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Characterisation of Pectinolytic *Bacillus pumilus* and *Paenibacillus amyloliticus* Strains, New Pathogens of Potato in Tunisia

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Abstract: Soft rot disease in potato is a major problem in fields and warehouses all over the world. Although it is known that bacteria from the genera *Pectobacterium* and *Dickeya* are the main causative agents of soft rot diseases, recent studies indicate the involvement of pectinolytic *Bacillus* and *Paenibacillus* in this disease. In the present research, samples of potato with soft rot symptoms were collected from eight governorates of Tunisia. Two hundred and seventy bacterial isolates were acquired from tubers. Twenty of the isolated strains indicted pectinolytic activity by forming deep cavities on crystal violet pectate medium. All pectinolytic isolates were able to macerate potato tuber tissue. Phenotypic characterisation showed that these isolates were Gram-positive bacilli, exhibiting pectinolytic, cellulolytic, proteolytic and amylolytic activity. The majority of the isolates indicated swimming and swarming motility. The application of API test, MALDI-TOF MS and 16S rDNA sequencing allowed for the assignment of nineteen of the tested isolates to the species *Bacillus pumilus* and one to the species *Paenibacillus amyloliticus*. To the best of our knowledge, this is the first report of soft rot in potato caused by pectinolytic *B. pumilus* in Tunisia.

Keywords: soft rot; pectinolytic bacteria; *Bacillus pumilus*; MALDI-TOF MS; emerging pests; epidemiology

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1. Introduction

Solanum tuberosum L. has been widely cultivated in Tunisia as an important vegetable crop. Potato is grown year-round as a seasonal, late-season, early-season and extra-early-season crop. In 2011, this crop covered about 23,200 hectares and the potato production rate in Tunisia reached about 367,000 tonnes [1]. Faced with the multiplicity of growing seasons and different climates in the cultivation areas, potato production is threatened by several serious diseases and pests in the field or during storage. The biotic factors responsible for soft rot diseases in this country are most commonly diagnosed visually, which leads to confusion between rots caused by bacteria in contrast to *Pythium ultimum*, *Fusarium* spp. and *Phytophthora* spp.

The most widely known agents responsible for potato blackleg and soft rot diseases are pectinolytic bacteria from the genera *Dickeya* and *Pectobacterium* [2–4]. Both genera

belong to the recently established *Pectobacteriaceae* family [5], and have been classified among the top ten bacterial pathogens responsible for severe economic damage in multiple crops [6]. The disease symptoms caused by pectinolytic bacteria during vegetation are brown to black discolouration of the stem base starting from below ground level, which is usually accompanied by wet rot. These disorders may aggravate and progress to the upper parts of the stem. In particular, brown discolouration may be recognised inside the stems with sagging pith [7].

Wet rot of the basal part of stem results in wilting, curling and yellowing of the leaves, and subsequently, leads to dieback. In the case of tuber tissue, a moist, grainy soft rot, white to cream in colour and that tends to blacken at the periphery, is observed. In addition, secondary invaders including bacteria and fungi may colonise the macerated tissue, culminating in the development of a characteristic odour [8].

Recent studies performed in Africa, Asia and Europe have shown that apart from *Dickeya* and *Pectobacterium* spp., bacteria from the genus *Bacillus*, especially pectinolytic *Bacillus pumilus*, are capable of causing soft rot in different crops and trees [9–12]. In more detail, *B. pumilus* infections led to the development of soft rot in stored potato tubers in Mali [10], bacterial blotch in peach in Egypt [13], wetwood disorder in young Scots pine trees of Ukraine and Belarus [12], ginger rhizome rot in China [11], leaf blight in mango trees in Egypt [9], bacterial fruit rot in muskmelon in China [14] and *Ficus lacor* in Pakistan [15], Persian oak decline in Iran [16], trunk bulges in rubber trees in Malaysia [17] and interveinal purple-brown leaf spots in common bean in Spain [18]. Additionally, the screening of Belarusian bacterial collections also revealed the occurrence of *B. pumilus*-triggered infections in *Cucumis sativus*, *Solanum lycopersicum*, *Linum usitatissimum*, *Solanum tuberosum* and *Pinus sylvestris* [19]. So far, little attention has been paid to the virulence factors responsible for the pathogenic potential of *B. pumilus* strains [9,10,12–14,17]. To the best of our knowledge, only Evdokimova at al. [18] have reported the activity of plant cell wall-degrading enzymes, i.e., pectinases, cellulases, proteases and lipases, among the investigated plant pathogenic *B. pumilus* isolates.

Considering that the previous research on major bacterial and fungal diseases in potato fields in Tunisia was conducted in the growing seasons of 1993–1994 [20], the objective of this work was to identify, via the MALDI-TOF MS- and 16S rDNA-based approaches, the currently dominating causative agents of soft rot in *S. tuberosum* in this region.

2. Materials and Methods

2.1. Potato Sampling and Isolation of Bacterial Strains and Their Growth Conditions

During the three growing seasons of 2018, 2019 and 2020, soft rot-affected potato tubers from the cultivars 'Spunta', 'Daiffla' and 'Donia' were collected in eight governorates of Tunisia, i.e., Nabeul and Bizerte situated in the north–east and the extreme north of the country, respectively; El Kef, Jendouba and Manouba in the north–west; Kairouan and Sidi Bouzid in the centre; and Gafsa in the south–west. All samples were collected from 12 arbitrarily selected potato fields from the listed governorates. The total number of collected samples was 1001 (126 in 2018, 525 in 2019 and 350 in 2020). During collection, the sampled soft rotten potato tubers were put into sterile plastic bags, properly labelled and transported to the Laboratory of Mycology, Pathologies and Biomarkers at the Faculty of Sciences of Tunis.

The collected tubers were disinfected with 0.5% sodium hypochlorite and subsequently washed with sterile distilled water. From each sample, 0.5 g of the edges of healthy and rotten tubers was cut out, homogenised in 8.75 g/l K_2HPO_4 (BIOMATIQ, Medchal, India) and 6.75 g/l KH_2PO_4 (BIOMATIQ, India) phosphate buffer at pH 7.5 and vortexed for 4 min. Each homogenate was diluted to 10^{-4}, and 100 µL of each dilution was spread on King's B medium (Biolife, Monza, Italy) supplemented with 200 mg/l of cycloheximide (SIGMA, Lezennes, France). After 2 days of incubation at 28 °C, the non-fluorescent colonies were subcultured on King B plates to obtain axenic bacterial colonies. A total of 270 isolates

were collected and kept for a long storage period in 40% glycerol at −80 °C and at 4 °C for short-term use as described before [21].

2.2. Isolation of Pectinolytic Bacteria

The collected bacterial strains were plated on a semi-selective crystal violet pectate (CVP) medium [7] and incubated at 28 °C for 48 h. Pectinolytic bacteria were detected by identifying the formation of cavities around colonies resulting from the degradation of polygalacturonic acid.

2.3. MALDI-TOF MS Analysis

Matrix Assisted Laser Desorption/Ionisation Mass Spectrometry with Time-of-Flight (MALDI-TOF MS) analysis was performed on a MALDI Biotyper® device (Bruker, Billerica, MA, USA) in the Laboratoria Medyczne Bruss (Gdansk, Poland) as a commercial service according to the procedure recommended by the manufacturer. The following classification criteria were applied: a score $\geq$ 2.0 allowed for identification of the species and a score between 1.7 and 1.9 supported genus identification, while a score < 1.70 was considered unreliable for identification purposes. All MALDI-TOF MS spectra for the studied strains were recorded, and two representative spectra for the isolates were identified.

2.4. Sequencing of 16S rDNA

The genomic DNA of the obtained pectinolytic isolates was extracted using a GF-1 Bacterial DNA Extraction Kit (Vivantis Technologies, Heeßen, Germany) according to the manufacturer's instructions. The DNA purity was assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA was stored at −20 °C for long-term preservation or at 4 °C for immediate use.

The 16S rRNA encoding region was PCR-amplified using the universal primer pair forward S1 (5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse S2 (5′-GGMTACCTTGTTACG AYTTC-3′) [22]. The PCR reaction was performed in a total volume of 50 µL containing 50 ng of the template DNA, 0.1 U *Taq* polymerase, 10× *Taq*-buffer, 25 mM $MgCl_2$, 25 mM dNTPs and 2 mM of each primer. The following thermal conditions were used: 95 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 55 °C for 45 s and 72 °C for 90 s, with a final extension at 72 °C for 2 min [22]. PCR was performed using a thermocycler (Biometra TRIO, Analytic Jena, Jena, Germany).

After electrophoretic separation, PCR products were visualised under UV light (Vilber Lourmat TFX-35.MC UV-Lamp, Vilber, Marne-la-Vallée, France) and the size of the amplicons was determined via comparison with a 1 kb DNA ladder (Promega, Madison, WI, USA) before they were sequenced. The obtained nucleotide sequences were compared with the sequences deposited in the GenBank database (National Center for Biotechnology Information; https://www.ncbi.nlm.nih.gov/, accessed on 5 July 2022) with the use of BLAST algorithm. The herein acquired 16S rDNA sequences of *B. pumilus* and *Paenibacillus* sp. strains were submitted to the GenBank database and deposited under the following accession numbers: ON898611, ON899811, ON899941, ON899942, ON900081-ON900086, ON921248-ON921252 and ON922531-ON922535.

2.5. Phenotypic Characterisation of the Isolated Strains

Out of 270 collected isolates, 20 showing pectinolytic activity were selected for further phenotypic studies involving: Gram staining, the examination of oxidase and catalase production, levan production from sucrose, bacterial abilities to macerate potato tuber tissue, the production of plant cell wall-degrading enzymes (PCWDEs), motility, and biochemical tests included in the commercial test kit API20E (bioMérieux, Craponne, France). Two reference strains of established causative agents of soft rot, *D. solani* IFB0099 and *P. parmentieri* IFB5308, originating from the bacterial collection of Laboratory of Plant Protection and Biotechnology IFB UG & MUG (Gdansk, Poland), were used for comparative purposes [23,24].

2.5.1. Bacterial Ability to Macerate Potato Tuber Tissue

Potato tubers were sterilised as mentioned above. Then, the tubers were aseptically cut into 10 mm slices. Subsequently, three holes (5 mm) per slice were made using a sterile cork borer. Bacterial suspensions of each isolate in M63 medium were adjusted to 0.5 McF, and 50 µL of the suspension was added to each hole in the potato slice. The tuber slices were placed in glass Petri dishes on absorbent paper, which had been previously moistened with sterile water. The dishes were incubated in plastic boxes at 28 °C for 48 h with 85–90% relative humidity. The holes designated as negative controls were filled with 50 µL of sterile 0.85% NaCl instead of the bacterial suspension. In terms of positive controls of efficient potato macerators, *Dickeya solani* IFB0099 and *Pectobacterium parmentieri* IFB5308 strains were applied. After 48 h of incubation, the rotten potato tissue was removed and weighed as performed before [25,26]. The experiment was repeated three times, each of which involved two technical replicates.

2.5.2. Production of Extracellular Enzymes and Bacterial Motility

The production of major PCWDEs (pectinases, cellulases, proteases and amylases) and cell motility of the selected isolates were assessed via semi-quantitative plate assays. *D. solani* IFB0099 and *P. parmentieri* IFB5308 were used as reference strains. For each assay, a single 2 µL drop of bacterial suspension, previously adjusted to 0.5 McF in 0.9% NaCl, was spotted on the below-listed solid media plates. All tests were performed three times with four technical replicates.

The activity of pectinases was tested on M63 agar medium supplemented with 0.25% polygalacturonate (PGA). After incubation for 48 h at 28 °C, the plates were flooded with 10% copper acetate, which forms a blue precipitate with PGA. The diameter of the pale white halo around the colony, corresponding to the PGA degradation capacity, was measured as mentioned in Reverchon et al. (1986) [27].

The activity of cellulases was evaluated on M63 agar medium supplemented with 1% carboxymethylcellulose (CMC). After incubation for 48 h at 28 °C, the plates were stained with 1% Congo red solution for 5 min and washed two times with 4 M NaCl. The diameter of the clear zone around the colony, corresponding to the exhibited cellulase activity, was measured [28].

Protease activity was tested on nutrient agar medium supplemented with 1% skim milk. After incubation for 48 h at 28 °C, the diameter of the clear zone around the colony was measured [29].

Amylase activity was evaluated on starch-containing medium. After incubation for 48 h at 28 °C, the amylase production was detected by adding an iodine solution. If the bacteria were able to degrade starch, clear zones around the bacterial colonies appeared [30]. The diameter of the clear zone around the colony was measured.

To evaluate swimming motility, bacterial strains were spotted on Tryptone Soy Agar (TSA) medium solidified with 0.3% agar. After 24 h incubation at 28 °C, the diameters of the bacterial colonies were measured [31].

To evaluate swarming motility, bacterial suspensions were spotted on TSA medium solidified with 0.6% agar. After 24 h of incubation at 28 °C, the diameters of the bacterial colonies were measured [31].

2.5.3. Biochemical Profiling

The oxidase and catalase assays were performed using bacterial colonies collected from TSA agar plates. For the oxidase test, filter paper soaked in a freshly prepared 1% Kovac's oxidase reagent was used [32]. For the catalase test, 3% H_2O_2 was added and examined for the formation of gas bubbles [32]. Levan production from sucrose was determined on nutrient agar containing 5% sucrose. A glucosidase activity test was performed according to Carrim et al. [30]. β-galactosidase, acetoin, gelatinase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, H_2S, urease, indole, acetoin, use of citrate and tartrate, fermentation of glucose, mannitol, inositol, sorbitol, rhamnose,

saccharose, melibiose, amygdalin and arabinose assays were performed using an API 20E test kit (bioMérieux, France) following the manufacturer's instructions.

2.6. Statistical Analysis

Statistical analysis involving analysis of variance (ANOVA), in addition to Pearson's test for the evaluation of the significance of the differences between parameters, was conducted using SAS Institute, Cary, NC USA 2008. The data were depicted as means ± standard deviations following Duncan's multiple-range tests at $p < 0.05$. For each measurement, at least three replicates were performed.

3. Results

3.1. Occurrence of Pectinolytic Bacteria in Potato Fields in the Territory of Tunisia

In a three-year survey (2018, 2019 and 2020), potato tubers with soft rot symptoms were collected from eight different governorates of Tunisia that are well-known for their high potato production rates (Figure 1).

Figure 1. Map of Tunisia showing origin of bacterial isolates acquired from potato tubers with soft rot symptoms. Red regions indicate locations from which pectinolytic bacterial isolates were obtained, green regions represent sites where no pectinolytic bacteria were isolated. GPS locations: (1) 37.17612, 10.00813/Bizerte; (2) 36.6103092, 8.9742237/Jandouba; (3) 36.46917, 10.78222/Nabeul; (4) 36.8506, 9.93608/Manouba; (5) 36.167965, 8.709579/El kef; (6) 35.675914, 10.091924/El Kairouan; (7) 35.035439, 9.483939/Sidi Bouzid; (8) 34.431140, 8.775656/Gafsa.

From a total of 1001 tuber samples obtained for study, 270 bacterial isolates were collected showing different morphological traits. In more detail, 26 strains were isolated in 2018, 160 in 2019 and 84 in 2020 (Supplementary Table S1). All bacterial isolates were screened for pectinolytic activity on a CVP medium. Twenty out of 270 isolates were able to form characteristic deep pits in this selective-differential medium (Figure 2C) and were chosen for further studies. These pectinolytic strains were isolated from soft-rotten potato tubers (cultivars 'Spunta' and 'Daifla') sampled in four Tunisian governorates (Bizerte, Jendouba, Nabeul and Sidi Bouzid) in 2019 and 2020 (Table 1). Most of the selected

pectinolytic isolates were obtained from tubers of the potato cultivar 'Spunta' collected in the Bizerte territory, i.e., 25% and 30% in 2019 and 2020, respectively.

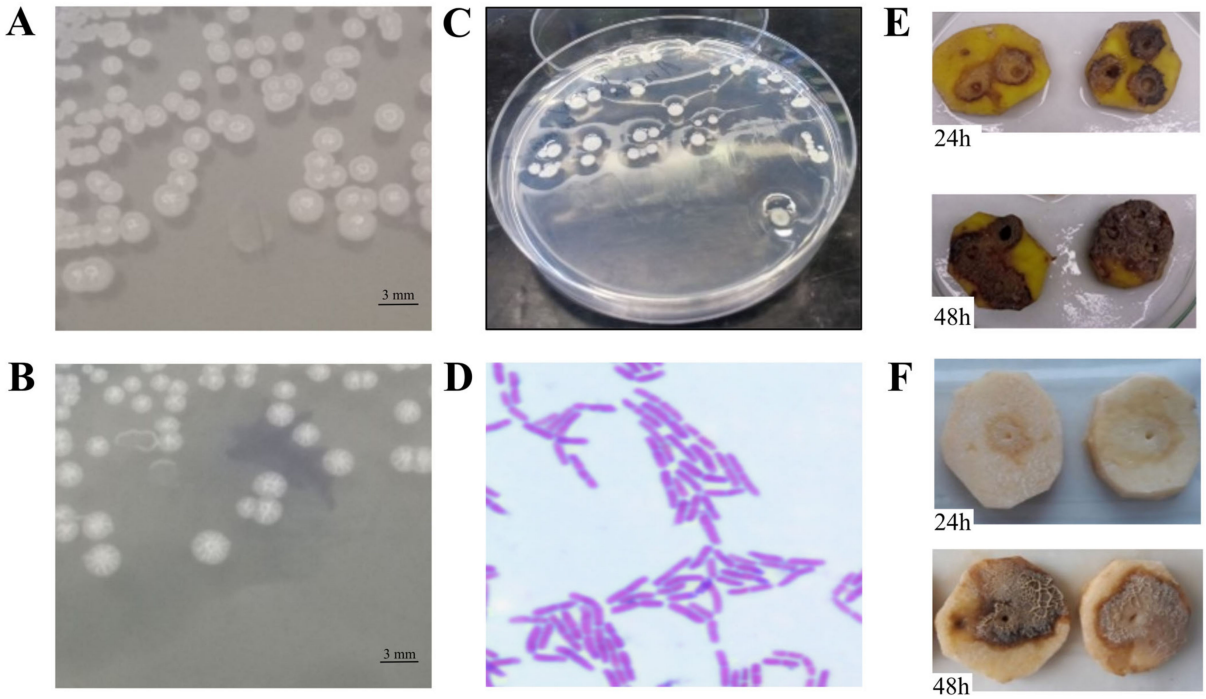

Figure 2. Phenotypic characterisation of the selected pectinolytic bacterial isolates. (**A**,**B**) Morphology of the colonies on TSA agar medium. (**C**) Cavity formation on CVP agar medium inoculated with a pectinolytic isolate and incubated at 28 °C for 48 h. (**D**) Cell morphology post-Gram staining, observations made using the ×100 oil immersion objective. (**E**) Bacterial soft rot on potato tuber slices of cv. 'Lilly' after 24 and 48 h incubation at 28 °C caused by the strain 'A10'. (**F**) Bacterial soft rot on potato tuber slices of cv. 'Spunta' after 24 and 48 h incubation at 28 °C caused by the strain 'A9'.

Table 1. Origin of the pectinolytic strains characterised in this study.

Code Strain	Governorate/ Region/Locality	GPS Location	Year/Month	Irrigation Type	Fertilisation	Cultivar	Organ/Symptom
A1	Bizerte/ Ras Djebel/Kichi	37.216273, 10.109030	2019/June	Drip irrigation	Biological and chemical	*Spunta*	Tuber/soft rot
A2	Bizerte/ Ras Djebel/Kichi	37.216273, 10.109030	2019/June	Drip irrigation	Biological and chemical	*Spunta*	Tuber/soft rot
A3	Nabeul/ Beni Khiar/Melliti	36.478755, 10.770850	2019/June	Drip irrigation	Biological and chemical	*Spunta*	Tuber/soft rot
A4	Bizerte/Ras Djebel/ Kichi	37.216273, 10.109030	2019/June	Drip irrigation	Biological and chemical	*Spunta*	Tuber/soft rot
A5	Bizerte/ Ghar Milh/Mohamed	37.169613, 10.147375	2019/June	Pond water	Chemical	*Spunta*	Tuber/soft rot
A6	Bizerte/ Ras Djebel/Kichi	37.216273, 10.109030	2019/June	Drip irrigation	Biological and chemical	*Spunta*	Tuber/soft rot
A7	Jandouba/ Bousalem/Bousalem1	36.612511, 9.000860	2019/February	Drip irrigation	Biological and chemical	*Daifla*	Tuber/soft rot
A8	Bizerte/ Ras Djebal/Kichi	37.216273, 10.109030	2020/June	Drip irrigation	Biological and chemical	*Spunta*	Tuber/soft rot
A9	Bizerte/ Ras Djebal/Kichi	37.216273, 10.109030	2020/June	Drip irrigation	Biological and chemical	*Spunta*	Tuber/soft rot
A10	Sidi Bouzid/ Bir El Hafey/Anonym	34.930545, 9.226859	2019/June	Drip irrigation	Biological	*Spunta*	Tuber/soft rot
A11	Nabeul/ Beni Khiar/Melliti	36.478755, 10.770850	2019/June	Drip irrigation	Biological and chemical	*Spunta*	Tuber/soft rot
A12	Jandouba/ Bousalem/Bousalem1	36.612511, 9.000860	2019/February	Drip irrigation	Biological and chemical	*Daifla*	Tuber/soft rot

Table 1. *Cont.*

Code Strain	Governorate/ Region/Locality	GPS Location	Year/Month	Irrigation Type	Fertilisation	Cultivar	Organ/Symptom
A13	Bizerte/ Ras Djebel/Kichi	37.216273, 10.109030	2020/June	Drip irrigation	Biological and chemical	*Spunta*	Tuber/soft rot
A14	Bizerte/ Ras Djebel/Kichi	37.216273, 10.109030	2020/June	Drip irrigation	Biological and chemical	*Spunta*	Tuber/soft rot
A15	Jandouba/ Bousalem/Bousalem 2	36.623775, 8.958674	2020/February	Drip irrigation	Biological and chemical	*Daifla*	Tuber/soft rot
A16	Jandouba/ Bousalem/Bousalem2	36.623775, 8.958674	2020/February	Drip irrigation	Biological and chemical	*Daifla*	Tuber/soft rot
A17	Jandouba/ Bousalem/Bousalem2	36.623775, 8.958674	2020/February	Drip irrigation	Biological and chemical	*Daifla*	Tuber/soft rot
A19	Bizerte/ Ras Djebel/Kichi	37.216273, 10.109030	2020/June	Drip irrigation	Biological and chemical	*Spunta*	Tuber/soft rot
A20	Bizerte/ Ghar Milh/Mohamed	37.169613, 10.147375	2020/June	Pond water	Chemical	*Spunta*	Tuber/soft rot
A22	Jandouba/ Bousalem/Bousalem2	36.623775, 8.958674	2020/June	Drip irrigation	Biological and chemical	*Spunta*	Tuber/soft rot

3.2. Identification of the Collected Pectinolytic Isolates

All pectinolytic strains included in this study were sub-cultured on TSA plates. Morphologically, the isolates showed some differences in the shape, edge, colour and size of their colonies on TSA medium. Nine isolates (A1–A6, A10, A19 and A22) formed round, transparent colonies of 1–3 mm in diameter with a white centre (Figure 2A), ten isolates (A7–A9, A11, A13–A17, A20) formed irregular colonies of 1–3 mm in diameter (Figure 2B), and one isolate (A12) exhibited a round, beige-coloured colony of 1–2 mm in diameter.

All pectinolytic isolates were rod-shaped and Gram-positive (Figure 2D), catalase-positive and oxidase-negative, and were able to grow at pH 7.5 both in the liquid and solid media at temperatures ranging from 14 °C to 37 °C, with an optimum at 28 °C.

Identification based on MALDI-TOF MS indicated that 19 pectinolytic strains (A1–A10, A13–A17, A19 and A22) belonged to the genus *Bacillus* spp. and one (A12) to the genus *Paenibacillus* spp. (Table 2). For all 20 isolates studied, the MALDI BioTyper scores exceeded 1.7, which supports bacterial identification at the genus level. Based on MALDI BioTyper identifications reaching scores ≥2.0, eight isolates (A1, A2, A6, A9, A13, A14, A18 and A20) were assigned to the species *Bacillus pumilus*, and one isolate (A12) to the species *Paenibacillus amyloliticus* (Table 2).

Table 2. Identification of the pectinolytic strains isolated from the diseased potato tubers in Tunisia based on MALDI-TOF MS and 16S rDNA gene sequencing evaluation.

Number	Isolate	MALDI-TOF MS-Based Classification	Log (Score) MALDI-TOF MS	Accession Number for the Sequenced 16S rDNA Gene Fragments	Final Identification
1	A1	*Bacillus pumilus*	2.02	ON898611	*Bacillus pumilus*
2	A2	*Bacillus pumilus*	2.03	ON899811	*Bacillus pumilus*
3	A3	*Bacillus pumilus*	1.82	ON899941	*Bacillus pumilus*
4	A4	*Bacillus pumilus*	1.99	ON899942	*Bacillus pumilus*
5	A5	*Bacillus pumilus*	1.96	ON900081	*Bacillus pumilus*
6	A6	*Bacillus pumilus*	2.03	ON900082	*Bacillus pumilus*
7	A7	*Bacillus pumilus*	1.86	ON900083	*Bacillus pumilus*
8	A8	*Bacillus pumilus*	1.82	ON900084	*Bacillus pumilus*
9	A9	*Bacillus pumilus*	2.04	ON900085	*Bacillus pumilus*
10	A10	*Bacillus pumilus*	1.90	ON900086	*Bacillus pumilus*
11	A11	*Bacillus altitudinis*	1.98	ON922531	*Bacillus pumilus*
12	A12	*Paenibacillus amyloliticus*	2.18	ON922535	*Paenibacillus amyloliticus*
13	A13	*Bacillus pumilus*	2.03	ON922532	*Bacillus pumilus*
14	A14	*Bacillus pumilus*	2.06	ON922533	*Bacillus pumilus*
15	A15	*Bacillus pumilus*	1.88	ON922534	*Bacillus pumilus*
16	A16	*Bacillus pumilus*	1.72	ON921248	*Bacillus pumilus*
17	A17	*Bacillus pumilus*	1.85	ON921249	*Bacillus pumilus*
18	A19	*Bacillus pumilus*	2.00	ON921250	*Bacillus pumilus*
19	A20	*Bacillus pumilus*	1.91	ON921251	*Bacillus pumilus*
20	A22	*Bacillus pumilus*	2.05	ON921252	*Bacillus pumilus*

According to the subsequently conducted alignment of the approx. 1100 bp high-quality fragments of the sequenced 16S rDNA of 20 analysed strains, the *Bacillus* sp. isolates previously not assigned to the species level share 100% homology in this 16S rDNA fragment with the strains undoubtedly assigned to *Bacillus pumilus* species based on the acquired MALDI-TOF MS spectra. Afterwards, the fragments of 16S rDNA sequenced for the herein collected strains were compared using a BLAST algorithm, with the reference sequences available in the GenBank database, which confirmed the former taxonomic assignments and allowed for recognition of the closest sequences to the ones tested. To conclude, the sequencing of 16S rDNA proved that nineteen pectinolytic strains (A1–A11, A13–A17, A19–A20 and A22) belong to the *Bacillus pumilus* species, while one strain (A12) belongs to the genus *Paenibacillus* (similarity: 99%) (Table 2).

3.3. Phenotypic Characterisation of the Collected Pectinolytic Isolates

3.3.1. Bacterial Ability to Macerate Potato Tuber Tissue

The maceration ability of the pectinolytic strains originating from different regions of Tunisia was tested on tuber slices of two potato cultivars: cv. 'Lilly', acquired in Poland (Figure 3A), and cv. 'Spunta', the most widely grown variety in Tunisia (Figure 3B).

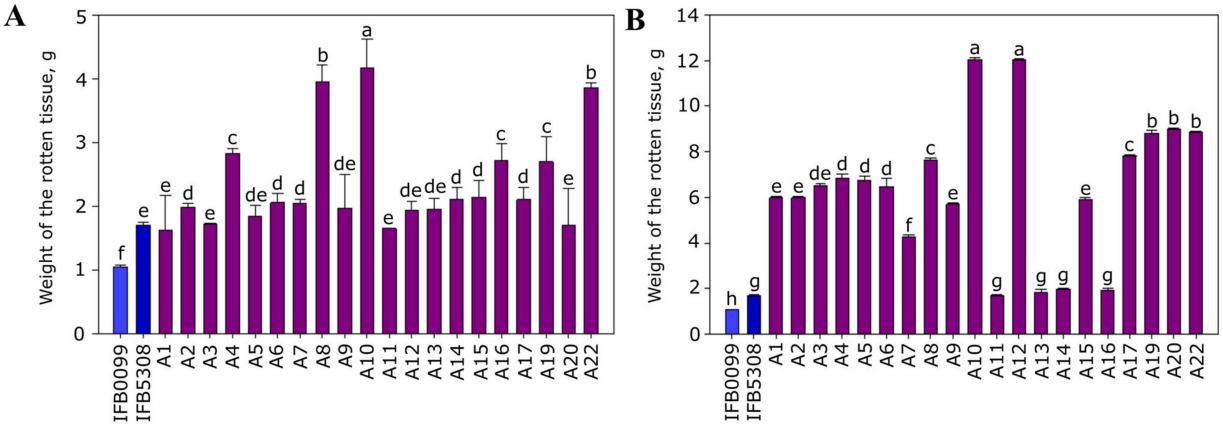

Figure 3. Comparison of the ability of the tested pectinolytic isolates to macerate potato tuber tissue of cultivars *Lilly* (**A**) and *Spunta* (**B**). Means with standard errors (n = 9) are depicted. Means with the same letter are not significantly different ($p > 0.05$). The experiment was repeated three times, each of which involved two technical replicates. Strains used were *D. solani* IFB0099 and *P. parmentieri* IFB5308 in addition to bacterial isolates analysed in this study (A1–A22).

The rotten tissue was collected and weighed 48 h after inoculation to establish the maceration capacity of the tested strains. All 20 pectinolytic strains showed the ability to macerate potato tissue, but differences in the potency between certain strains were noted. In more detail, the highest bacterial ability to macerate the potato tuber tissue of cv. 'Lilly' was recorded for isolates A4, A8, A10 and A22 (Figure 3A), and in the case of cv. 'Spunta', the most prominent maceration potencies were shown by isolates A10 and A12 (Figure 3B).

It needs to be acknowledged that potato tubers of cv. 'Spunta' indicated a higher level of susceptibility to bacterial infection, as the recorded weights of the macerated tuber tissue of cv. 'Spunta' were approximately four times higher than those of cv. 'Lilly' and ranged from 2 to 12 g (Figure 3A,B).

Inoculation of the tuber slices with bacterial suspensions of *D. solani* strain IFB0099 and *P. parmentieri* strain IFB5308, used for comparison purposes, revealed that some pectinolytic strains collected in Tunisia have a higher ability to macerate potato tissue than the reference strains (Figure 3A,B). The presented data indicated that pectinolytic bacteria isolated from the rotten potato tuber in Tunisia were capable of macerating plant tissue under laboratory conditions.

3.3.2. Major Pathogenicity-Associated Features

The analysis of pectinase activity on the M63 PGA plates revealed that the majority of the tested strains exhibited comparable pectinolytic activity to the reference strain *D. solani* IFB0099 (Figure 4A). Isolate A22 indicated the highest activity among the studied strains, similar to that of *P. parmentieri* strain IFB5308. Several isolates (A6–A7 and A9–A11) revealed significantly lower pectinolytic activity than the included reference strains (Figure 4A).

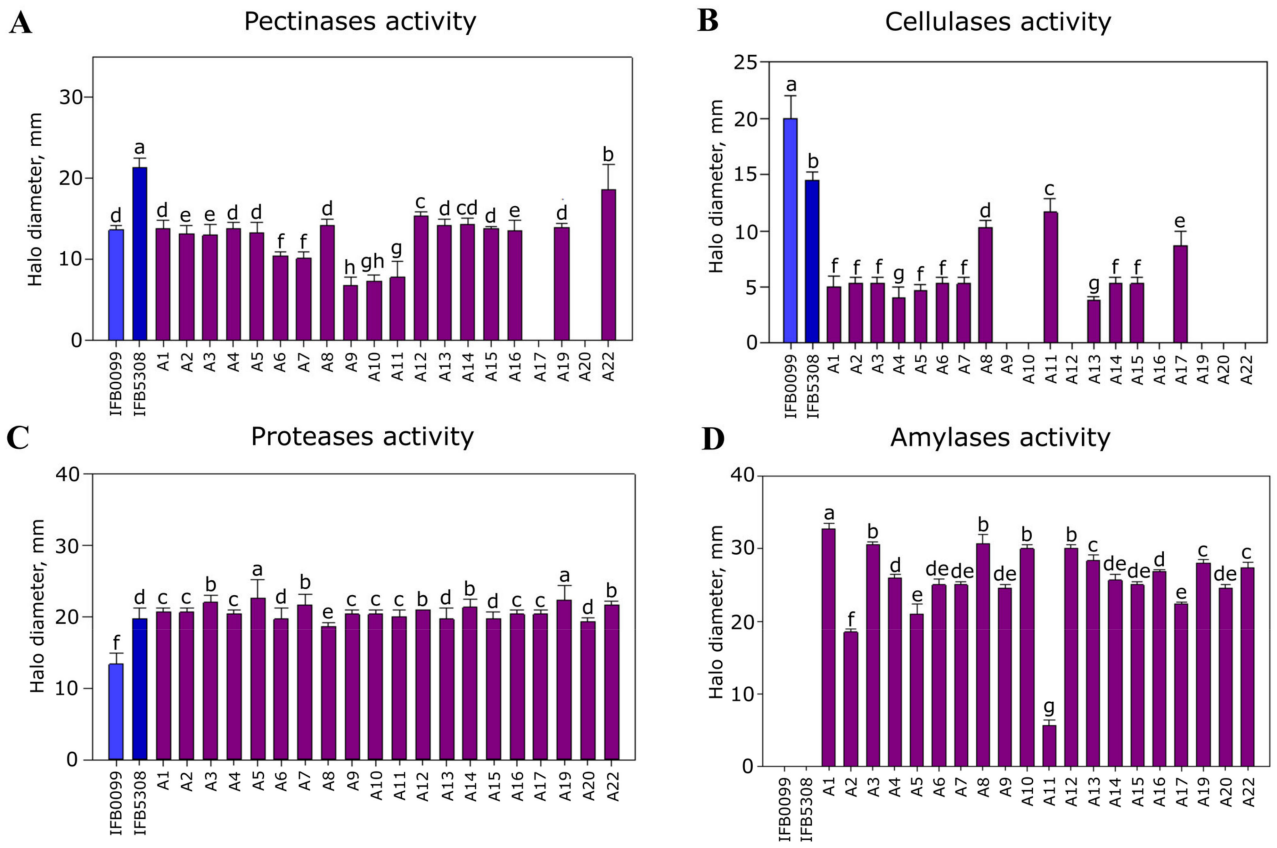

Figure 4. Comparison of the plant cell wall-degrading enzyme activity of pectinolytic strains isolated in Tunisia. Pectinase (**A**), cellulase (**B**), protease (**C**) and amylase (**D**) production was estimated by determining the diameter (mm) of the haloes observed on the detection plates. All the experiments were performed three times with four technical replicates. Means with standard errors (N = 3) were depicted. Means with the same letter are not significantly different ($p > 0.05$). Strains used were *D. solani* IFB0099, *P. parmentieri* IFB5308 and bacterial isolates analysed in this study (A1–A22).

All tested pectinolytic isolates showed significantly lower cellulolytic activity than the reference strains *D. solani* IFB0099 and *P. parmentieri* IFB5308 (Figure 4B). Some of the characterised pectinolytic strains, i.e., A9, A10, A16, A19, A20, A22 and A12, did not exhibit any cellulolytic activity (Figure 4B).

All tested pectinolytic isolates originating from different regions of Tunisia revealed protease activity comparable to *P. parmentieri* IFB5308, reaching the highest value of 22 mm. Isolates A1–A5, A7, A9–A12, A14–A17 and A19 showed the highest protease activity (20–22 mm), and strains A6, A8, A13 and A19 exhibited the lowest protease activity (18–19 mm).

All investigated isolates showed significantly higher protease activity than the reference strain *D. solani* IFB0099, for which this trait approximated a value of 13 mm (Figure 4C).

The majority of the characterised pectinolytic *Bacillus* and *Paenibacillus* spp. strains yielded amylolytic activity (Figure 4D). Significantly lower amylase activity was shown by the A11 strain. In addition, it was confirmed that *D. solani* IFB0099 exhibited no amylase activity (Figure 4D).

All strains of pectinolytic *Bacillus* and *Paenibacillus* spp. isolated in Tunisia revealed similar swimming motility, which, at the same time, was higher than that of the reference strains, reflected by the colonies' diameters on TSA supplemented with 0.3% agar, which ranged from 78 to 67 mm (Figure 5A).

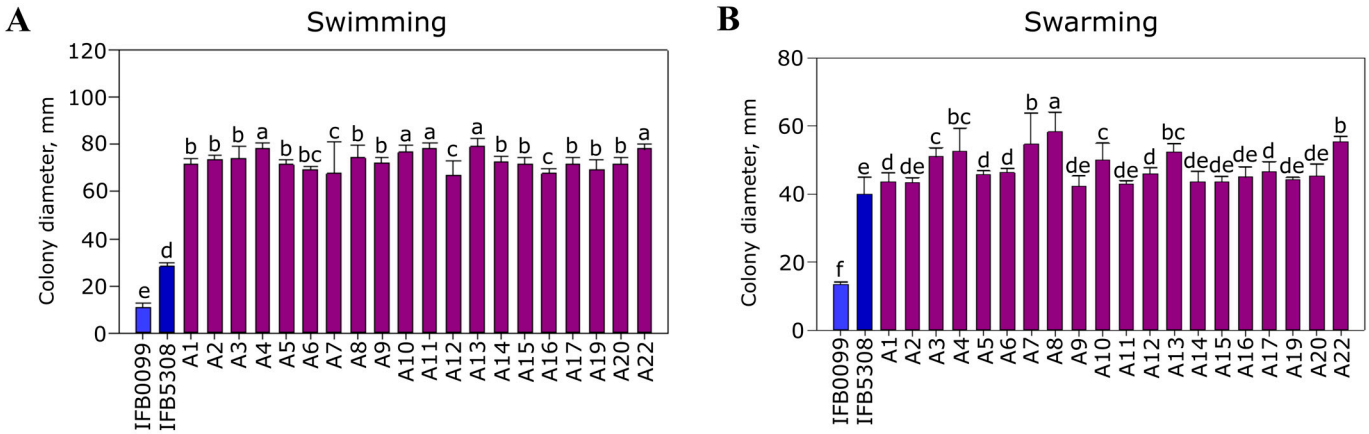

Figure 5. Comparison of the swimming and swarming motilities of pectinolytic strains isolated in Tunisia. (**A**) Swimming motility was estimated by the diameter (mm) of the spread of the colonies on plates containing 0.3% agar. (**B**) Swarming motility was estimated by the diameter (mm) of the spread of the colonies on plates containing 0.6% agar. All the experiments were performed three times with four technical replicates. Means with standard errors (N = 3) were depicted. Means with the same letter are not significantly different ($p > 0.05$). Strains used were *D. solani* IFB0099 and *P. parmentieri* IFB5308 in addition to bacterial isolates analysed in this study (A1−A22).

In terms of swarming motility examined on TSA supplemented with 0.6% agar, all the investigated strains showed potency comparable to the reference *P. parmentieri* IFB5803 strain as the noted colonies' diameters were within 43–58 mm. The performed analysis revealed that strains A7, A8 and A22 indicated the highest swarming motility among the investigated isolates (Figure 5B).

3.3.3. Biochemical Profiling

All tested pectinolytic isolates produced β-galactosidase and gelatinase in addition to fermented saccharose and amygdalin. Moreover, each of the studied bacterial strains lacked lysine decarboxylase and urease activity and was incapable of H_2S, indole or levan production, in addition to showing an inability to utilize tartrate.

Based on the API 20E assay, it was found that the pectinolytic isolates were capable of β-galactosidase, saccharose and amygdalin fermentation and utilising D-mannitol. The examined isolates were also capable of producing gelatinase. Some isolates (A7–A10, A12–A17, A20 and A22) managed to degrade L-arabinose, L-rhamnose and melibiose. In addition, several strains (A1–A6, A11, A12 and A19) were shown to be unable to utilise sorbitol (Table 3).

Interestingly, the sole isolate of *Paenibacillus* spp. differed from all the analysed *Bacillus* spp. in terms of lack of capacity to ferment mannitol and glucose. It is also important to acknowledge that strain-dependent variation was identified among the herein investigated *B. pumilus* and *Paenibacillus* spp. strains in relation to the production of arginine dihydrolase, ornithine decarboxylase and acetoin, in addition to the utilisation of citrate, inositol, sorbitol, rhamnose, melibiose and arabinose.

Table 3. Biochemical characteristics of pectinolytic bacterial isolates using API 20E identification system.

Isolate	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	IN	SOR	RHA	SAC	MEL	AMY	ARA	OX
A1	+	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	+	−	+	−	−
A2	+	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	+	−	+	−	−
A3	+	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	+	−	+	−	−
A4	+	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	+	−	+	−	−
A5	+	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	+	−	+	−	−
A6	+	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	+	−	+	−	−
A7	+	+	−	+	+	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	−
A8	+	+	−	+	+	−	−	−	−	+	+	+	+	+	+	+	+	+	+	−	−
A9	+	+	−	+	+	−	−	−	−	+	+	+	+	+	+	+	+	+	+	−	−
A10	+	−	−	+	+	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	−
A11	+	−	−	−	−	−	−	−	−	+	+	+	+	−	−	+	+	−	+	−	−
A12	+	+	−	+	+	−	−	−	−	+	+	−	−	−	−	+	+	+	+	+	−
A13	+	+	−	+	+	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	−
A14	+	+	−	+	+	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	−
A15	+	+	−	+	+	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	−
A16	+	+	−	+	+	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	−
A17	+	+	−	+	+	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	−
A19	+	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	+	−	+	−	−
A20	+	+	−	+	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	−
A22	+	+	−	+	+	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	−

+ indicates a positive test result; − indicates a negative test result. ONPG (β-galactosidase production), CIT (utilisation of citrate), VP (production of acetoin), GEL (gelatinase production), ADH (arginine dihydrolase), LDC (lysine decarboxylase), ODC (ornithine decarboxylase), H_2S (H_2S production), URE (urease production), TDA (utilisation of tartrate), IND (indole production), VP (acetoin production), GEL (gelatinase production), GLU (glucose fermentation), MAN (mannitol fermentation), INO (inositol fermentation), SOR (sorbitol fermentation), RHA (rhamnose fermentation), SAC (saccharose fermentation), MEL (melibiose fermentation), AMY (amygdalin fermentation), ARA (arabinose fermentation), and OX (oxidase).

4. Discussion

Over the past 10 years, an increasing incidence of soft rot or blackleg caused by *Erwinia* spp. has been reported. This disease is responsible for extensive damage in the field and in potato storage in Tunisia. Moreover, *Erwinia* spp. has been isolated from 20% of rotten imported seed tubers [20]. It has also been noted that latent infections with *Erwinia* spp. not only occurs in 90% of imported seed potatoes, but also does not decrease with successive progenies, thus posing a real threat to the cultivation and storage of potatoes [33]. Contrarily, previous research on major bacterial and fungal diseases in potato fields in Tunisia conducted in the growing seasons of 1993–1994 pointed to predominance of dry rot, with 87–90% of the isolates classified as *Fusarium solani*, followed by minor contributions of *F. sambucinum* in addition to either *Verticillium dahliae* and *Phoma exigua* or *Sclerotina minor*, depending on the year of study [20]. From the 1993 collections, only five isolates of *E. caratovora* were acquired among the 141 samples plated on CVP medium [20]. No members of the current family *Pectobacteriaceae* were identified during the survey performed in 1994 [20]. The current study, conducted in the 2018–2020 growing seasons, revealed that just 20 bacterial strains showed pectinolytic properties on CVP medium, and no soft rot *Pectobacteriaceae* were detected among the 270 isolates obtained from the diseased potatoes picked up in distinct Tunisian regions; this agrees with the data of Priou and Mahjoub collected over 20 years ago. Similarly to the previous suggestions of Priou and Mahjoub [20], the climatic conditions present in Tunisia do not seem to favour development of disease symptoms by *Pectobacterium* and *Dickeya* spp., in contrast to the common occurrence of these bacteria in temperate climates [26,34,35]. Thus, as shown in

the current study, there was an ecological niche accessible to a different plant pathogen, reported herein for the first time as a causative agent of bacterial soft rot in potato in Tunisia.

Of the 20 pectinolytic isolates originating from the diseased potatoes collected in the territory of Tunisia, 19 were assigned as *Bacillus pumilus*, while 1 was assigned to the genus *Paenibacillus*. Interestingly, strains classified as *Bacillus pumilus* (former member of the *Bacillus subtilis* group) indicate various features, such as plant growth promotion [36,37], the potential to cause disease symptoms in plants [9–12], and the ability to cause human diseases [38,39], to spoil food [40], or to sustain themselves in demanding ecosystems [41,42]. *B. pumilus* strains are particularly resistant to environmental stressors, such as low nutrient availability, UV irradiation, desiccation, high salinity or various oxidisers [41], which may be the reason for their higher resilience and better fitness in the Tunisian climate of *Bacillus pumilus* in contrast to *Dickeya* and *Pectobacterium* spp. Additionally, the genus *Paenibacillus* encloses ubiquitous strains of over 200 species [43] of notable relevance to plants, humans, animals or the environment, which have been isolated from highly diverse habitats, e.g., polar regions, tropics, aquatic ecosystems or even deserts. These bacteria draw interest due to their plant growth-promoting properties, potential uses in bioremediation and effective production of enzymes, such as lipases, pectinases, amylases, cellulases or hemicellulases, which exhibit higher activity, stability and cost-effectiveness in contrast to the currently utilised alternatives [43]. However, the production of proteases, lipases and phospholipases, in addition to the ability of *Paenibacillus* spp. to multiply at low temperatures, contributes to the food spoilage process.

In this research, we have proven that all the investigated *Bacillus* and *Paenibacillus* spp. strains are able to cause disease symptoms on potato slices and that the vast majority of these isolates exhibit the activity of plant cell wall-degrading enzymes, i.e., pectinases, cellulases, proteases and amylases, which significantly contribute to the decay of plant tissue [44]. The manifestation of disease symptoms triggered by *Bacillus pumilus* in potato, referred to as water-soaked or yellowish-brown rot, agrees with the former observations of Bathily et al. [10] and further supports the previously reported [9–12] pathogenic potential of this species.

Contrarily, the pathogenic potential of *Paenibacillus* spp. is not commonly raised as, to the best of our knowledge, there are only two reports on the isolation of *Paenibacillus* spp. strains from diseased plants, i.e., wheat and barley, showing leaf deformation, wilting, spotting, strokes and stripes [45], in addition to symptomatic *Solanum lycopersicum*, *Alium cepa*, *Pelargonium* sp., *Lilium* sp., *Begonia obliqua*, *Anthurium* sp., *Dianthus caryophyllus* and *Hypericum perforatum*, which were sent to the Plant Disease Clinic in Poland in 2013–2019 for examination [46]. Considering that in this study, the initially observed disease symptoms in potato were reproduced under laboratory conditions post artificial inoculation of two potato cultivars ('Lilly' and 'Spunta'), the pathogenicity of *Paenibacillus* spp. strains should be taken into consideration and further investigated. It is especially imperative to do so, as the recorded potato maceration capacities of the vast majority of the studied *Bacillus* and *Paenibacillus* spp. strains turned out to exceed the potency of the included reference *Dickeya* and *Pectobacterium* spp. strains, which are recognised as the main causative agents of soft rot and blackleg diseases [44,47].

Subsequently, we studied the activity of pivotal virulence factors associated with the development of soft rot symptoms among the 20 isolated *Bacillus* and *Paenibacillus* spp strains. So far, little information has been published on the activity of most significant plant cell wall-degrading enzymes among plant pathogenic strains of *Bacillus pumilus* [9,10,12–14,17]. Evdokimova et al. [19] are the sole reporters of lipase and protease action among all the investigated plant pathogenic *B. pumilus* strains. Notably, some intraspecies variation was noted in cellulase and pectinase activity among phytopathogenic *B. pumilus* by Evdokimova et al. [19], though the capacity to produce these enzymes dominated among the included isolates. Likewise, the common production of pectinases, cellulases, proteases and amylases was exhibited by *Bacillus* and *Paenibacillus* spp. strains for industrial use [43,48–52]. In terms of plant pathogens, Od23 *Bacillus pumilus* characterised by Bathily et al. [10], the GR8 isolate obtained by Peng et al. [11] and all the strains examined by Kovaleva et al. [12] were reported to be inca-

pable of hydrolysing starch, in contrast to all the herein investigated strains showing amylase activity. Putatively, these discrepancies result from the application of diverse methodologies, as the action of amylases of *Bacillus* and *Paenibacillus* spp. found confirmation in research focused on industrial processes or the characterisation of this group of enzymes in the listed bacterial taxa [43,50,52]. It is also worth noting that we identified two *Bacillus pumilus* strains lacking the action of pectinases, and six *B. pumilus* and one *Paenibacillus* spp. deprived of cellulase activity. In spite of the absence of these virulence factors, all the herein investigated *Bacillus* and *Paenibacillus* spp. strains efficiently macerated potato tissue. Additionally, all isolates exhibited high swimming and swarming capacities, which confirmed former reports on the motility of plant pathogenic *Bacillus pumilus* strains [9,10,12–14,17]. Curiously, the intensities of the swimming and swarming of *Bacillus* and *Paenibacillus* spp. strains exceeded the ability to move of the two analysed *Dickeya* and *Pectobacterium* strains.

Biochemical characterisation of the phytopathogenic *Bacillus* and *Paenibacillus* spp. isolated in Tunisia was performed in order to reveal intra-species diversity. Similarly to the current research, each of the five *Bacillus pumilus* strains studied by Galal et al. [9] fermented glucose and mannitol, though they were incapable of gelatin liquefaction, contrarily to our work and the studies of Peng et al. [11,13,19]. Moreover, the inability of *B. pumilus* isolates to produce indole in the herein presented study agreed with that of Bathily et al. [10]. However, the Od23 strain investigated by the latter group gave inconsistent results in terms of H_2S production, and was incapable of producing acetoin and fermenting arabinose, in opposition to the outcomes collected for some of the herein analysed isolates. Notably, the lack of oxidase activity for all the herein investigated strains is confirmed in several reports [13,14,17,19]. It is likewise worth mentioning that the abilities of phytopathogenic *Bacillus pumilus* to utilise mannitol, glucose and saccharose agreed with the study of Saleh et al. [13]. Furthermore, the raised intraspecies variability among *B. pumilus* strains regarding biochemical features is consistent with former research, which revealed such phenomena, for instance, in terms of glycerol and D-cellobiose utilisation [11], trehalose metabolisation [13] and arabinose degradation [9,10], in addition to H_2S production [9].

To summarise, we identified pectinolytic plant pathogenic *B. pumilus* and *Paenibacillus* sp. as being responsible for potato diseases in the fields of Tunisia. As the studied strains macerated potato tissue, in addition to exhibiting activity of major virulence factors, their pathogenic potential seems well founded. We want to underline the high significance and novelty of the current research as there are few previous reports that describe the phytopathogenic properties of *B. pumilus* strains in diverse plant hosts throughout the world, and even less data concerning the plant disease-causing potential of *Paenibacillus* sp. Most of all, the pathogenic properties of *B. pumilus* and *Paenibacillus* sp. should be taken into consideration in view of the attempts to utilise the members of these taxa as biocontrol and/or plant growth-promoting agents. Previously, the beneficial effects of *B. pumilus* inoculations against fungal pathogens were associated with the production of peptide antibiotics, biosurfactants, chitinases, other fungus cell wall-degrading enzymes and volatiles, in addition to diverse stimulants of plant defence systems [36]. Moving to the plant growth-promoting *Paenibacillus* spp., these isolates may secrete phytohormones, antimicrobials or insecticides, induce plant systemic resistance or solubilise inaccessible forms of phosphorous or iron, and some strains even exhibit nitrogen-fixation properties [53]. Based on the data shown herein and the previous reports of Peng et al. [11], Galal et al. [9], Bathily et al. [10] and Kovaleva et al. [12], we suggest devoting the highest attention to both preventing fungal-derived diseases and stimulating plant growth with bacterial strains with disease-causing potential. This matter should be thoroughly investigated in the future.

5. Conclusions

The occurrence of new, virulent pectinolytic phytopathogenic bacteria from the genera *Bacillus* and *Paenibacillus*, in particular, *B. pumilus* and *P. amyloliticus*, is a serious problem for the producers of seed potatoes, as well as for the farmers in Tunisia. To the best of our knowledge, this is the first report on the occurrence of *Bacillus* and *Paenibacillus* spp.

capable of causing soft rot in potato in Tunisia. It is important to conduct a complementary study on the interactions between pathogenic bacteria associated with soft rot during the different seasons and cultivar susceptibility in order to develop a potential strategy for controlling this disease in the field and in postharvest storage.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13061275/s1, Table S1: Characteristics of the Tunisian orchards surveyed for bacterial soft rot and blackleg diseases in 2018, 2019 and 2020.

Author Contributions: Conceptualisation, A.Y., M.O., A.M.-P., W.S., E.L. and N.S.-Z.; methodology, A.Y., A.M.-P., W.S., N.K., E.L. and B.T.; sampling, A.Y. and M.R.H.; conducting experiments, A.Y.; supervision, E.L. and N.S.-Z.; project administration, N.S.-Z.; writing—original draft, A.Y., M.O., A.M.-P., N.K., E.L. and N.S.-Z.; writing—review and editing, N.S.-Z. and E.L. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Not applicable.

Data Availability Statement: The collected data is contained within the article or Supplementary Materials. Raw data presented in this study are available on reasonable request from the corresponding authors. The herein acquired 16S rDNA sequences of *B. pumilus* and *Paenibacillus* sp. strains are publicly available in the GenBank database under the following accession numbers: ON898611, ON899811, ON899941, ON899942, ON900081-ON900086, ON921248-ON921252 and ON922531-ON922535.

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