products were run on 1.5% agarose gel and analysed as described above. The positions of unequivocally scorable RAPD bands were transformed into a binary character matrix ("1" for the presence and "0" for the absence of a band at a particular position). The genetic similarity among strains was calculated based on Jaccard's coefficient with the SIMQUAL program of NTSYS pc software v. 2.1 (Rohlf, 2000). A dendrogram was generated using the unweighted pair group method with arithmetic average (UPGMA) and the sequential agglomerative hierarchical nested cluster analysis (SHAN) methods in the NTSYS software.

3 | RESULTS

3.1 | Isolation, biochemical and nutritional characterization

Atypical fluorescent *Pseudomonas* colonies with LOPAT profiles resembling that of *P. viridiflava*, except for morphology and pigmentation in the L test, were exclusively isolates from symptomatic plants. These colonies were convex with mucoid material of yellowish colour on hypersucrose medium, in contrast to the white flat colonies of the *P. viridiflava* typical strains NCPPB 635 (type strain) and DISSPA Pv37 (Table 2). The colony morphology of the isolates differed also from the levaniform colonies of *P. marginalis* (DiSSPA Pm8) and *P. syringae* pv. *syringae* (type strain NCPPB 281) and from the flat and white colonies of *P. cichorii* strains DiSSPA Pci5 and DiSSPA Pci6 (Table 2). The atypical and typical *P. viridiflava* isolates and strains were oxidase negative, potato rot positive, arginine dihydrolase negative and tobacco hypersensitive reaction (HR) positive (Table 2). Furthermore, the same isolates and strains hydrolysed gelatin, were not able to reduce nitrates or produce 2-ketogluconate and were able to use L(−)lactate, D(−)tartrate, sorbitol, erythritol, mannitol, betaine, and malonate, but not L(+)tartrate, cellobiose, trehalose, rhamnose, D(−)arabinose, and adonitol (Table 2). Using the Biolog Microbial Identification System, after 48h at 25°C, the atypical isolates and the typical strains showed a similarity index ranging from 0.60 to 0.70 with *P. viridiflava*.

3.2 | Molecular identification

For all isolates, 16S rDNA amplification generated a fragment of 1059bp (NCBI accession nos. OR295617–OR295624). According to BLASTn analysis, the 16S rDNA of the *P. viridiflava* typical strain DiSSPA Pv37 and seven atypical isolates showed 99.8%–100% identity with the type strain NCPPB 635 (NR_117825.1).

The atypical and typical isolates yielded a profile more like that of *P. viridiflava* than those of *P. syringae* pv. *syringae* (NCPPB 281) and *P. marginalis* (DiSSPA Pm8) in the ranges 700–1500bp and 300–1500bp in the SacI- and the HinfI-mediated RFLP assays of 16S rDNA. In addition, *P. cichorii* (DiSSPA Pci5 and DiSSPA Pci6) showed the same profile as *P. viridiflava* (Figure 2).

The *rpoB* sequences of both *P. viridiflava* atypical isolates and the typical local isolate DiSSPA Pv37 (1042bp, Table 1) showed 98.4%–99.0% identity with the type strain NCPPB 635 (AJ717483.1) and identities between 99.2% and 100% with other *P. viridiflava* strains (e.g., JQ267560.1, JQ267558.1, JQ267554.1 and JQ267555.1).

3.3 | Pathogenicity test

All the LOPAT-atypical *P. viridiflava* isolates reproduced the symptoms observed in the field on their respective hosts at 5 dpi on Catalogna chicory, 7 dpi on curly type lettuce and sugarloaf chicory and 20 dpi on safflower. No symptoms were observed in the control mock-inoculated plants. Moreover, the same isolates caused more severe symptoms on common chicory than the typical strains DiSSPA Pv37 and NCPPB 635. In particular, among the atypical isolates, DiSSPA Pv1, isolated from safflower, caused the most severe symptoms (Figure 3). Colonies reisolated from artificially inoculated plants exhibited the same morphological, biochemical and molecular traits as those used for inoculation.

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TABLE 2 Comparison of atypical *Pseudomonas viridiflava* isolates recovered from different host plants in southern Italy with typical strains and other fluorescent *Pseudomonas* species through differential nutritional and biochemical tests.

Test	*Pseudomonas viridiflava*												*Pseudomonas* spp. strains[a]			
	Atypical isolates										Typical strains					
	DiSSPA Pv1	DiSSPA Pv1R[b]	DiSSPA Pv28	DiSSPA Pv29	DiSSPA Pv30	DiSSPA Pv31	DiSSPA Pv33	DiSSPA Pv33R[c]	DiSSPA Pv35	DiSSPA Pv35R[c]	DiSSPA Pv37	NCCPB 635[c]	DiSSPA Pci5	DiSSPA Pci6	DiSSPA Pm8	NCCPB 281[c]
Yellowish mucoid material[d]	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–
LOPAT																
Levan formation	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+
Oxidase reaction	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–
Potato rot capability	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–
Arginine dihydrolase	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Tobacco hypersensitivity	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
Gelatin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+
2-ketogluconate production	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Nitrate reduction	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
L (–) lactate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D (–) tartrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–
L (+) tartrate	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–
Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+
Used for growth																
Inositol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Erythritol	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cellobiose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	na[e]
Trehalose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	na
Rhamnose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	na
D (–) arabinose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Adonitol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Betaine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Malonate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	na

[a]Isolates belonging to *Pseudomonas cichorii* (DiSSPA Pci5 and DiSSPA Pci6), *Pseudomonas marginalis* (DiSSPA Pm8) and *Pseudomonas syringae* pv. *syringae* (NCCPB 281).
[b]Reisolated from artificially inoculated host plants.
[c]Type strains.
[d]On SNA medium.
[e]na: not assayed.

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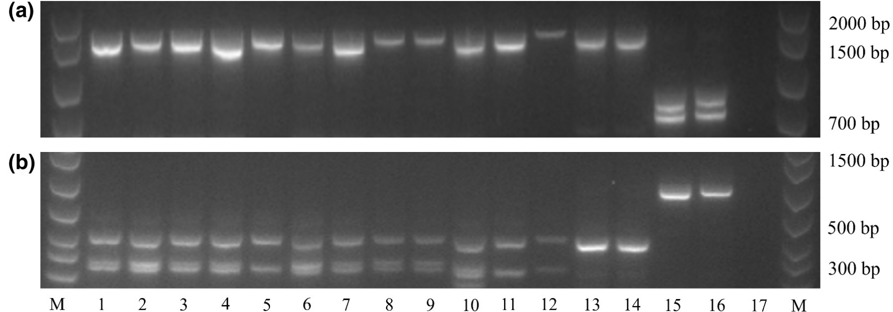

FIGURE 2 Restriction profiles of SacI (a) and HinfI (b) digestion of 16S rDNA fragments from isolates of *Pseudomonas viridiflava* (lane 1: DiSSPA Pv37, typical isolate; Lanes 2–11: DiSSPA Pv1, DiSSPA Psv1R, DiSSPA Pv28, DiSSPA Pv29, DiSSPA Pv30, DiSSPA Pv31, DiSSPA Pv33, DiSSPA Psv33R, DiSSPA Pv35, DiSSPA Psv35R, atypical isolates; Lane 12: NCPPB 635 type strain), *P. cichorii* (lanes 13, 14: DiSSPA Pci5, DiSSPA Pci6), *P. marginalis* (lane 15: DiSSPA Pm8), and *P. syringae* pv. *syringae* (lane 16: NCPPB 281). Lane M: 50–2000 bp DNA ladder Bio-Rad molecular standard; lane 17: Negative control.

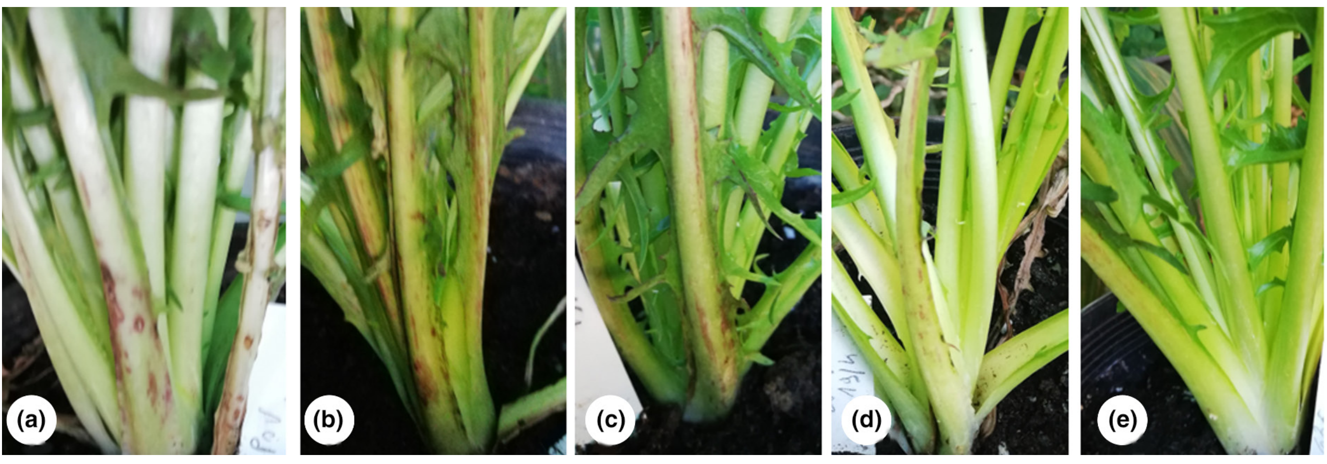

FIGURE 3 Symptoms induced by prick-inoculated *Pseudomonas viridiflava* atypical isolates DiSSPA Pv1 (a) and DiSSPA Pv29 (b) and the typical strains DiSSPA Pv37 (c) and NCPPB 635 (d) on common chicory plants compared with mock-inoculated control (e).

3.4 | Analysis of genetic variability

According to *rpoB*-based phylogenetics using the ML method, a tree with the highest log likelihood of 1669.63 was obtained. The most parsimonious tree obtained with MP (tree length = 140; consistency index = 0.900; retention index = 0.939; composite index = 0.852) was like that obtained with ML (Figure 4). Branch lengths were calculated based on 34 nucleotide substitutions. Both analyses showed that the atypical isolate from safflower (DiSSPA Pv1) did not group with any other Italian isolates collected in this study. In more detail, the other Italian isolates subclustered as follows: (i) the isolates DiSSPA Pv30 and DiSSPA Pv31 (atypical, lettuce), DiSSPA Pv35 (atypical, chicory) and DiSSPA Pv37 (typical, potato) formed a clade with the Iranian isolates KB13 (apricot) and KB20 (peach); (ii) the isolate DiSSPA Pv28 (atypical, chicory) formed a clade with the Greek isolates PV272, PV272a and PV274 (the so-called celery group 1 by Sarris et al., 2012); (iii) the isolates DiSSPA Pv29 and DiSSPA Pv33 (atypical, lettuce) clustered together (Figure 4).

All the products amplified with the Op-primers showed polymorphic and distinguishable banding patterns. A total of 100 reproducible and scorable markers were generated by RAPD-PCR with Op-primers. The highest number of bands (6) was obtained with the primer Opd-2, while the lowest (1) was achieved using the Opa-17 primer (Figure 5a). The Jaccard's similarity index was estimated among the seven *P. viridiflava* atypical isolates; the two typical strains (DiSSPA Pv37 and NCPPB 635) and the strains of *Pseudomonas* species (*P. cichorii*, *P. marginalis* and *P. syringae* pv. *syringae*) used as outgroup. The similarity coefficient obtained by examination of RAPD profiles of all sets ranged from 0.08 to 0.84. The similarity coefficient ranged from 0.50 to 0.84 for the *P. viridiflava* isolates (Figure 5b). According to the dendrogram, a greater similarity was observed for the atypical isolates obtained from lettuce in Polignano a Mare (DiSSPA Pv29, DiSSPA Pv30, DiSSPA Pv31 and DiSSPA Pv33), from safflower in Gaudiano (DiSSPA Pv1), and the typical local strain (DiSSPA Pv37) obtained from potato in Conversano. This group of isolates was distinguishable from the type strain NCPPB 635 and the atypical isolates DiSSPA Pv28 and

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FIGURE 4 Phylogenetic tree of *rpoB* sequences of *Pseudomonas viridiflava* isolates generated from maximum likelihood (ML) and maximum parsimony (MP) analysis. Figures on the node represent the percentages (ML/MP) of concordant trees in the bootstrap test with 1000 replicates. *Pseudomonas marginalis* DiSSPA Pm8, *P. cichorii* DiSSPA Pci5 and *P. syringae* pv. *syringae* NCPPB 281 were used as outgroup. Isolates from this study are highlighted in bold.

DiSSPA Pv35 obtained from chicory in Metaponto and Molfetta, respectively (Figure 5b).

4 | DISCUSSION

The fluorescent gram-negative bacterium *P. viridiflava* has a worldwide distribution and is well known as a multihost pathogen affecting over 50 different plants in the monocot and dicot clades (Lipps & Samac, 2022). The wide host range of *P. viridiflava* is partially explained by the production of pectate lyases, encoded by the *pel* genes, as pathogenicity factors causing pectin degradation in plant cell walls, soft rot and maceration of plant tissues (Liao, 1991; Liao et al., 1988; Lipps & Samac, 2022). *P. viridiflava* pathogenicity in some cases was, however, reported as strain-specific. For example, a *P. viridiflava* strain obtained from the dark-reddish spot in soybean was unable to cause disease on common bean (González et al., 2012), the opposite to *P. viridiflava* isolates obtained from other host plants that were non-host-specific (Morris et al., 2019). In addition, examples of cultivar-specific resistance to *P. viridiflava* have been reported for plum cultivars, with Sapphire and Songold being susceptible (Bophela et al., 2020), in contrast to the cv. Marina GF8-1, in which no disease was observed in a host range test (Morris et al., 2019).

During the present survey, isolates from diseased plants of safflower, curly type lettuce, sugarloaf and Catalogna chicory grown on SNA were exclusively atypical fluorescent *Pseudomonas* species. The LOPAT profiles of these isolates were similar to *P. viridiflava* except for their colony morphology and pigmentation in the L test; they were characterized by convex colonies with yellowish mucoid material on SNA in contrast to the white flat colonies of the typical *P. viridiflava* strains. These atypical isolates were confirmed as belonging to the *P. viridiflava* species by differential nutritional and biochemical tests, the Biolog microbial identification system, and BLASTn analyses of 16S rDNA and *rpoB* sequences. Indeed, atypical and typical *P. viridiflava* isolates and strains showed the same biochemical and

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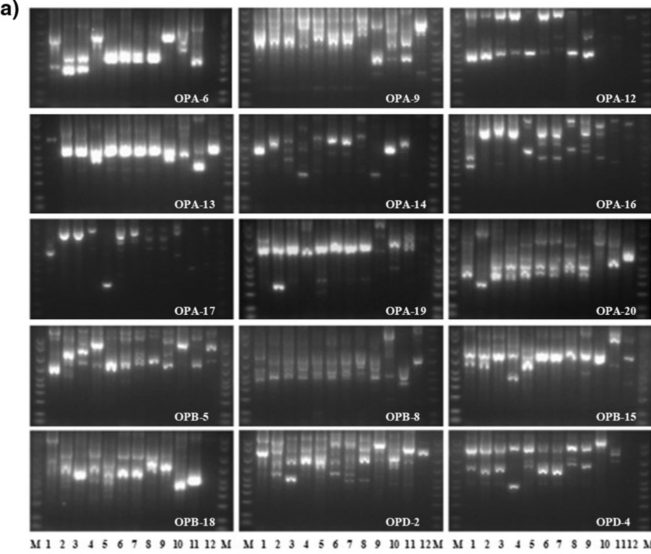

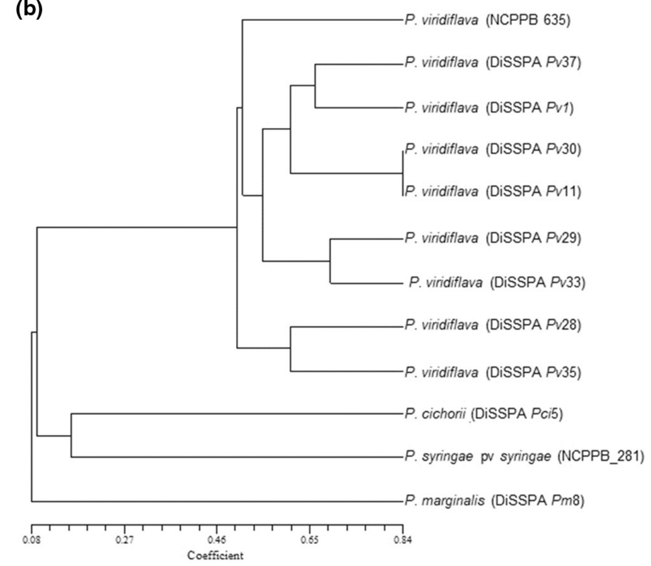

FIGURE 5 RAPD profiles (a) and phylogenetic tree (b) of *Pseudomonas viridiflava*. (a) Lanes 1–9: NCPPB 635 (type strain), DiSSPA Pv37 (typical isolate), and atypical isolates DiSSPA Pv1, DiSSPA Pv28, DiSSPA Pv29, DiSSPA Pv30, DiSSPA Pv31, DiSSPA Pv33 and DiSSPA Pv35; lane 10: *P. cichorii* (DiSSPA Pci5), lane 11: *P. marginalis* (DiSSPA Pm8) and lane 12: *P. syringae* pv. *syringae* (NCPPB 281). M: 50–2000 bp DNA ladder Bio-Rad molecular standard. (b) Jaccard's similarity coefficient was estimated among the *P. viridiflava* isolates and strains by examination of RAPD profiles, and *P. cichorii*, *P. marginalis*, and *P. syringae* pv. *syringae* were used as the outgroup. The dendrogram was generated using the unweighted pair group method with arithmetic average (UPGMA) and the sequential agglomerative hierarchical nested cluster analysis (SHAN) methods.

nutritional profile. In the same way, the Biolog microbial identification system yielded a similarity index from 0.60 to 0.70 with *P. viridiflava*. Partial 16S rDNA sequences of both atypical and typical *P. viridiflava* strains showed BLASTn identities higher than 99% with other *P. viridiflava* strains (e.g., 10Kf2, CFBP 1590 and X1) well characterized in other studies (Jakob et al., 2002; Liu et al., 2019; Ruinelli et al., 2017). In agreement with González et al. (2003), SacI- and the HinfI-mediated RFLP patterns of 16S rDNA showed a profile similar to *P. viridiflava*. The *rpoB* gene sequences showed 98.7%–99.0% identity with the type strains NCPPB 635 (AJ717483.1) and 99.2%–100% with the *P. viridiflava* strains PV3006, PV570, PV574a and PV272a available in GenBank.

Pathogenicity assays performed with seven representative isolates of the atypical *P. viridiflava* isolates confirmed their role as responsible for the disease on their respective hosts, and reisolated bacteria exhibited the same morphological, biochemical and molecular traits as those used for inoculation.

In addition to differing in their pathogenicity and host range, *P. viridiflava* isolates differ from *P. syringae* in two of the tests (L and P) in the LOPAT scheme. *P. viridiflava* is typically levan-negative, forming flat colonies on hypersucrose medium, and potato rot positive, whereas *P. syringae* is levan-positive, forming “levan type” colonies on hypersucrose medium, and potato rot negative. Nevertheless, the production of exopolysaccharides by the atypical mucoid *P. viridiflava* isolates may increase their tolerance to plant defence mechanisms; in addition, the pectinolytic ability of these strains could play an important role in releasing sugars to support bacterial colonization (Bartoli et al., 2014). It has been hypothesized that *P. viridiflava* strains are probably plastic in their mucoid and nonmucoid phenotypes, but the genetic mechanisms underlying phase switching are unknown (Lipps & Samac, 2022).

Atypical *P. viridiflava* isolates were first reported in northern Spain on common bean and weed plants present in the same fields, kiwifruit and lettuce (Fernández-Sanz et al., 2022; González et al., 2003) and in South Korea on rape (Myung et al., 2010). *P. viridiflava* was recently isolated from leaf spots of common and spiny chicory in Crete (Greece), but those isolates showed a typical LOPAT profile (Trantas et al., 2022). To the best of our knowledge, the atypical *P. viridiflava* isolates described in this study have not been previously reported in Italy. Moreover, this is the first report of safflower as a new natural host of *P. viridiflava*. In addition, we report for the first time the occurrence of *P. viridiflava* as an aetiological agent of bacterial leaf spot on potato (*Solanum tuberosum*).

As to the origin of the atypical lineage in *P. viridiflava* populations, it can be supposed that parental levan-negative bacteria could have acquired the ability to produce exopolysaccharide, which may improve epiphytic fitness and play a role as a virulence factor (González et al., 2003). These features might be perhaps attribute to horizontal gene transfer that occurs frequently for most strains of the *P. syringae* complex (Lipps & Samac, 2022).

González et al. (2003) and Myung et al. (2010) reported that atypical *P. viridiflava* isolates showed variable pectinolytic activity ranging from negative or very weak to relatively strong. All our atypical strains were positive in the potato rot test, showing a pectinolytic activity like that of typical strains of the pathogen. Our atypical isolates were more virulent than typical *P. viridiflava* strains. This was highlighted by the pathogenicity test on common chicory in which all the atypical *P. viridiflava* isolates caused

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more serious symptoms than the typical strains. Moreover, we recorded a disease incidence greater than 60%–70% in all the surveyed fields where atypical *P. viridiflava* isolates were found and the symptoms were particularly severe in safflower crops, where the disease induced plant death. In the fields where atypical isolates were obtained, the disease severity was much higher than that observed on the potato field, where only typical isolates were found. In addition, in the pathogenicity assay on common chicory, the atypical isolates were more aggressive than the typical strains NCPPB 635 and DiSSPA Pv37.

The genetic variability in phytopathogenic bacteria can be explored using a multitude of molecular approaches, such as REP-PCR, ERIC-PCR or BOX-PCR, RAPD-PCR, analysis of conserved sequences (e.g., *gyrB*, *rpoB* and *rpoD*), and comparative genomic analysis (Djitro et al., 2022; Gilbert et al., 2009; Prencipe et al., 2018; Sarris et al., 2012). Among the analyses of conserved sequences, 16S rDNA is frequently used for molecular identification at the level of species, but the degree of resolution obtained is not sufficient to reveal intraspecific variations due to the extremely slow rate of rDNA evolution (Yamamoto et al., 2000). We used the *rpoB* gene sequences to compare atypical isolates with *rpoB* gene sequences available in GenBank. From all the examined sequences, 34 informative single-nucleotide polymorphisms (SNPs; two at the same position) were detected. Only small differences were recorded among the atypical *P. viridiflava* isolates from lettuce and chicory, which clustered in a different group from the atypical isolates from safflower and the type strain NCPPB 635. Most of the *P. viridiflava* sequences available in GenBank and used to build the *rpoB*-based MP/ML trees were from Greek isolates, but no differences associated with the country could be found.

RAPD analysis, a common method for obtaining a snapshot of the whole bacterial genome, is suitable for intraspecific population analyses (Martins et al., 2006) and has already been applied to determine genetic diversity among *P. viridiflava* isolates in Iran (Heydari et al., 2014). In our study, a total of 100 reproducible and scorable markers were generated by RAPD PCR with 15 random primers. The dendrogram obtained showed a broad diversity among *P. viridiflava* isolates, which agrees with results previously obtained with different molecular approaches (Goss et al., 2005; Sarris et al., 2012). Nevertheless, a greater similarity was observed among the atypical isolates collected from the same host (lettuce) and location (Polignano a Mare) in two consecutive years (2016 and 2017).

Finally, our results corroborate the broad genetic diversity of *P. viridiflava* that includes bacteria showing endophytic, epiphytic, saprophytic, as well as phytopathogenic habits on a variety of agricultural and wild host plants (Bartoli et al., 2014; Lipps & Samac, 2022). The occurrence of atypical *P. viridiflava* isolates on different hosts highlights the opportunistic ability of the bacterium and its ability to modify virulence and pathogenicity depending on the host plants. This behaviour is suggestive of variation in genes involved in virulence and/or pathogenicity. Recombination and horizontal gene transfer could be the drivers for the broad intraspecific diversity occurring in *P. viridiflava* and the whole *P. syringae* species complex (Dillon et al., 2019; Lipps & Samac, 2022).

Curly type lettuce and chicory represent economically important crops in the Apulia and Basilicata regions of Italy and worldwide. The emergent and very aggressive mucoid variant of *P. viridiflava* could present a problem in crop management due to its destructive effects. The modified environmental conditions induced by climate changes, such as more prolonged periods of mild temperatures in the seasons, frequent rainfalls and high relative humidity, might favour the spread of the bacteria and disease development. Therefore, new, effective, and sustainable crop protection strategies are required to prevent heavy yield losses on susceptible crops caused by *P. viridiflava* and the new variant of the bacterium.

In conclusion, in this study we reported the occurrence of atypical and virulent *P. viridiflava* isolates on chicory, lettuce and safflower. Moreover, this is the first report of the pathogen on safflower and potato. Genetic variability among typical and atypical *P. viridiflava* isolates was assessed by analysis of partial sequences of the *rpoB* gene and RAPD analysis. This last approach, especially, highlighted a broad intraspecific diversity that is worth investigating in more detail. Severe symptoms caused by *P. viridiflava* were recently observed even in the years 2021 and 2022 in common chicory crops in the Lecce province (Apulia, southern Italy), where atypical isolates were obtained and characterized. The results obtained so far suggest a fairly rapid spread of atypical isolates in the field, probably due to their high fitness and virulence compared to the typical strains of the pathogen. Therefore, the preliminary results of this study suggest that atypical isolates are probably replacing at least partially pre-existing typical individuals in the *P. viridiflava* populations; the first obvious outcome of this is a broadening of the host plant spectrum that now also includes potato and safflower. Further monitoring on different host plants is required to confirm the prevalence of *P. viridiflava* atypical strains and their relationship with the higher disease severity observed in this study. Hence, the findings reported in this paper highlight *P. viridiflava* as a relevant threat to sustainable growth of horticultural crops in southern Italy.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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