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Characterisation of fungal pathogens causing basal rot of lettuce in Belgian greenhouses

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Abstract Basal rot is a common disease in lettuce greenhouses. A 3-year study on the diversity of pathogens associated with basal rot in Belgium was carried out. A total of 150 isolates were collected originating from 56 greenhouses. Four pathogens appeared to be involved. *Rhizoctonia solani* was found to be the causal agent at 23 locations, *Sclerotinia* spp. at 14, *Botrytis cinerea* at 17 and *Pythium* spp. at seven. The isolates of *R. solani* were further characterised to anastomosis groups and subgroups using morphological characteristics, pectic zymogram and PCR-RFLP. Five anastomosis groups could be distinguished: AG1-1B, AG4 HGI, AG10, AG2-1, AG2-1 Nt and AG3, with isolates of AG4 HGI and AG1-1B being the most prevalent and the most aggressive. *Sclerotinia sclerotiorum* was found at 13 locations, while *S. minor* was found at only one location. Based on ITS-sequencing *Pythium* isolates were assigned to three different species. At 20°C, isolates of all pathogens were able to cause lesions on detached lettuce leaves, except isolates of *R. solani* AG3 and AG2-1 Nt. A correlation could be found between the occurrence of the pathogens and the growing season. *Botrytis cinerea* was the most common pathogen in winter, whereas *R. solani* was most frequently isolated in summer. *Sclerotinia* spp. and *Pythium* spp. were isolated in spring, summer and autumn. The information obtained in this study will be most useful in the development of an alternative control strategy for causal agents of basal rot.

Keywords Anastomosis group · *Botrytis cinerea* · Bottom rot · *Pythium* spp. *Rhizoctonia solani* · *Sclerotinia* spp.

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Introduction

Belgium is one of the major lettuce (*Lactuca sativa*) producing countries of the world (Subbarao 1998). The typical butterhead lettuce is usually cultivated in greenhouses in a continuous monoculture or in crop rotation with tomato. This intensive management has lead to an increase in incidence of soilborne pathogens. Soilborne diseases in lettuce used to be managed by methyl bromide fumigation. In Belgium, the use of methyl bromide has been completely prohibited since 2006 and effective alternatives are urgently needed. Increased environmental considerations have stimulated research on non-chemical and integrated control strategies for soilborne pathogens. When developing integrated control strategies the knowledge of the causal pathogens is crucial. Unfortunately, research

on identification has been neglected in the past, partially due to the broad spectrum approach of methyl bromide (Martin 2003).

Worldwide various soilborne pathogens are known to affect lettuce, including *Sclerotinia* spp. (lettuce drop), *Rhizoctonia solani* (bottom rot), *Botrytis cinerea* (grey mold) and *Pythium* spp. (Davis et al. 1997). These pathogens are responsible for serious yield losses, causing symptoms described as basal rot, i.e. rotting of the leaves in contact with soil. Heavily infested crops will collapse completely, whereas the loss of lower leaves in less infested crops will make them often unmarketable, because of the decrease in size and weight (Wareing et al. 1986).

Sclerotinia sclerotiorum and *S. minor* have been observed worldwide as the causal agents of lettuce drop, but at most locations only one species or the other is responsible for losses (Subbarao 1998). In 2000, a third species which also causes lettuce drop symptoms, was detected in China, namely *S. nivalis* (Li et al. 2000).

Rhizoctonia solani is a species complex and based on hyphal anastomosis reactions it can be subdivided into 13 anastomosis groups (AGs): AG1 to 13 (Carling et al. 2002). Members of different AGs can react differently to control measurements (Kataria and Gisi 1996), stressing the importance of assigning isolates to AGs. Most AGs are further divided into subgroups; isolates belonging to the same subgroup have high similarity in host spectrum, colony morphology, nutritional requirements, biochemical and molecular characteristics (Cubeta and Vilgalys 1997; Kuninaga et al. 1997; Ogoshi 1987). Previous studies in Germany, the United States and Brazil showed AG1-1B as the predominant AG causing bottom rot in field-grown lettuce (Grosch et al. 2004; Herr 1992; Kuramae et al. 2003). In greenhouses in the UK and the Netherlands, isolates of AG1, AG2 and AG4 were detected (Kooistra 1983; Wareing et al. 1986).

Botrytis cinerea is recorded wherever lettuce is grown, often in combination with *R. solani*, *Sclerotinia* spp. or *Pythium* spp. (Davis et al. 1997; Wareing et al. 1986). In the Netherlands several species of *Pythium* have been observed associated with basal rot: *P. tracheiphilum*, *P. incinulatum* and *P. sylvaticum* (Blok and Vanderplaats-Niterink 1978).

Although basal rot is very common in Belgian lettuce greenhouses, no previous research has been conducted on the possible causal agents. Therefore, the aims of this study were (a) to identify and characterise isolates collected from symptomatic lettuce crops, using morphological, biochemical and molecular techniques, and (b) to determine their relative importance, their pathogenic potential and aggressiveness. This information will be of great importance in the design of alternative methods for controlling basal rot.

Materials and methods

Pathogen isolation

From 2004 to 2007, lettuce plants with symptoms of basal rot were collected from greenhouses in the major lettuce producing regions in Belgium. In every greenhouse, three to five symptomatic plants were selected randomly. Isolation was achieved by cutting eight pieces (±1 cm^2) on the margin of healthy and diseased tissue. The pieces were first rinsed in tap water, surface-sterilised in 1% NaOCl for 1 min and rinsed in 0.3% NaCl (Grosch et al. 2004). Four pieces per plant were plated on water agar (WA) containing 50 μg ml^{-1} streptomycin and 50 μg ml^{-1} carbenicillin (WA+), the other four on a basidiomycete selective medium (WA+ amended with 4 μg ml^{-1} prochloraz (as Sporgon WP, BASF Belgium S.A.) (WASP); all plates were incubated at 20°C. If sclerotia were present on the diseased plants, they were collected, surface–sterilised (2 min in 1% NaOCl followed by 2 min in 70% ethanol), rinsed in sterile demineralised water, bisected and plated on WA+. After 1–4 days, hyphae growing out of the tissue pieces or sclerotia were transferred to potato dextrose agar (PDA) for further purification and identification. The isolates were identified to genus level, based on microscopic and cultural characteristics and stored in the laboratory collection. The isolates of *Rhizoctonia*, *Botrytis* and *Sclerotinia* were maintained on PDA slants at room temperature. *Pythium* isolates were conserved by placing mycelium plugs in plastic tubes containing sterile demineralised water.

Isolate characterisation

Isolates of *Rhizoctonia* were characterised to anastomosis groups and subgroups based on pectic zymograms, PCR-RFLP and sequencing of the rDNA-ITS region, following the same procedure as Pannecouc-

que et al. (2008). Isolates of *Sclerotinia* and *Botrytis* were characterised based on cultural characteristics and SDS-PAGE. In order to compare cultural characteristics, isolates were grown on PDA at 20°C for three weeks. Sclerotial characteristics were used to distinguish between *S. sclerotiorum* and *S. minor*, according to Tariq et al. (1985). The sclerotial proteins of *Sclerotinia* and *Botrytis* isolates were compared by a one-dimensional SDS-PAGE (Li et al. 2000; Tariq et al. 1985). Five sclerotia were ground in liquid nitrogen to a fine powder by a mortar and pestle. The powder was suspended in 400 µl extraction buffer (0.05 M Tris–Glycine, pH 8.3) and after homogenisation, centrifuged at 13,000 rpm for 5 min. The supernatant was stored at −20°C until further use. The protein concentration was estimated with a spectrophotometer at 280 nm. A stock solution of the sample buffer was prepared, containing 0.01 g bromophenol blue, 4.8 ml Tris–HCl (0.5 M, pH 6.8), 8 ml of 10% SDS (*w*/*v*), 4 ml glycerol and 21.2 ml H_2O and stored at −20°C. Before use, 475 µl of the sample buffer was amended with 25 µl β-mercapto-ethanol. The supernatant was dissolved in the sample buffer, in order to obtain a final concentration of 1 µg protein μl^{-1}. The samples were denaturated for 5 min at 95°C. Ten microliters was loaded in a polyacrylamide gel, consisting of a 4% stacking gel (0.5 M Tris–HCl, pH 6.8) and a 12% resolving gel (1.5 M Tris–HCl, pH 8.8). Electrophoresis was conducted at room temperature under a constant voltage of 150 V, using 0.025 M Tris–glycine buffer (pH 8.3) containing 0.1% SDS (*w*/*v*) as running buffer. The gels were stained in coomassie blue.

Identification of the *Pythium* isolates was done by sequencing of the rDNA-ITS region (Levesque and De Cock 2004). Each isolate was grown in 25 ml of liquid medium (10 g sucrose, 1 g yeast extract, 1 l distilled water) for 7 days at 25°C (Wang and White 1997). Mycelium mats were harvested by filtration, blotted dry and ground in liquid nitrogen. The next steps in the DNA-extraction were carried out using the DNeasy Plant Mini Kit (Qiagen) according to manufacturer's instructions. The ITS region was amplified using primers ITS1 and ITS4 (White et al. 1990). The total reaction volume was 50 µl, containing 28.5 µl sterile highly purified water, 10 µl DNA (10 ng μl^{-1}), 5 µl 10× reaction buffer, 3 µl $MgCl_2$ (25 mM), 1 µl dNTPs (10 mM), 1 µl each of 10 µM primers, 0.5 µl Taq DNA polymerase (5 units μl^{-1}; Sigma-Aldrich). Amplification was performed in a Tetrad Thermal Cycler (Model: MJ Research PTC-200; GMI Inc.) with an initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 90 s, 55°C for 90 s, 72°C for 2 min, and a final extension at 72°C for 10 min. PCR products were analysed by electrophoresis (100 V, 30 min) on 1% agarose gels in TAE buffer. Purification of PCR products was carried out to remove the excess of primers and dNTPs. Sequencing was performed using the CEQ DTCS sequencing chemistry (Beckman Coulter Inc.) and run on an automated DNA capillary sequencer (Model: CEQ 2000-XL; Beckman Coulter Inc.).

Pathogenicity determination

A detached leaf test was conducted to determine the pathogenicity of the isolates towards butterhead lettuce (Thornton et al. 1999). Detached leaves from an adult lettuce plant (*Lactuca sativa* cv. Alexandria, Rijk Zwaan, De Lier, The Netherlands) were inoculated with two mycelial plugs (4 mm diam), one on each site of the leaf; control leaves were inoculated with sterile PDA plugs. The leaves were placed in sealable plastic containers with moistened tissue paper and incubated at 20°C in the dark. There were four replicate samples for each isolate tested. The lesion size was measured after 3 days for *Sclerotinia* spp., after 4 days for *B. cinerea* and after 5 days for *Pythium* spp. For the *Rhizoctonia* isolates the lesion size was measured at two different temperatures: after 6 days at 20°C and after 4 days at 28°C. The software programme ASSESS (Image analysis software, The American Phytopathological Society, 2002) was used for plant disease quantification.

Additionally, pathogenic potential of *R. solani* isolates towards lettuce seedlings (*L. sativa* cv. Suzan, De Bolster, Kielwindeweer, The Netherlands) and tomato seedlings (*Lycopersicon esculentum* cv. Moneymaker) was evaluated according to an *in vitro* bioassay developed by Keijer et al. (1997). Before use, the seeds were disinfected in 0.5% NaOCl for 1 min and rinsed three times in sterile demineralised water. The bioassay was conducted in square Petri dishes (12×12 cm) on B5 medium (basal salt mixture including vitamins, Duchefa Biochemie). Six seeds were placed at equal intervals of 2 cm; two mycelial plugs (7 mm) were placed between the seeds. Sterile PDA plugs were used as control. The plates were

sealed with Parafilm and wrapped in aluminium foil in order to protect the roots from light. First, the plates were incubated in the dark for 48 h, followed by incubation in 12 h daylight at 21°C. Disease severity was assessed using the score described by Carling et al. (1999), with minor modifications: 0=no damage, 1=minor discolouration on stem, hypocotyl, root or leaf, 2=discolouration plus small necrotic lesions on stem, hypocotyl, root or leaf (<1 mm diam), 3=discolouration plus large necrotic lesion on stem, hypocotyl, root or leaf (≥1 mm diam), 4=seedling dead.

Statistical analysis

All data were analysed using the software package SPSS 15.0 for windows. Data of the detached leaf tests were analysed by a one-way ANOVA. Homogeneity of variance was assessed using Levene's test. As variances and sample size were unequal, differences between groups were tested using Tamhane's *T*2 test (P=0.05) (Tamhane 1979). The ordinal data of the pathogenic potential test were analysed using non-parametric statistics. If data of a same AG were not statistically different according to the Kruskal–Wallis test (P=0.05), data were pooled per group/subgroup. Differences between groups/isolates were detected based on Mann–Whitney comparisons (P=0.05).

Results

Pathogen isolation

From 2004 to 2007, a total of 150 isolates were collected from lettuce crops with basal rot symptoms, originating from 56 greenhouses in Belgium. For each crop, based on morphological characteristics one isolate was retained (see Table 1). Morphological and microscopic characteristics revealed the occurrence of four genera: *Rhizoctonia*, *Sclerotinia*, *Botrytis* and *Pythium* isolates, originating from 23, 14, 17 and seven greenhouses, respectively. In almost all cases, the symptoms were assigned to one pathogen, except in five greenhouses where two different pathogens were detected at the same time; combinations of *Sclerotinia* and *Botrytis* (two locations), *Rhizoctonia* and *Sclerotinia* (one location), *Botrytis* and *Pythium* (one location), and *Pythium* and *Rhizoctonia* (one location) were found. Although different pathogens occurred in one greenhouse, they were never isolated from the same crop. Symptoms caused by the four pathogens were slightly different, but based on symptoms alone a correct diagnosis could rarely be made. A remarkable correlation was found between the occurrence of the pathogens and the time of the year (see Table 2). Most isolates of *Rhizoctonia* (78.3%) were collected in summer, whereas *Botrytis* occurred more in winter (58.8%). Problems caused by *Pythium* and *Sclerotinia* were more common in spring, summer and autumn; during winter *Pythium* was not reported and *S. sclerotiorum* was only isolated once.

Isolates of *R. solani* were further characterised to anastomosis group and subgroup level (see Table 1). Using pectic zymograms, 18 isolates, 22 isolates and two isolates shared the same zymogram pattern with tester-isolates of AG1-1B, AG4 HGI and AG10, respectively. RFLP-analysis of the ITS-region using enzymes *Mse*I, *Mun*I, *Ava*II and *Hin*cII confirmed these results (that 18 isolates belonged to AG1-1B, 22 to AG4 HGI and two to AG10). Additionally, one isolate could be assigned to AG3 and one to AG2-1. Based on the RFLP technique, three isolates, S066-1, S042-2 and S042-5, could not be identified. Isolate S066-1 showed the same PCR-RFLP patterns as AG9 and AG2-1 Nt, and it is impossible to distinguish between these two groups using this RFLP-method (Guillemaut et al. 2003). Two other isolates originating from the same greenhouse (S042) possessed an RFLP-pattern which did not correspond with one of the tester isolates used in this study. The ITS-regions of isolates S066-1 and S042-2 were sequenced and compared to the NCBI database using Megablast. S066-1 had 99% similarity with isolate RT-23 (AB054853), an AG2-1 Nt isolate from tobacco. Based on this result, S066-1 was assigned to the AG2-1 Nt group. S042-2 showed 95% similarity with AG2-1 (DQ355133), AG10 (DQ356410) and AG9TP (AY154315). No higher similarity could be detected with isolates present in GenBank.

Based on morphological characteristics, i.e. sclerotial size and sclerotial distribution, 17 *Sclerotinia* isolates were assigned to the species *S. sclerotiorum* and one isolate to the species *S. minor*. The isolates identified as *S. sclerotiorum* produced large sclerotia (2–20 mm), which grew in concentric circles; the *S. minor* isolate produced smaller sclerotia (0.5–2 mm),

Table 1 Identification of the pathogens isolated from lettuce with basal rot symptoms, with RS: *R. solani*, BC: *B. cinerea*, SS: *S. sclerotiorum*, SM: *S. minor*, PC: *P. cylindrosporum/P.(ir)regulare*, PU: *P. ultimum*, PS: *P. sylvaticum*

Greenhouse	Isolates	Pathogen	AG	Isolation date
S001	S001-1, S001-2	RS	AG1-1B	3-06-05
S002	S002-1, S002-2, S002-3	RS	AG1-1B	4-08-05
S003	S003-1	RS	AG10	13-07-04
S004	S004-1	RS	AG4 HGI	11-08-04
S005	S005-1, S005-2	RS	AG4 HGI	11-08-04
S006	S006-1, S006-2, S006-3	SS		11-08-04
S007	S007-1, S007-2, S007-3, S007-4	BC		15-11-04
S008	S008-1, S008-3, S008-4, S008-5	RS	AG4 HGI	26-08-05
S010	S010-1, S010-2, S010-3, S010-5	RS	AG4 HGI	8-09-05
S011	S011-1, S011-2	RS	AG1-1B	9-09-05
S012	S012-1, S012-3	RS	AG4 HGI	13-09-05
S013	S013-2	RS	AG2-1	14-09-05
S014	S014-1, S014-3	SS		14-09-05
S014	S014-2	RS	AG1-1B	14-09-05
S016	S016-1	RS	AG4 HGI	20-09-05
S017	S017-1	BC		20-09-05
S020	S020-2	BC		20-09-05
S025	S025-2, S025-3, S025-5, S025-6	RS	AG4 HGI	20-09-05
S025	S025-4	RS	AG1-1B	20-09-05
S026	S026-1	SS		29-08-05
S028	S028-1	SS		6-10-05
S029	S029-1	SS		6-10-05
S030	S030-1	SS		4-10-05
S033	S033-1	SS		13-03-05
S034	S034-1	SS		1-04-05
S036	S036-1	SS		20-10-05
S037	S037-1	SS		20-10-05
S037	S037-2, S037-4	BC		20-10-05
S038	S038-3	PC		18-11-05
S040	S040-2, S040-3, S040-4	PC		28-11-05
S042	S042-2, S042-5	RS	unknown	30-11-05
S042	S042-4	RS	AG3	30-11-05
S044	S044-1, S044-2, S044-3, S044-5	BC		3-01-06
S045	S045-3, S045-6, S045-7	BC		5-01-06
S047	S047-1, S047-2, S047-4, S047-5	BC		9-01-06
S048	S048-5	BC		17-01-06
S049	S049-2, S049-3, S049-5	BC		17-01-06
S050	S050-1, S050-2	BC		1-03-06
S058	S058-1, S058-2	BC		2-03-06
S060	S060-3	BC		17-03-06
S061	S061-3	BC		5-04-06
S061	S061-4	PS		5-04-06
S062	S062-1, S062-4	BC		7-04-06
S063	S063-2, S063-4	SS		18-04-06
S063	S063-1, S063-3	BC		18-04-06
S064	S064-1, S064-3	RS	AG1-1B	12-05-06
S065	S065-1, S065-3	RS	AG1-1B	16-05-06
S066	S066-1	RS	AG2-1 Nt	5-07-06
S067	S067-1	PS		5-07-06
S068	S068-1	RS	AG1-1B	5-07-06

Table 1 (continued)

Greenhouse	Isolates	Pathogen	AG	Isolation date
S068	S068-2, S068-3	PU		5-07-06
S069	S069-1, S069-2	RS	AG1-1B	5-07-06
S070	S070-1, S070-2, S070-3, S070-4	PU		5-07-06
S071	S071-1	SS		11-07-06
S072	S072-2, S072-4	RS	AG4 HGI	31-07-06
S075	S075-1, S075-2	RS	AG4 HGI	10-08-06
S076	S076-3, S076-4	RS	AG1-1B	10-08-06
S077	S077-1	SS		21-09-06
S080	S080-2	RS	AG-10	9-11-06
S081	S081-2, S081-3	PC		25-10-06
S082	S082-1, S082-3	BC		5-01-07
S085	S085-1	BC		15-01-07
S086	S086-3	SM		19-04-07

which were scattered over the plate. Using one dimensional SDS-PAGE to compare the protein pattern of the sclerotia, *S. minor* could be distinguished from *S. sclerotiorum* by means of the small protein band of approximately 18 kDa (see Fig. 1).

The sclerotia of isolates identified as *B. cinerea* all showed identical protein patterns, which appeared to be different from the pattern of the *Sclerotinia* isolates (see Fig. 1). As the *Botrytis* isolates collected in this study did not sporulate in the dark, morphological differentiation between *Botrytis* and *Sclerotinia* isolates was done by studying the morphology of the sclerotia. Sclerotia of *B. cinerea* are planoconvex and attached to the substrate, whereas the tuberoid sclerotia of *Sclerotinia* spp. are formed free of the substrate (Willetts 1997).

In total 14 *Pythium* isolates were collected at seven locations. One isolate per location was randomly chosen for further characterisation. Comparison of ITS sequences of these isolates to sequences in GenBank, revealed the occurrence of *P. sylvaticum* at two locations and *P. ultimum* var. *ultimum* at two other locations. Three isolates could not be assigned to one species; S038-3, S040-4 and S081-3 showed a high similarity (>97%) with *P. cylindrosporum* (AY598643), *P. regulare* (AF492018) and *P. irregulare* (AB108000).

Table 2 Number of greenhouses with recovery of *B. cinerea*, *R. solani*, *S. sclerotiorum* and *Pythium* spp. in spring (21/3–21/6), summer (21/6–21/9), autumn (21/9–21/12) or winter (21/12–21/3)

Pathogen[a]	Spring	Summer	Autumn	Winter	Total
B. cinerea	3 (17.6)[b]	2 (11.8)	2 (11.8)	10 (58.8)	17 (100)
R. solani	3 (13.0)	18 (78.3)	2 (8.7)	0 (0.0)	23 (100)
S. sclerotiorum	2 (15.4)	4 (30.8)	6 (46.2)	1 (7.7)	13 (100)
Pythium spp.	1 (14.3)	3 (42.9)	3 (42.9)	0 (0.0)	7 (100)
Total	9	27	13	11	

[a] Fisher's exact test (P=0.05) showed a relation between pathogen recovery and season

[b] Values in parentheses are relative percentages of pathogen recovery in the different seasons

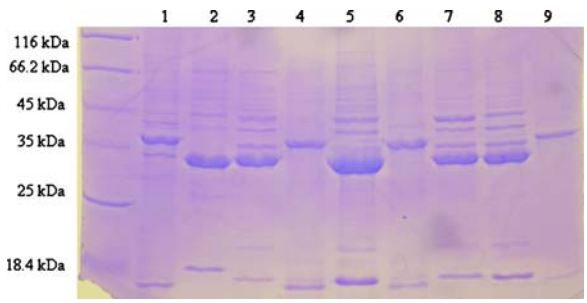

Fig. 1 Protein pattern of the sclerotia obtained with one dimensional SDS-PAGE. Bands were visualised using coomassie blue. From *lane 1–9*: S007-2, MUCL38484, S006-2, S020-2, S036-1, S037-4, S028-1, S037-1, S017-1. *Sclerotinia minor* (*lane 2*) can be distinguished from *S. sclerotiorum* (*lane 3, 5, 7 and 8*) by protein band at ±18.4 kDa. *Botrytis cinerea* (*lane 1, 4, 6 and 9*) and *Sclerotinia* spp. differ in the major protein band (±35 kDa)

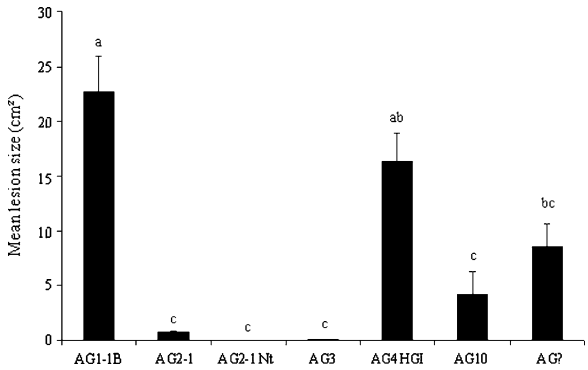

Fig. 2 Mean lesion size produced by isolates belonging to different AGs/subgroups, measured after 6 days incubation at 20° C. Tests were conducted with following isolates: S001-2, S002-1, S011-2, S014-2, S064-1, S065-1, S068-1 and S076-4 representing *AG1-1B*; S066-1 (*AG2-1 Nt*); S013-2 (*AG2-1*); S042-4 (*AG3*); S004-1, S005-1, S008-1, S010-1, S012-1; S016-1, S025-3, S072-2, S075-1 representing *AG 4 HGI*; S003-1 and S080-2 (*AG10*); S042-2 (*unknown AG*). Data within the same AG/subgroup did not show statistical differences and were pooled. *Bars* indicated with the *same letter* are not statistically different based on Tamhane's *T2* test (P=0.05). Standard errors of mean are indicated

Pathogenicity: detached lettuce leaves

To verify if the collected isolates were pathogenic towards lettuce, tests were conducted on detached lettuce leaves. Per location one isolate representing one of the different groups (genus/species/AG) was selected randomly: 25 *R. solani* isolates (belonging to AG1-1B (ten isolates), AG2-1 (one), AG3 (one), AG4 HGI (nine), AG10 (two), AG2-1 Nt (one), and the unknown AG (one)); 13 *S. sclerotiorum* isolates, one *S. minor* isolate; 17 *B. cinerea* isolates; and seven *Pythium* isolates (belonging to *P. ultimum* (two isolates), *P. (ir)regulare/cylindrosporum* (three) and *P. sylvaticum* (two)). Per genus, lesion sizes of the isolates were compared. Lesions produced by *R. solani* isolates were irregularly shaped. No statistical differences in pathogenicity on detached lettuce leaves could be detected among isolates of the same group (anastomosis group/subgroup), i.e. among isolates belonging to AG1-1B, AG4 HGI and AG10 (data not shown). As a result, data of isolates of the same AG were pooled and compared (Fig. 2). Isolates of AG1-1B and AG4 HGI were the most aggressive, followed by isolate S042-2. The isolates of AG10 appeared to be slightly aggressive as real lesions were not always formed; in some cases the symptoms were even restricted to a few brown spots. Isolates S042-4 (AG3) and S013-2 (AG2-1) gave rise only to brown spots, and isolate S066-1 (AG2-1 Nt) appeared to be non-pathogenic (Fig. 2). For a smaller subset of *Rhizoctonia* isolates the pathogenicity test was also conducted at 28°C (Fig. 3). Again no statistical differences could be detected among isolates of the same group. Isolates of AG1-1B and AG4 HGI were the most aggressive, followed by S003-1 (AG10). Isolates S042-4 (AG3), S013-2 (AG2-1) and S042-2 were slightly aggressive at this temperature.

All tested *Sclerotinia* spp. isolates were able to form lesions on detached lettuce leaves. Most *B. cinerea* isolates formed regularly shaped lesions, except for isolates S058-1, S017-1 and S037-2, which turned out to be not or slightly aggressive, producing no or very small lesions (data not shown). *Pythium* isolates also differed in aggressiveness. The lesion size produced after five days was highly variable per isolate. The three isolates from the *P. (ir)regulare/cylindrosporum* group (mean lesion size 4.38–13.14 cm^2) and the two isolates of *P. ultimum* (7.08–7.7 cm^2) were highly aggressive, while the isolates of *P. sylvaticum* were less aggressive (0.02–3.77 cm^2).

Pathogenic potential test *Rhizoctonia solani*

The most current crop rotation in Belgian greenhouses is lettuce–tomato. As isolates of *R. solani* can

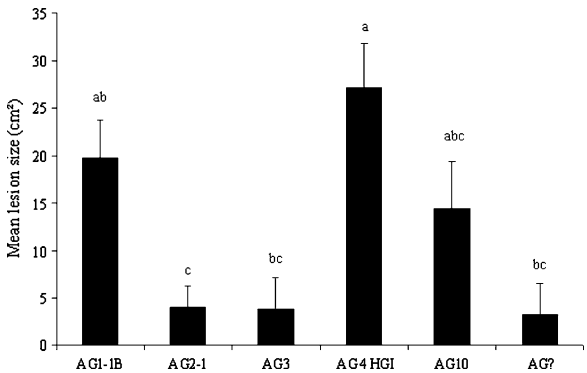

Fig. 3 Mean lesion size produced by isolates belonging to different AGs/subgroups at 28°C, measured after 4 days incubation at 28°C. Tests were conducted with following isolates: S001-2, S002-1, S011-2, S014-2 representing *AG1-1B*; S013-2 (*AG2-1*); S042-4 (*AG3*); S005-1, S008-1, S010-1, S012-1; S016-1, S025-3 representing *AG 4 HGI*; S003-1 (*AG10*); S042-2 (*unknown AG*). Data within the same AG/subgroup did not show statistical differences and were pooled. *Bars* indicated with the *same letter* are not statistically different based on Tamhane's *T2* test (P=0.05). Standard errors of mean are indicated

have a different host preference and as this is often associated with the anastomosis group or subgroup; a subset of isolates representing different anastomosis groups/subgroups were compared in their pathogenic potential towards lettuce and tomato seedlings (Table 3). *In vitro* seedling tests are commonly used to verify the pathogenic potential of *R. solani* isolates. All isolates were pathogenic towards lettuce seedlings. The isolates of AG4 HGI were the most aggressive, killing nearly all seedlings. The aggressiveness of the AG1-1B isolates was more variable; disease severity indices fluctuated between 2.09 and 3.58. The isolates S042-2 (unknown AG) and S013-2 (AG2-1) appeared also to be highly aggressive, whereas the isolates of AG10, AG3 and AG2-1 Nt were less aggressive, showing disease severity indices between 1.65 and 2.13.

Isolates belonging to AG3, AG4 HGI, AG2-1 and AG2-1 Nt were the most aggressive to tomato. The isolates of AG1-1B and AG10 had a mean disease severity index of 2.74 and 1.94, respectively. The isolate S042-2 of the unknown AG turned out to be only slightly aggressive.

Table 3 Disease severity index obtained by *in vitro* bio-assay of *R. solani* isolates belonging to different anastomosis groups (AGs) towards lettuce and tomato seedlings

AG/subset	Isolate	Disease severity index			
		Lettuce		Tomato	
1-1B	S002-1	3.17	bcd	2.70	
	S014-2	3.58	b	2.91	
	S025-4	3.46	b	2.38	
	S011-2	3.42	b	2.41	
	S001-2	2.09	def	1.75	
	S065-3	2.39	cdef	2.19	
	S064-1	3.17	bcd	3.00	
	S076-4	3.25	bcde	3.55	
	–[a]			2.74	cd
4-HGI	S010-1	4.00		3.43	
	S025-3	4.00		3.30	
	S012-1	4.00		3.54	
	S008-1	3.88		3.26	
	S004-1	4.00		3.74	
	S016-1	4.00		3.75	
	S005-1	4.00		3.95	
	S072-4	4.00		3.91	
	–[a]	3.99	a	3.61	ab
10	S003-1	2.13		2.26	
	S080-2	2.13		1.62	
	–[a]	2.13	f	1.94	d
3	S042-4	1.65	f	3.78	a
?	S042-2	3.17	bcd	0.91	e
2-1	S013-2	3.29	bc	3.04	ab
2-1Nt	S066-2	1.83	f	3.11	bc

Plants were scored using a scale ranging from 0 (no damage) to 4 (seedling dead). Experiments were repeated once and data were statistically analysed using non-parametric statistics. Values followed by different letters are statistically different

[a] Data were pooled when the isolates within an AG did not statistically differ in aggressiveness (Kruskal–Wallis analysis; $P=0.05$)

Discussion

This is the first time that pathogens causing basal rot of lettuce in Belgium have been described. Isolates collected belonged to four genera: *Rhizoctonia*, *Sclerotinia*, *Botrytis* and *Pythium*, with *R. solani* being the predominant pathogen, followed by *B. cinerea*, *S. sclerotiorum*, *Pythium* spp. and *S. minor*.

The diversity of pathogens found in our study is similar to that found in previous studies, but the relative proportion seems to be different. Kooistra (1983) found *Botrytis* as the most common basal rot pathogen in Dutch greenhouses. *Rhizoctonia solani*, *S. sclerotiorum*, *S. minor* and *Pythium* spp. were also detected, but to a lesser extent. Wareing et al. (1986) also reported *B. cinerea* to be the most frequently isolated pathogen in outdoor and protected lettuce crops in the UK. They considered *R. solani* to be more common in protected crops, and *Sclerotinia* spp. more common in outdoor crops. Wareing et al. (1986) did not detect *S. minor* or, *S. sclerotiorum*; *Pythium* spp. were only isolated erratically. The correlation between the occurrence of the pathogens and the time of the year, as observed in this study, is in agreement with Kooistra (1983), who found that *B. cinerea* was the only pathogen isolated in winter crops, whereas *R. solani* favoured summer crops.

The major *R. solani* anastomosis groups causing basal rot of lettuce in Belgian greenhouses appeared to be AG1-1B and AG4 HGI. AG1-1B was most commonly observed in field–grown lettuce in Germany and Ohio (Grosch et al. 2004; Herr 1992). In the Netherlands and the UK, AG1 was also frequently isolated, but the subgroup was never specified (Kooistra 1983; Wareing et al. 1986). AG4 causing bottom rot has been sporadically detected in the UK,

the USA and the Netherlands, but not to such a high extent. In the former studies the AG4 isolates were never assigned to a subgroup. Isolates of AG2-1 appeared to be of lesser importance in Belgium, whereas this AG was very common in greenhouses in the UK (Wareing et al. 1986). Remarkable in this study was the isolation of AG10 from symptomatic lettuce crops located in two different greenhouses. To our knowledge, this is the first time that AG10 has been associated with bottom rot of lettuce. Although our pathogenicity tests revealed that isolates of AG10 were only slightly aggressive at 20°C, a higher aggressiveness could be observed at 28°C. In previous research, AG10 has been isolated from cereals, legumes and canola, but up until now this AG was only considered to be a weak pathogen of crucifers (Eken and Demirci 2003; Khangura et al. 1999; MacNish et al. 1995; Ogoshi et al. 1990). Two isolates from greenhouse S042, which were moderately pathogenic on lettuce, could not be identified based on zymogram or PCR-RFLP. The ITS sequence showed only 95% similarity with known anastomosis groups in GenBank. According to Kuninaga et al. (1997), isolates of the same subgroup within one AG have a similarity >96%, isolates of different subgroups within one AG can be 66–100% similar, and isolates of different AG show a similarity of 55–96%. Therefore, it remains to be determined whether isolates S042-2 and S042-5 belong to a new *R. solani* subgroup or anastomosis group. Remarkably, these isolates, collected in November, were more aggressive at 20°C than at 28°C, whereas isolates belonging to other anastomosis groups generally showed an increase in aggressiveness at higher temperatures.

Both in the pathogenicity test on detached lettuce leaves, as in the *in vitro* pathogenic potential test on lettuce seedlings, a correlation was found between the AG and the aggressiveness of the isolates. However, the results obtained in both tests were not completely equivalent, probably because different symptoms are evaluated: the ability to cause lesions on adult leaves, and the ability to cause damping-off on seedlings. Isolates belonging to AG4 are known to cause damping-off on a wide range of hosts (Sneh et al. 1991); this can explain the relatively higher aggressiveness on seedlings compared to detached leaves. The detached leaf test is perhaps more appropriate than the seedling test when determining the ability to cause basal rot. However, greenhouse tests should be conducted to confirm these results. On tomato AG4 HGI and AG3 were highly aggressive, followed by AG2-1 and AG2-1 Nt. This is in agreement with findings in previous studies. AG4 HGI and AG2-1 are known to be pathogenic against tomato (Kuramae et al. 2003; Montealegre et al. 2003) and AG3 is considered to be a pathogen of *Solanaceae* (Tu et al. 1996). Isolates of AG1-1B appeared to be less aggressive against tomato. This indicates that when serious losses occur in lettuce, in some cases problems in the following tomato crop can also be suspected, this depending on the AG, underlining the importance of determining the AG when identifying the causal agent.

Compared to *S. sclerotiorum*, *S. minor* appeared to be uncommon in Belgian greenhouses. The identification of the *Sclerotinia* and *Botrytis* based on morphological characteristics was always confirmed by SDS-PAGE, making this technique redundant in most cases. However, the sclerotial formation of the *Botrytis* isolates and the absence of sporulation when incubated in the dark, may lead to misidentification. Yet, sclerotial characteristics should be sufficient to distinct between the two genera (Willetts 1997), but when having less experience or in case of doubt, SDS-PAGE can be an easy tool to distinguish between *Sclerotinia* spp. and *B. cinerea*. The isolates of *S. sclerotiorum* could be readily separated from *S. minor* by means of the protein band with molecular weight of approximately 18.4 kDa, which is in agreement with the findings of Tariq et al. (1985). However, the differences between the two species based on the major protein bands, also described by Tariq et al. (1985), could not be observed. The distinction between *B. cinerea* and *Sclerotinia* spp. was made based on the major protein band of approximately 35 kDa. The difference in molecular weight of the sclerotial proteins has been reported by Novak and Kohn (1991).

The role of *Pythium* species in causing basal rot in lettuce is not well documented. The only report dates from 1978 in the Netherlands; different *Pythium* species were isolated from lettuce with basal rot symptoms: *P. sylvaticum*, *P. tracheiphilum* and *P. uncinulatum* (Blok and Vanderplaats-Niterink 1978). Wareing et al. (1986) and Kooistra (1983) detected *Pythium* spp. in their surveys as well, but did not characterise them to species level. The isolates collected in the present study were identified by ITS

sequencing, which has been shown to be a useful tool for rapid identification of *Pythium* species (Levesque and De Cock 2004; Moorman et al. 2002). The occurrence of *P. sylvaticum* at two locations is in agreement with the findings of Blok and Vanderplaats-Niterink (1978). However, the ability of this species to cause basal rot was never investigated in detail. *Pythium ultimum* was found at two other locations in the present study. *Pythium ultimum* is also known to be a pathogen of lettuce, but only as causal agent of damping-off (Watson 1970). At three locations *Pythium* ITS-sequences appeared to be highly similar in *P. cylindrosporum*, *P. regulare* and *P. irregulare*. These three species have been shown to be closely related and were classified in clade F, the same clade of *P. sylvaticum*, by Levesque and De Cock (2004). The ITS-sequence of *P. cylindrosporum* and *P. regulare* is identical (Levesque and De Cock 2004), which makes it impossible to distinguish between these two species. *Pythium cylindrosporum* and *P. regulare* are both rarely–occurring species (Levesque and De Cock 2004). *Pythium cylindrosporum* has been isolated from soil and *Antirrhinum* (snapdragon) (Levesque and De Cock 2004; Moorman et al. 2002); *P. regulare* is a relatively new species isolated from soil in the Canary Islands and first described by Masih and Paul (2003). *Pythium irregulare*, on the other hand, is a widespread species, which consists of a heterogeneous group of isolates (Levesque and De Cock 2004). Matsumoto et al. (2000) divided this species in four subgroups based on ITS-RFLP analysis and RAPD analysis. The three isolates found in this research are closely related with the isolates of subgroup II.

This study demonstrates that in Belgian greenhouses four pathogens are important in causing basal rot of lettuce: *R. solani*, *S. sclerotiorum*, *B. cinerea* and *Pythium* spp. The occurrence of the four pathogens and the similarity of the symptoms emphasise the importance of a correct diagnosis, especially when working with narrow spectrum pesticides or biological control agents. For example, in the case of the fungal antagonist *Coniothyrium minitans*, only sclerotia of *Sclerotinia* spp. are parasitised and killed, while sclerotia of *Rhizoctonia* spp. or *Botrytis* spp. remain unaffected (Whipps et al. 1991). The correlation found between the occurrence of the pathogen and time of the year can lead to a reduction in preventive application of pesticides. In winter, fungicide use can be focused on controlling *B. cinerea*, whereas in summer especially *R. solani*, *Sclerotinia* spp. and *Pythium* spp. need to be controlled. The knowledge obtained in this study can be considered as a first step in the development of an integrated control strategy.

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