1 # *Pseudomonas cichorii* as causal agent of midrib rot, an
2 # emerging disease of greenhouse-grown butterhead lettuce
3 # in Flanders

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1 **Abstract**

2 Bacterial midrib rot of greenhouse-grown butterhead lettuce (*Lactuca sativa* L. var. *capitata*)
3 is an emerging disease in Flanders (Belgium). Fluorescent pseudomonads are suspected to
4 play an important role in the disease. Isolations from infected lettuces collected from 14
5 commercial greenhouses in Flanders yielded 149 isolates that were characterised
6 polyphasically including morphological characteristics, pigmentation, pathogenicity tests by
7 injection and spraying of lettuce, LOPAT characteristics, FAME analysis, BOX-PCR
8 fingerprinting, 16S rRNA and rpoB gene sequencing, and DNA-DNA hybridization. Ninety-
9 eight isolates (66%) exhibited a fluorescent pigmentation and were associated with the genus
10 *Pseudomonas.* Fifty-five of them induced an HR^+ (hypersensitive reaction in tobacco leaves)
11 response. The other 43 fluorescent isolates are most probably saprophytic bacteria and about
12 half of them were able to cause rot on potato tuber slices. BOX-PCR genomic fingerprinting
13 was used to assess the genetic diversity of the *Pseudomonas* midrib rot isolates. The
14 delineated BOX-PCR patterns matched quite well with *Pseudomonas* morphotypes defined on
15 the basis of colony appearance and variation in fluorescent pigmentation. 16S rRNA and rpoB
16 gene sequence analyses allowed to allocate most of the fluorescent isolates into *Pseudomonas*
17 as either belonging to the *P. fluorescens* group, *P. putida* group, or the *P. cichorii/syringae*
18 group. In particular the isolates allocated to this last group constituted the vast majority of
19 HR^+ isolates and were identified as *P. cichorii* by DNA-DNA hybridization. They were
20 demonstrated in spray-inoculation tests on greenhouse-grown lettuce to induce the midrib rot
21 disease and could be re-isolated from lesions of inoculated plants. Four HR^+ non-fluorescent
22 isolates associated with one sample that showed an atypical midrib rot were identified as
23 *Dickeya* sp..
24 ***Keywords:*** bacterial rot, butterhead lettuce, greenhouse, *Pseudomonas cichorii*, pectolytic
25 fluorescent pseudomonads, *Dickeya* sp..
26

Introduction

Bacterial midrib rot of butterhead lettuce has emerged as an economical threat for greenhouse lettuce production in Flanders, Belgium. Sporadic outbreaks of any economic importance in lettuce production were first observed in the mid-1990s, and the disease has since increased to a continuous problem. This increase is possibly related to the general shift in lettuce production in Flanders from the field to the greenhouse with lettuces being grown under intensive production systems for a continuous supply to the market. Symptoms of bacterial midrib rot consist of a dark-brown to greenish-black rot along the midrib of one or more middle to inner head leaves, often extended into flanking tissue of the leaf blade. Damage can be extensive and usually results in partial or total loss of crops. Disease build-up can be rapid and the problem is especially evident on lettuce approaching harvest, which impedes early detection of the disease. With approximately 3294 acres planted annually and an estimated production value of € 50 million in 2006, greenhouse butterhead lettuce (*Lactuca sativa* L. var. *capitata*) is the most important leafy vegetable produced in Flanders. Quality considerations are critical in the marketing of leaf lettuce and the presence of rotted inner leaves or other blemishes generally destroys marketability of the crop.

The etiology of bacterial rot of vegetables is complex and commonly associated with *Erwinia carotovora* and several fluorescent pseudomonads with pectolytic and surfactant-like activity [21,29]. Pectolytic fluorescent pseudomonads have been reported as the causal organisms of bacterial rot on head lettuce [4], broccoli [5,21] and cauliflower [30]. Further, a preliminary study [3] on greenhouse lettuce rots in Flanders indicated that fluorescent pseudomonads are suspected to play an important role in the disease.

The purpose of this study was to isolate and characterise the bacteria that are associated with dark-brown midrib rot of greenhouse-grown butterhead lettuce in the region of Flanders in order to identify the causal agent(s) of this disease, and to reproduce the symptoms under greenhouse conditions.

Materials and methods

Isolation of bacteria and used reference strains

Bacteria were isolated from symptomatic butterhead lettuces collected from 14 commercial greenhouses in the region of Flanders in Belgium from September 2004 to November 2005

1 (Table 1). Collected samples were routinely examined under a stereomicroscope for fungal
2 growth to exclude symptoms possibly attributed to *Rhizoctonia solani* or *Botrytis cinerea*.
3 One sample consisted of three nearly mature lettuce plants with one or more midrib rot
4 infected inner head leaves. Leaves with midrib rot symptoms were briefly surface disinfected
5 in 70% ethanol, and rinsed in sterile tap water. Leaf midrib pieces were excised from the
6 margins of lesions and macerated in 2 ml sterile 50 mM potassium phosphate buffer (PB, pH
7 7.0). Fifty microliter aliquots of tenfold serial dilutions in PB (10^{-3}, 10^{-4} and 10^{-5}) were
8 replicate spread on Difco™ Pseudomonas Agar F (PAF; Becton Dickinson and Company,
9 MD, USA) supplemented with 0.1 g l^{-1} cycloheximide (Sigma-Aldrich N.V., Belgium) to
10 prevent fungal contamination. Plates were incubated at 28 °C and examined for bacterial
11 growth after 4 to 7 days. All visibly different colony types were isolated from the plates
12 inoculated with the highest dilutions and if less than three were present, also colonies
13 dominantly present on the second highest dilution plates were taken. One hundred forty-nine
14 colonies were purified by re-streaking on PAF medium. All isolates were kept on Difco™
15 tryptic soy agar (TSA; Becton Dickinson and Company, MD, USA) slant tubes for routine
16 use, or in PB with 20% glycerol at –70 °C for long-term storage.
17 Representatives of each morphotype group (see below) were deposited in the Belgian
18 Coordinated Collections of Microorganisms/bacteria collection Laboratory of Microbiology
19 (BCCM/LMG), Ghent University, Belgium as: LMG 24428 (SF1047-01, R-27204,
20 morphotype group C1), LMG 24427 (SF0057-02, R-26430, morphotype group C2), LMG
21 24440 (SF0119-01, R-33145, morphotype group C3), LMG 24426 (SF1012-01, R-24816,
22 morphotype group E1), LMG 24436 (SF1045-01, R-27199, morphotype group F1), LMG
23 24435 (SF0041-07, R-26745, morphotype group F2), LMG 24433 (SF0057-01, R-26429,
24 morphotype group F3), LMG 24434 (SF0039-02, R-26735, morphotype group F4), LMG
25 24438 (SF0080-03, R-29016, morphotype group F5), LMG 24437 (SF0077-03, R-29008,
26 morphotype group F6), LMG 24431 (SF0055-02, R-26424, morphotype group F7), LMG
27 24432 (SF0056-03, R-26428, morphotype group F8), LMG 24430 (SF0055-01, R-26423,
28 morphotype group F9), and LMG 24439 (SF0120-01, R-32840, morphotype group F10).
29 The following strains of *Pseudomonas cichorii* were included for comparative analysis: LMG
30 2162^{T} and LMG 2163 (PC26, Burkholder); the 'lettuce varnish spot' strain 9D42 [16]
31 obtained from the University of California, Davis, U.S.A.; and strain IVIA 154 3.1-1 causing
32 black streak of endive [14] obtained from the Instituto Valenciano de Investigaciones
33 Agrarias, Valencia, Spain. We were not able to obtain 'lettuce tar' *P. cichorii* strains from

Opmerking [b1]: Insert Table 1

1 Kochi University, Monobe, Japan.

2 **Characterization of the isolates**

3 The lettuce isolates were tested for morphological, biochemical, and physiological
4 characteristics. Colony morphology (colour, texture, colony size and shape) was determined
5 visually and under the dissecting microscope after 5-days of growth on PAF at 28 °C and was
6 used to classify the isolates into morphotype groups. The isolates were further characterised
7 by: i) the presence of oxidase with Bactident Oxidase test strips (Merck, Germany) according
8 to manufacturer's instructions; ii) the potato rot test for which 300 µl of an aqueous bacterial
9 suspension (approximately 10^6 cells ml^{-1}) was spread on potato tuber slices (*Solanum*
10 *tuberosum* L. cv. Bintje) placed on moistened filterpaper in Petri-dishes, and examined for
11 soft rot after 2 days at 28 °C, iii) the tobacco hypersensitive reaction (HR) [25] for which 200
12 µl of an aqueous suspension (approximately 10^8 cells ml^{-1}) was infiltrated by hypodermic
13 syringe into alternate leaf panels of expanded tobacco leaves (*Nicotiana tabacum* L. cv.
14 Xanthi) and scored positive if the zone of infiltrated leaf tissue collapsed and became brown
15 and papery after 72 hours, and iv) visual evaluation of fluorescent pigment on PAF [24] under
16 UV light at 366 nm. Finally, the fluorescent isolates were further evaluated for additional
17 LOPAT characteristics including levan formation from sucrose and production of arginine
18 dihydrolase as described by Lelliot and Stead [27].

19 **Fatty acid methyl ester analysis**

20 A qualitative and quantitative analysis of cellular fatty acid compositions was performed with
21 the gas-liquid chromatographic procedure as described by Dawyndt et al. [9]. The resulting
22 profiles were identified with the Microbial Identification software (MIDI) using the TSBA
23 database version 5.0 (Microbial ID, Newark, DE, USA). An overview of the fatty acid content
24 of the *P. cichorii* isolates is provided as extra table in the supplementary data.

25 **Assay of pectate lyase (PL) activity**

26 Isolates assayed for the production of pectate lyase were grown with shaking (200 rpm) for 72
27 h at 28 °C in 5 ml MMY broth medium [28] without addition of $CaCl_2$. After centrifugation of
28 the bacterial suspensions at 6,000 g for 10 min, the supernatant was filtered through a 0,2-µm
29 filter (Sartorius Biotech GmbH, Goettingen, Germany). Further steps (sample preparation,
30 measuring and calculation) were performed as described in Membré & Burlot [35].
31 Absorbance (235 nm) was measured at a temperature of 30 °C by use of an Uvikon 932
32 spectrophotometer (Kontron Instruments, Groß-Zimmern, Germany). Enzyme activity values
33 below 5 units were scored as weak positive, values above 5 units as positive. One unit is

defined as the amount of enzyme that produces 1 µmol of unsaturated product per minute [35].

Pathogenicity tests

As a routine test, all 149 isolates were assessed for the ability to cause symptoms on lettuce by injection into the leaf midrib. Bacterial suspensions in sterile PB (50 mM, pH 7) containing approximately 10^6 cells ml^{-1} prepared from 48-h-old cultures on PAF were used. The midrib of leaves of 6-weeks-old lettuce (*Lactuca sativa* L. var. *capitata* cv. Burgia) was injected in duplicates with 200 µl of suspension by a hypodermic syringe. Negative control leaves were similarly inoculated with sterile PB (50 mM, pH 7). After inoculation, plants were kept in a greenhouse and examined for disease symptoms after two to five days.

The 81 isolates that caused symptoms of rotting upon injection of the midrib were further tested by spray-inoculation on butterhead lettuces (cv. Burgia) at heading stage maintained in the greenhouse at night and day temperatures of about 8-12 °C and 15-20 °C, respectively. Bacterial suspensions in tap water containing approximately 10^6 cells ml^{-1}, prepared from 16-h-old cultures grown in Difco™ tryptic soy broth (TSB; Becton Dickinson and Company, MD, USA) on an orbital shaker at 28 °C, were used. Per isolate, two lettuce plants were uniformly sprayed between the leaves with 200 ml of suspension using a hand-held sprayer. Control lettuce plants were similarly treated with tap water. The inoculated plants were periodically irrigated with overhead sprinklers and examined daily for 20 days for midrib rot development. Bacteria were re-isolated from lesions and characterised with BOX-PCR to confirm the presence of inoculated bacteria in association with the disease symptoms.

DNA extraction and DNA-DNA hybridization

Total genomic DNA was purified for BOX-PCR, 16S rRNA and rpoB gene sequencing using a slight modification of the method of Pitcher et al. [43], as described by Heyndrickx et al. [17]. For DNA-DNA hybridization, approximately 1 g of biomass (wet weight) was harvested from agar plates. DNA was purified by a combination of the protocols of Marmur [33] and Pitcher et al. [43], as described by Logan et al. [31].

The G+C content of the DNA was determined by HPLC [36] using the further specifications given by Logan et al. [31]. DNA-DNA hybridization was performed using a modification of the microplate method of Ezaki et al. [13], as described by Willems et al. [53]. A hybridization temperature of 45 °C (calculated with correction for the presence of 50% formamide) was used.

Repetitive sequence-based polymerase chain reaction (rep-PCR)

Detailed characterization of the genetic variability among isolates belonging to *Pseudomonas* was achieved by DNA fingerprinting based BOX-PCR using the primer BOXA1R [34]. PCR conditions were as described by Rademaker and de Bruijn [44]. Electrophoresis was performed as described by Heyrman et al. [18]. Patterns were normalized and clustered according to the Pearson correlation coefficients by unweighted pair group method with arithmetic averages (UPGMA) and analyzed with the cophenetic correlation method in BioNumerics version 4.6 (Applied Maths, Sint-Martens-Latem, Belgium).

16S rRNA gene sequence analysis and phylogenetic analysis

PCR amplification was performed as described by Heyrman & Swings [19]. Amplicons were purified using the Nucleofast® 96 PCR system (Millipore). Sequence reaction mixtures contained 3 µl purified and concentrated PCR product, 1 µl of BigDye™ Termination RR mix version 3.1 (Perkin Elmer), 1.5 µl of BigDye™ buffer (5x), 1.5 µl sterile milliQ water and 3 µl (20 ng/µl) of primer. The primers for partial sequencing according to the *E. coli* numbering system (reverse 358-339 and reverse 536-519) and PCR program were previously described by Heyrman & Swings [19]. The sequencing products were cleaned up, as described by Naser et al. [39]. Sequence analysis of the partial 16S rRNA gene was performed using an Applied Biosystems 3100 DNA Sequencer according to protocols provided by the manufacturer.

Reverse strands of 16S rRNA genes were assembled with the BioNumerics 4.6 software and were aligned with 16S rRNA gene sequences retrieved from the EMBL database using ClustalX [49]. The 16S rRNA gene fragment used for phylogenetic analysis ranged from position 13 to 399, according to reference sequence numbering of *Pseudomonas aeruginosa* LMG 1242^T (accession number Z76651). Phylogenetic analyses were performed with Treecon [51]. Trees were constructed with the neighbour joining algorithm without corrections. Statistical evaluation of the tree topologies was performed by bootstrap analysis with 1000 resamplings. Also, sequences were compared towards the EMBL prokaryotes database using a FASTA search [40].

rpoB gene sequence analysis and phylogenetic analysis

PCR amplification was performed as described by Tayeb et al. [47]. Clean-up, sequencing PCR and sequence analysis were identical to the protocols for 16S rRNA gene. The amplification primers were used for sequencing. Forward and reverse strands of rpoB were assembled with the BioNumerics 4.6 software. Sequence authenticity was checked through translation with Transeq and domain assessment through pBLAST. Sequences were aligned

with other rpoB gene sequences retrieved from the EMBL database using ClustalX [49]. An alignment of 981 bp ranging from position 1465 to 2431 according to reference sequence numbering of *Pseudomonas aeruginosa* PAO1, was used to perform phylogenetic analyses with Treecon [51]. Trees were constructed with the neighbor joining algorithm without corrections. Statistical evaluation of the tree topologies was performed by bootstrap analysis with 1000 resamplings. It should be noted that reference sequence *P. aeruginosa* PAO1 was included as an inverted repeat in the public databases. Therefore, the positions of the newly generated rpoB gene fragments do not agree with the primer positions given by Tayeb et al. [47], according to the same reference strain.

Nucleotide sequence accession numbers

The sequence data generated in this study have been deposited in Genbank/EMBL/DDBJ. The accession numbers of the 16S rRNA gene sequences and the rpoB sequences are given in Figure 4 and Figure 5, respectively.

Results and discussion

Isolation and characterization of isolates

All collected samples were greenhouse-grown butterhead lettuce cultivars approaching harvest with midrib rot symptoms on several inner head leaves. An overview of the procedure used for the grouping of isolates is given in Fig. 1 (see supplementary data).

This study has focused on characterization of isolates obtained from midrib rot lesions following a short disinfection of leaf surfaces with 70% ethanol, merely to diminish interference of fast growing saprophytes on the dilution plates. A total number of 149 bacteria was isolated from diseased leaf midrib tissue of lettuces collected from 14 commercial greenhouses sampled at different sites in Flanders (Belgium). These 149 isolates consisted of 98 fluorescent (66%) and 51 non-fluorescent (34%) bacteria as determined on PAF under UV_{366nm} radiation. Of the non-fluorescent isolates, 43 were Gram-negative bacteria and based on FAME analysis assigned to the classes *Alphaproteobacteria* (4 isolates), *Betaproteobacteria* (15 isolates), and *Gammaproteobacteria* (24 isolates). The remaining eight non-fluorescent isolates belonged to Gram-positive bacteria.

The majority of bacteria isolated were fluorescent and based on FAME analysis assigned to the genus *Pseudomonas*. Further differentiation of these 98 fluorescent isolates, based on the ability to elicit the nonhost hypersensitive reaction (HR), showed 55 isolates to be HR^+ while 43 were HR^-. The HR^- isolates were further distinguished by the ability to rot potato tuber

slices into 22 pectolytic and 21 non-pectolytic isolates. The non-pectolytic isolates represented a morphologically diverse set of bacteria belonging to LOPAT groups Va and Vb of fluorescent pseudomonads [26]. As these isolates, as well as most of the non-fluorescent isolates, did not induce apparent symptoms on lettuce in the initial pathogenicity tests by leaf midrib injection, they will not be further discussed. The pectolytic fluorescent isolates were differentiated based on colony appearance into 9 morphotype groups designated as F1 to F9 in Table 2. In LOPAT tests, four morphotype groups (F2, F4, F5 and F6) belonged to group IVa, and five morphotype groups (F1, F3, F7, F8 and F9) to group IVb of fluorescent pseudomonads [26]. Pectate lyase is believed to be the principal enzymatic factor involved in tissue maceration by pectolytic fluorescent pseudomonads [28]. Activities of pectate lyase were detected in culture supernatants of the isolates belonging to morphotype groups F1, F2, F3 and F7 and of one isolate (SF0067-04) of morphotype group F6. No pectate lyase was produced by the other isolates belonging to morphotype groups F4, F5, F6, F8 and F9. Overall, these pectolytic HR^- isolates were obtained from 9 of the 14 samples. None of the morphotypes F1 to F9 was recovered from more than two symptomatic samples except F2, which was shown to be present in three samples.

The main group of isolated fluorescent bacteria consisted of 55 HR^+ isolates that elicited a clear hypersensitive reaction in tobacco leaves. Two isolates, recovered from the same sample (SF0119-0120; Table 1), formed mucoid pale lemon-yellow colonies on PAF producing a dim whitish-yellow fluorescence under UV light. They were designated as morphotype group F10 (Table 2); tested positive for the production of pectate lyase; and belonged to LOPAT group II, which is indicative for *P. viridiflava*.

The remaining 53 HR^+ isolates formed greenish-yellow colonies on PAF differentiated into morphotype groups C1, C2 and C3 (Table2) distinct in shade and fullness of pigmentation (Fig. 2). They were isolated from 13 of the 14 midrib rot samples. Morphotype groups C1, C2 and C3 included, respectively, 29 isolates obtained from seven samples (SF0034-0039, SF0040-0042, SF0058-0059, SF1040-1047, SF0066-0067, SF0068-0069, SF0075-0080; Table 1), 12 isolates obtained from another three samples (SF0053-0057, SF0063-0065, SF0091-0093), and 12 isolates obtained from still another three samples (SF0119-0120, SF0125-0126, SF0129-0131). None of these C-morphotype isolates produced pectate lyase. In LOPAT tests they belonged to group III, indicative for *P. cichorii*. However, it should be noted that, whereas a negative reaction in the potato rot test is typical for *P. cichorii* [27], isolates of morphotype group C2 tested positive. The isolates of morphotype group C1 formed

Opmerking [b2]: Insert Table 2

Opmerking [b3]: Insert Fig. 2

colonies on PAF that were translucent with low convex elevation, serrate margins and an uneven surface, producing a strong bluish (young colonies) to dim yellowish-green (older colonies) fluorescence under UV light. Isolates of morphotype group C3 formed similar colonies but were slightly darker greenish-yellow, more opaque and sticky. The isolates of both morphotypes sometimes appeared as slimy whitish-yellow colonies on PAF, particularly after being kept in the refrigerator. Colonies formed by the isolates of morphotype group C2 were clearly distinct in that they produced an orange-yellow pigment after four to five days on PAF and, in contrast to C1 and C3 isolates, were weakly blue fluorescent under UV light.

One sample with atypical midrib rot

The diseased lettuces obtained from one greenhouse (sample SF1012-1017; Table 1) showed atypical midrib rot symptoms characterized by a dry and mauve-brownish appearance (Fig. 3, up left). Isolations of these lesions yielded dominant bacteria that were non-fluorescent, oxidase negative, pectolytic and HR^+. In pathogenicity tests on lettuce, they caused brown wilting of the leaf after midrib injection but did not incite symptoms after spray-inoculation. Colonies on PAF were translucent pale greenish cream, raised with lobate margins and a rough surface. The four isolates were designated as morphotype group E1 in Table 2, and by fatty acid analysis tentatively identified as belonging to the *Enterobacteriaceae*, which was confirmed by an identification as *Dikeya* sp. through 16S rRNA gene sequence analysis. Indeed, isolate SF1012-01 (LMG 24426) showed 98.2% 16S rRNA gene sequence similarity with *Dikeya dieffenbachiae* CFBP 2051^T (accession number AF520712), *Dikeya zeae* CFBP 2052^T (accession number AF520711), and *Dikeya chrysanthemi* CFBP 1270 (accession number AF520709), previously *Pectobacterium chrysanthemi* [46].

Pathogenicity tests and re-isolation

In the initial pathogenicity tests, all isolates were screened for the ability to produce symptoms on lettuce after injection of the leaf midrib. Eighty-one out of the 149 isolates produced symptoms that ranged from pale brown to dark brown rot of the midrib, to wilting of the entire leaf, depending on the isolate tested. No symptoms were observed on control leaves injected with sterile PB (50 mM, pH 7.0). These 81 isolates with pathogenic potential corresponded to the 14 morphotype groups given in Table 2, and included all the HR^+ (55 isolates) and pectolytic HR^- (22 isolates) fluorescent isolates, and the four non-fluorescent isolates of morphotype group E1.

In subsequent pathogenicity tests, these isolates were further assessed by spray-inoculation on nearly mature greenhouse-grown lettuce. Only isolates with phenotype HR^+ that belonged to

morphotype groups C1, C2 and C3 incited within 2 weeks after spraying leaf midrib rot symptoms similar to those observed on the diseased lettuces from commercial greenhouses (Fig. 3, up right and down left). No symptoms were observed on control plants treated with tap water. Re-isolations from lesions of spray-inoculated plants demonstrated the presence of bacteria with similar colony morphology as the inoculated isolates, and were confirmed to be identical to the inoculated isolates by BOX-PCR genomic fingerprinting (not shown).

Opmerking [b4]: Insert Fig. 3

Genotypic characterization

All 81 isolates that in the initial inoculation tests done by injection were shown to cause rot of the leaf midrib, were further genotypically identified. As mentioned previously, fatty acid analysis assigned all fluorescent isolates of morphotype groups F1 to F10, and C1 to C3, to the genus *Pseudomonas*, while the four non-fluorescent isolates of morphotype group E1 belonged to the family *Enterobacteriaceae*.

Phylogenetic analysis in the genus *Pseudomonas* is universally considered strenuous, with different described intragenic clusters, lineages and groups, based on 16S rRNA gene [1,38], gyrB and rpoD [54] and more recently rpoB [47] sequencing. In general, only small groups of strains clustered together with high bootstrap values. It is clear that in nearly all analyses one rather stable intragenic cluster delineates around *P. aeruginosa* while the other clusters are less pronounced. Tayeb et al [47] suggested not drawing detailed conclusions on relationships above species level based on phylogenetic trees of one or two genes. In addition, the same study considered rpoB sequencing as a useful application for identification of *Pseudomonas* strains at species level. Here, phylogenetic analysis of both 16S rRNA (Fig. 4) and rpoB gene sequences (Fig. 5) were used to identify one representative of each morphotype group preliminary identified as *Pseudomonas* sp. Both genes were analyzed, as the 16S rRNA gene is still the reference gene used for identification. Publicly available sequences of type strains of species belonging mostly to the *P. fluorescens* intragenic cluster were included for phylogenetic analysis of both genes. *Pseudomonas pertucinogena* was chosen as out-group, because this species was found on the borderline of the genus [1,47].

Opmerking [bc5]: Insert Figs. 4 and 5

Both gene sequence analyses showed two intragenic clusters (one around *P. aeruginosa* and one around *P. fluorescens*), supported with high bootstrap values. Within the *P. fluorescens* intragenic cluster, 16S rRNA gene sequence analysis (Fig. 4) showed three groups, containing either *P. fluorescens*, *P. syringae/cichorii*, and *P. putida*, supported with high bootstrap values, while rpoB gene sequence analysis (Fig. 5) could not dinstinguish the *P. putida* group. For both genes, representatives of most pectolytic HR$^-$ pseudomonads – belonging to

1 morphotype groups F2 (R-26745), F3 (R-26429), F4 (R-26735), F5 (R-29016) and F6 (R-
2 29008) – grouped within the *P. fluorescens* group, supported with high bootstrap values. After
3 comparison of the 16S rRNA gene sequences with entries in the EMBL database (Table 2),
4 most isolates within the *P. fluorescens* group revealed high sequence similarities (> 99%)
5 with species previously isolated from aquatic environments such as *P. cedrella*, *P. veronii*,
6 and *P. extremorientalis* [8,11,22]. Thus, strains of morphotype groups F2, F3, F4, F5 and F6
7 could be assigned to the *P. fluorescens* group, although identification onto species level was
8 not possible. Whether these strains are resident epiphytes or could have been introduced on
9 the lettuce plants in association with the irrigation water needs to be further confirmed.
10 The sequence analysis of 16S rRNA and rpoB genes for representatives of morphotype
11 groups F1 (R-27199), F7 (R-26424), F8 (R-26428) and F9 (R-26423) were not congruent. So,
12 assignment to an intragenic or species level is difficult. The representative of morphotype
13 group F10 (R-32840) clustered closely with the type strain of *P. viridiflava* LMG 2352^T for
14 both genes with high bootstrap values which corroborates its allocation to LOPAT group II.
15 Furthermore, comparison of the 16S rRNA gene sequences with entries in the EMBL
16 database (Table 2) showed 100% sequence similarity with the type strain of *P. viridiflava*. It
17 is possible that members of morphotype group F10 belong to this species, but this should be
18 confirmed. It should be noted that in the 16S rRNA gene analysis, *P. viridiflava* clustered
19 within a distinct cluster of *P. fluorescens*, while in the rpoB analysis, *P.viridiflava* is a
20 member of the *P. syringae/cichorii* group. Further research is needed to clarify this uncertain
21 position.
22 Representatives of the midrib rot causing HR^+ isolates, belonging to morphotype groups C1
23 (R-27204), C2 (R-26430) and C3 (R-33145), clearly grouped closely with *P. cichorii* LMG
24 2162^T based on both gene sequence analyses, supported with high bootstrap values. DNA-
25 DNA hybridizations were performed between all three representatives and the type strains of
26 *P. syringae* LMG 1247^T and *P. cichorii* LMG 2162^T, which confirmed the assignment of
27 these pathogenic isolates to the species *P. cichorii* with a DNA relatedness of at least 70% (+/-
28 5%) given in Table 3.

Opmerking [b6]: Insert Table 3

29 For genotypic characterization, both 16 rRNA and rpoB gene sequence analyses were applied
30 for trying to assign representatives of different morphotype groups to certain *Pseudomonas*
31 species. We found that although rpoB gene sequence analysis has been described as a good
32 identification tool in this genus, a large database of rpoB gene sequences of named
33 *Pseudomonas* strains is imperative for species assignment due to unresolved genus

phylogeny. Therefore, presumptive identification at species level still needs to be confirmed by the laborious DNA-DNA hybridization, as was done here for three strains assigned to *P. cichorii*.

BOX-PCR genomic fingerprinting

Repetitive sequence-based PCR genomic fingerprinting (rep-PCR) with the BOXA1R primer [34] was used to assess the genetic diversity of the fluorescent pseudomonads belonging to morphotype groups F1 to F10 and C1 to C3. Rep-PCR is a useful and reliable technique to assess the bacterial diversity at the species, subspecies, or isolate level; and its applications to environmental microbiology have been reviewed [32].

The generated genomic patterns consisted of 20 or more DNA fragments ranging in size from approximately 0.2 to 5.0 kb. Cluster analysis of the BOX-PCR patterns of the 22 pectolytic fluorescent pseudomonads indicated an important genetic heterogeneity. At a cut-off value of 80% similarity (Pearson coefficient), a clustering was obtained that was not in contradiction with the morphotype grouping (designated F1 to F9) (Fig. 6, see supplementary data). The internal genetic homogeneity of each of the morphotype groups as reflected by their BOX clusters seemed mostly relevant when isolates originating from a different infected sample were present (F1, F2, F6 and F8). Although there are no clear-cut guidelines on the minimal number of strains that should be used to cover genetic variability within a bacterial 'type', we are aware that the number of isolates studied per morphotype group was not large enough for definite conclusions on the genetic diversity of the pectolytic fluorescent pseudomonads found on butterhead lettuce in Flanders. Nevertheless, our data suggest a rather genetically heterogeneous group of these pseudomonads present on lettuce. Furthermore, the two *P. viridiflava* isolates of morphotype group F10 yielded an identical BOX-PCR pattern that was distinct from all others including the *P. cichorii* isolates and the HR^- pectolytic pseudomonads (Fig. 6, see supplementary data).

Cluster analysis of the BOX-PCR patterns generated for the 53 *P. cichorii* isolates revealed three distinct but rather genetically homogeneous groups when delineated at 80% similarity. The BOX-PCR groups (BOX I to III, Fig. 7) corresponded remarkably well to the three morphotype groups C1, C2 and C3, which demonstrates the usefulness of a detailed description of colony morphology for the grouping of these plant pathogenic bacteria. Representatives of more than one C-morphotype group were never recovered from the same sample although they were found in 13 of the 14 midrib rot samples, suggesting that the infection within a greenhouse originated from a single inoculum source. BOX-PCR

Opmerking [b7]: Insert Fig. 7

comparative analysis with a number of strains previously reported as causing 'varnish spot' [16] and brown leaf lesions on lettuce [4] or endive [14] revealed that LMG 2163 and IVIA 154 3.1-1 grouped together in the BOX-PCR dendrogram with the isolates of morphotype group C2, whereas the 'varnish spot' strain 9D42 occupied a separate position in the dendrogram. The BOX-PCR genomic fingerprint of the *P. cichorii* type strain LMG 2162 was too distinctive to allocate to a BOX group in Fig. 7 (not shown).

P. cichorii **as a plant pathogen**

P. cichorii has been reported as a leaf pathogen on a broad range of host plants. It causes leaf spot and blight diseases of ornamentals [6,7,12,15,23,37,50], grasses [42], and vegetable crops [41,45,48,52].

On field-grown lettuce, *P. cichorii* has been reported to produce dark-brown, firm, necrotic spots on the blades and petioles of lettuce head leaves, which was referred to as 'varnish spot' in the United States [16] and Italy [2], and 'tar' in Japan [20]. In our greenhouse tests, scattered brown spots were visible on the leaf blades of inner head leaves at the early stage of infection after spray-inoculation (Fig. 3, down right), the spots eventually coalesced into irregular dark rotted lesions but were of minor importance in comparison to the dark brown midrib rot at the later infection stage. In an earlier study by Burkholder [4] on rots of head lettuce, supposedly distinct from 'varnish spot' [16], three bacterial pathogens were found associated each with a distinct rot of which *P. cichorii* was obtained from brown lesions. Further, a report from Canada on damage caused by *P. cichorii* on greenhouse-grown lettuce described symptoms as dark-brown stem rot of inner leaves [10], which seem to resemble the midrib rot symptoms observed in Flanders. Strain variation and lettuce type or cultivar may be responsible for the distinct symptoms but the major factor that might play a role is the different environment of greenhouse *versus* field.

Conclusion

Our approach demonstrated that predominant HR^+ isolates obtained from diseased butterhead lettuces from 13 of the 14 sampled greenhouses in Flanders belong to *Pseudomonas cichorii*. When they were spray-inoculated on greenhouse-grown lettuce, typical midrib rot symptoms developed that were similar to those observed in affected commercial greenhouses. Compared to 'varnish spot' in the field, midrib rot is another manifestation of the infection caused by the same bacterial pathogen on the same host but under greenhouse environmental conditions. The characterization of the pectolytic HR^- isolates revealed a morphologically diverse group of fluorescent pseudomonads, which belonged to the overall intragenic *P. fluorescens* group

1 based on 16S rRNA and rpoB gene sequence analysis. They probably are resident epiphytes
2 on lettuce or partially being introduced in the greenhouse via irrigation water, and their
3 disease potential as soft rot bacteria remains unclear.

4 ## Acknowledgements

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8 for supplying *P. cichorii* strain IVIA 154 3.1-1, and C. Kado (Department of Plant Pathology,
9 University of California, Davis, U.S.A.) for supplying the 'lettuce varnish spot' *P. cichorii*
10 strain 9D42.

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Figure Legends

Fig. 1. Sent to file with supplementary data.

Fig. 2. Appearance on PAF medium of the three *P. cichorii* morphotypes C1 (left), C2 (up) and C3 (down) after 5 days of incubation at 28° C.

Fig. 3. Upper panel: two kinds of leaf midrib rots on middle or inner head leaves were observed among the infected samples collected from 14 lettuce production greenhouses in Flanders. Left = mauve-brownish, dry midrib rot lesions were found in only one greenhouse (sample SF1012-1017), Right = dark-brown, moistened midrib rot lesions were found in the other 13 greenhouses.

Lower panel: symptoms similar to those observed on the lettuces collected from 13 greenhouses were produced by the *P. cichorii* isolates (morphotype groups C1, C2 and C3) after spray-inoculation on greenhouse-grown lettuce plants. Left = typical dark brown midrib rot lesion on an inner head leaf two weeks after inoculation, Right = small, brown spots on the leaf blades one week after inoculation.

Fig. 4. Phylogenetic analysis of 16S rRNA gene. Neighbour-joining tree based on a 386 bp alignment of 16S rRNA gene sequences of representatives of the studied fluorescent pseudomonads from lettuce midrib rot symptoms, and type strains of related *Pseudomonas* species. The 16S rRNA gene fragment used for phylogenetic analysis ranged from position 13 to 399, according to reference sequence numbering of *Pseudomonas aeruginosa* LMG 1242^{T} (accession number Z76651). EMBL accession numbers are shown in parenthesis. The symbols F1 to F10 and C1 to C3 refer to the morphotype groups distinguished among the isolates on the basis of colony appearance. *Pseudomonas pertucinogena* was included as out-group. Bootstrap values (expressed as percentages of 1000 replicates) are shown at the branch points. Bar represents 0.02 substitutions per nucleotide position.

1 **Fig. 5.** Phylogenetic analysis of *rpoB*. Neighbour-joining tree based on a 981 bp alignment of
2 rpoB gene sequences, showing the relationships of representatives of the studied fluorescent
3 pseudomonads from lettuce midrib rot symptoms within a subset of closely related
4 *Pseudomonas* species. The rpoB gene fragment used for phylogenetic analysis ranged from
5 position 1465 to 2431, according to reference sequence numbering of *Pseudomonas*
6 *aeruginosa* PAO1 (accession number AE004091). Values (expressed as percentages) are the
7 number of times that a branch appeared in 1000 bootstrap replications. EMBL accession
8 numbers are given in parenthesis. F1 to F10 and C1 to C3 refer to the morphotype groups
9 defined among the isolates on the basis of colony appearance. *Pseudomonas pertucinogena*
10 was included as out-group. Bar represents 0.05 substitutions per nucleotide position.
11 *Note: accession number AJ717459 refers to *P. stutzeri* strain LMG 11199^T and has been
12 wrongly registered as CIP 11199^T in the EMBL database.
13 **Fig. 6.** Sent to file with supplementary data.
14 **Fig. 7.** Grouping of BOX-PCR patterns of the 53 *P. cichorii* isolates from lettuce midrib rot
15 and known reference strains (9D42, IVIA154 3.1-1 and LMG 2163) previously reported as
16 causing similar leaf symptoms on lettuce or endive [4,14,16]. The dendrogram was
17 constructed by UPGMA clustering with Pearson's correlation similarity coefficients using
18 BioNumerics version 4.6. C1 to C3 refer to the three morphotype groups distinguished among
19 the *P. cichorii* isolates based on colony appearance. The cophenetic correlation tool was used
20 for cluster significance analysis.

Fig. 2.

B. Cottyn, K. Heylen, J. Heyrman, K. Vanhouteghem, E. Pauwelyn, P. Bleyaert, J. Van Vaerenbergh, M. Höfte, P. De Vos, M. Maes

1

2

3 **Fig. 3.**

4 B. Cottyn, K. Heylen, J. Heyrman, K. Vanhouteghem, E. Pauwelyn, P. Bleyaert, J. Van

5 Vaerenbergh, M. Höfte, P. De Vos, M. Maes

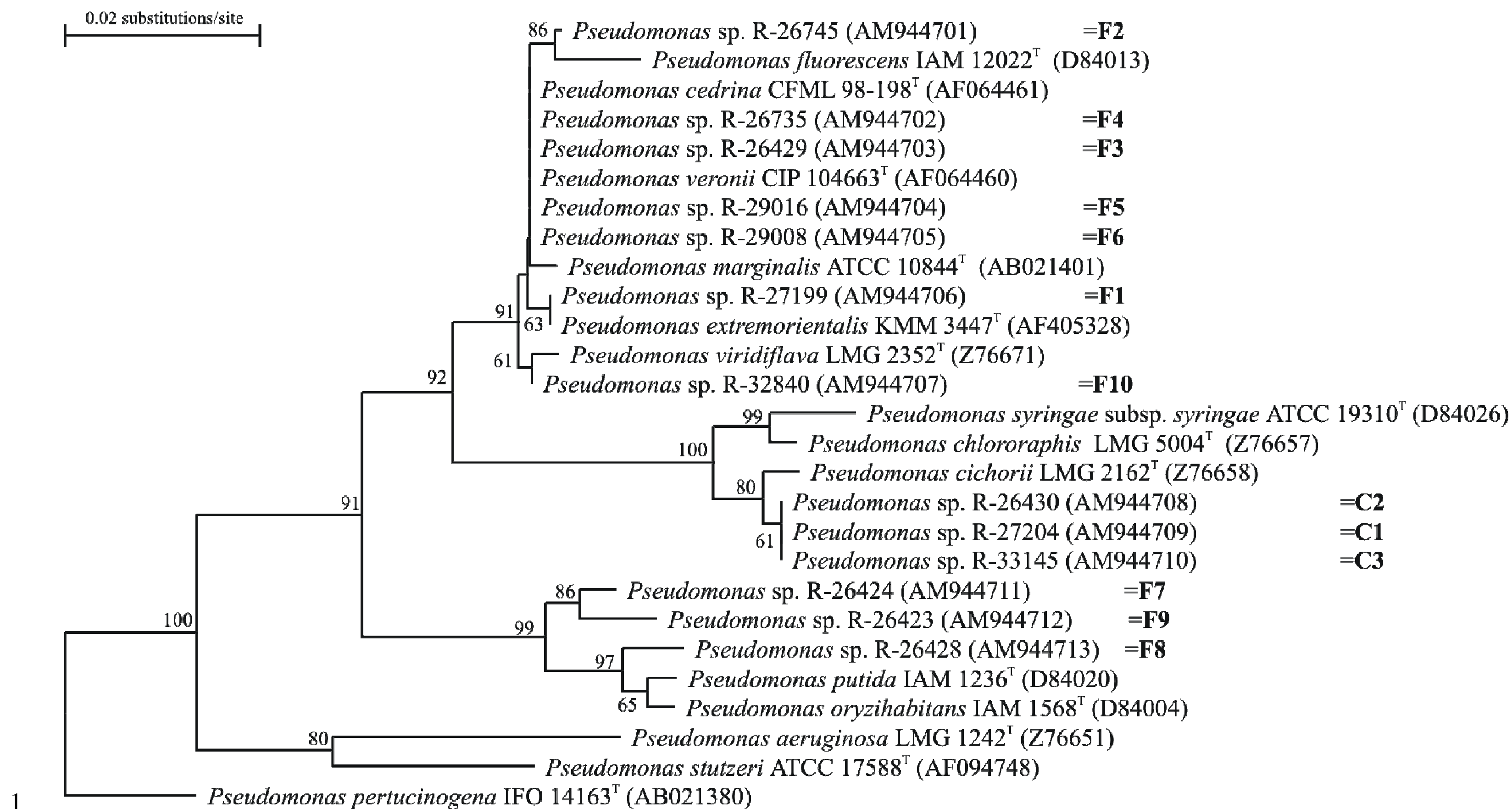
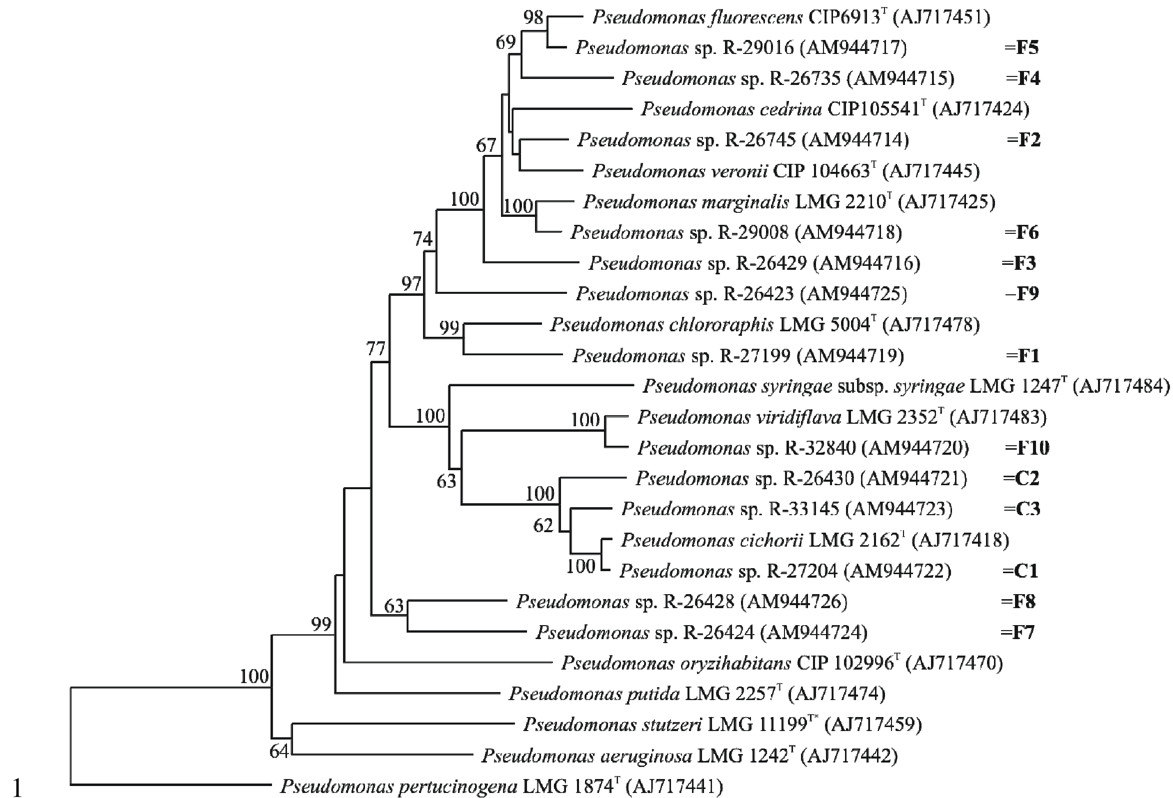


Fig. 4.

B. Cottyn, K. Heylen, J. Heyrman, K. Vanhouteghem, E. Pauwelyn, P. Bleyaert, J. Van Vaerenbergh, M. Höfte, P. De Vos, M. Maes



1

2 **Fig. 5.**

3 B. Cottyn, K. Heylen, J. Heyrman, K. Vanhouteghem, E. Pauwelyn, P. Bleyaert, J. Van Vaerenbergh, M. Höfte, P. De Vos, M. Maes

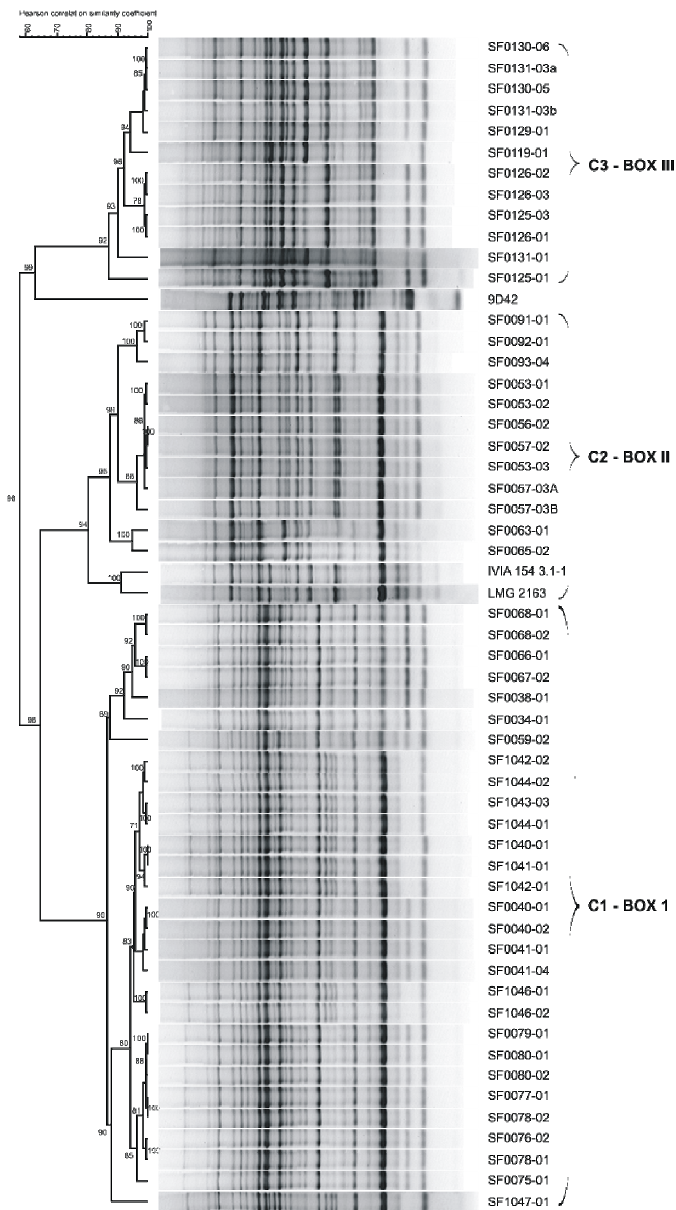


Fig. 7.

B. Cottyn, K. Heylen, J. Heyrman, K. Vanhouteghem, E. Pauwelyn, P. Bleyaert, J. Van Vaerenbergh, M. Höfte, P. De Vos, M. Maes

1 Table 1. Samples of symptomatic butterhead lettuces collected from 14 lettuce production

2 greenhouses in the region of Flanders

Isolation date	Sample No.	No. of isolates	Butterhead lettuce cultivar	Location
09/2004	SF1012-1017	11	n.a.	Haasdonk
10/2004	SF0034-0039	19	Flandria	Houthulst
10/2004	SF0040-0042	11	Flandria	Koolskamp
11/2004	SF0053-0057	15	Burgia	Reninge
12/2004	SF0058-0059	2	Lollo bionda	Berlaar
02/2005	SF1040-1047	26	Burgia	Lemberge
02/2005	SF0063-0065	5	Hofnar	Putte
02/2005	SF0066-0067	6	Hofnar	St-Kathelijne Waver
02/2005	SF0068-0069	7	Hofnar	St-Kathelijne Waver
04/2005	SF0075-0080	16	Lollo bionda	Torhout
06/2005	SF0091-0093	8	Flandria	Ingelmunster
09/2005	SF0119-0120	5	n.a.	Gits
10/2005	SF0125-0126	6	Zendria	Ardooie
11/2005	SF0129-0131	12	Lollo rossa	Oostnieuwkerke

3 n.a. = not available

Table 2. Characterization of the morphotype groups distinguished among the 81 midrib rot isolates that caused symptoms in initial inoculations by injection of the leaf midrib of lettuce

Group[a]	Occurrence[b]	Representative isolates[c]			Colony appearance[d]	Fluorescence[e]	LOPAT grouping[f]						PL[g]	16S rRNA gene sequence similarity[h]			
		Original no.	Synonymous no.				L	O	P	A	T			Closest relatives	Strain no.	%	Acc. no.
E1 (4)	1 site	SF1012-01	R-24816 (AM945592)	LMG 24426	Translucent pale greenish cream, circular, lobate margins, raised	none	nd	-	+	nd	+		+	*Dickeya zeae*	CFBP 2052^{T}	98.2	AF520711
														D. dieffenbachiae	CFBP 2051^{T}	98.2	AF520712
														D. chrysantemi	CFBP 1270	98.2	AF520709
F1 (5)	2 sites	SF1045-01	R-27199 (AM944706)	LMG 24436	Translucent dry greenish-white, circular, serrate margins, low convex	blueish	-	+	+	+	-	IVb	+	*Pseudomonas veronii*	CIP 104663^{T}	99.8	AB021411
														P. extremorientalis	KMM 3447^{T}	99.8	AF405328
F2 (3)	3 sites	SF0041-07	R-26745 (AM944701)	LMG 24435	Translucent pale yellowish-green, circular, serrate margins, raised	pale yellowish-green	+	+	+	+	-	IVa	(+)	*P. veronii*	CIP 104663^{T}	100	AF064460
														P. cedrella	CFML 96-198^{T}	100	AF064461
														P. chlororaphis	DSM 6698	100	AY509898
F3 (1)	1 site	SF0057-01	R-26429 (AM944703)	LMG 24433	Translucent rough yellowish-white, circular, undulate margins, flat	dim greyish-blue	-	+	+	+	-	IVb	+	*P. fluorescens*	ATCC 13525^{T}	99.3	AF094725
														P. corrugata	ND9L	99.3	AF348508
F4 (3)	1 site	SF0039-02	R-26735 (AM944702)	LMG 24434	Butyrous bright yellowish-green, circular, serrate margins, convex	bright yellowish-green	+	+	+	+	-	IVa	-	*P. veronii*	CIP 104663^{T}	100	AF064460
F5 (2)	1 site	SF0080-03	R-29016 (AM944704)	LMG 24438	Pale olive-yellow, circular, entire margins, convex	yellowish-green	+	+	+	+	-	IVa	-	*P. veronii*	CIP 104663^{T}	100	AF064460
														P. cedrella	CFML 96-198^{T}	100	AF064461
														P. chlororaphis	DSM 6698	100	AY509898
F6 (3)	2 sites	SF0077-03	R-29008 (AM944705)	LMG 24437	Butyrous brown yellowish-green, circular, serrate margins, convex	bright yellowish-green	+	+	+	+	-	IVa	-	*P. veronii*	CIP 104663^{T}	100	AF064460
														P. cedrella	CFML 96-198^{T}	100	AF064461
														P. chlororaphis	DSM 6698	100	AY509898
F7 (1)	1 site	SF0055-02	R-26424 (AM944711)	LMG 24431	Butyrous green beige, circular, serrate margins, convex	blueish-white	-	+	+	+	-	IVb	(+)	*P. xanthomarina*	KMM 1447^{T}	99.5	AB176954
														P. stutzeri	DSM 50227	99.3	U26415

													P. chloritidismutans	AW-1^T	99.3	AY017341
F8 (2)	2 sites	SF0056-03	R-26428 (AM944713)	LMG 24432	Butyrous sand-yellow, circular, entire margins, convex	dim greyish-blue	-	+	+	+	-	IVb	-	*P. oryzihabitans*	HAMBI 2374	99.3	AF501336
														P. putida	ATCC 17494	99.0	AF094740
														P. asplenii	ATCC 23835^T	98.6	AB021397
F9 (2)	2 sites	SF0055-01	R-26423 (AM944712)	LMG 24430	Slimy pale greenish yellow, circular, entire margins, droplike	yellowish-green	-	+	+	+	-	IVb	-	*P. putida*	ATCC 11250	99.5	AF095892
														P. fulva	NRIC 0180^T	99.5	AB046998
														P. parafulva	AJ 2129^T	99.5	AB060132
F10 (2)	1 site	SF0120-01	R-32840 (AM944707)	LMG 24439	Mucoid pale lemon yellow, circular, serrate margins, low convex	dim pale yellow	-	-	+	-	+	II	+	*P. migulae*	CIP 105470^T	100	AF074383
														P. viridiflava	KNOX3.4a	100	AY574907
														P. corrugata	ND9L	100	AF348508
C1 (29)	7 sites	SF1047-01	R-27204 (AM944709)	LMG 24428	Butyrous pale greenish yellow /Slimy whitish-yellow	yellowish-green	-	+	-	-	+	III	-	*P. cichorii*	ATCC 10857^T	100	AB021398
														P. syringae	MAFF 302259	98.6	AB001442
														P. congelans	LMG 21466^T	98.6	AJ492828
C2 (12)	3 sites	SF0057-02	R-26430 (944708)	LMG 24427	Mucoid greenish-white turning to orange-yellow	dim blueish	-	+	+	-	+	III	-	*P. cichorii*	ATCC 10857^T	99.7	AB021398
														P. syringae	MAFF 302259	98.6	AB001442
														P. congelans	LMG 21466^T	98.6	AJ492828
C3 (12)	3 sites	SF0119-01	R-33145 (AM944710)	LMG 24440	Butyrous greenish yellow /Slimy whitish-yellow	yellowish-green	-	+	-	-	+	III	-	*P. cichorii*	ATCC 10857^T	100	AB021398
														P. syringae	MAFF 302259	98.6	AB001442
														P. congelans	LMG 21466^T	98.6	AJ492828

[a] The 81 isolates that showed to have ‘pathogenic potential’, were classified into 14 morphotype groups on the basis of similarities in colony appearance (colony shape, texture, and colour). Number of isolates representing each morphotype group is given in parenthesis. The isolates of morphotype group E1 belonged to the *Enterobacteriaceae* based on whole cell fatty acid methyl ester (FAME) analysis, isolates of the other 13 morphotype groups were assigned to *Pseudomonas*. The isolates of morphotype groups C1, C2 and C3 produced midrib rot symptoms in subsequent spray-inoculation experiments, the others did

1 not.

2 [b] Indicates the number of sites, on 14 greenhouses sampled, from where isolates of a morphotype group were found.

3 [c] Original no. = isolate number; Synonymous no. = representative isolates were deposited in the Research Collection (R-accession numbers) and the

4 BCCM/LMG Bacteria Collection (LMG accession numbers) of the University Ghent, Belgium, EMBL accession numbers of deposited partial 16S rRNA gene

5 sequences are given in parenthesis.

6 [d] The colony shape, texture and colour of 5-day-old cultures grown at 28° C on PAF medium was examined visually and under the dissecting microscope at

7 12-power magnification.

8 [e] Fluorescent pigment production on PAF medium under UV light (366 nm).

9 [f] The LOPAT tests for the grouping of fluorescent pseudomonads [26] include the following physiological characteristics: L = levan formation from sucrose, O

10 = oxidase reaction, P = ability to rot potato, A = arginine dihydrolase, T = hypersensitive reaction in tobacco leaves. n.d. = not determined

11 [g] PL = extracellular pectate lyase activity measured spectrophotometrically in culture supernatans. + = positive, (+) = weak positive, - = negative

12 [h] Characterization of the isolates on the basis of 16S rRNA gene sequence analysis. Closest relatives obtained by comparison to the EMBL database using the

13 FASTA search option [40]. Similarity percentages, strain and accession numbers of the closest related database entries are given.

1 Table 3. DNA-DNA hybridization results between representatives of morphotype groups C1

2 (R-27204, SF1047-01), C2 (R-26430, SF0057-02), C3 (R-33145, SF0119-01) and the type

3 strains of *P. cichorii* LMG 2162 and *P. syringae* LMG 1247

	DNA reassociation values (%)				
	R-27204	R-26430	R-33145	LMG 2162^{T}	LMG 1247^{T}
R-27204	100				
R-26430	75	100			
R-33145	82	65	100		
P. cichorii LMG 2162^{T}	95	67	82	100	
P. syringae LMG 1247^{T}	32	32	34	34	100

4