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Epidemiology and biology of soil-borne pathogens affecting glasshouse-grown butterhead lettuce

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Thesis submitted in fulfilment of the requirements for the degree of

Doctor in Bioscience Engineering

Dutch translation of the title

Epidemiologie en biologie van grondgebonden pathogenen in kropsla onder glas

Cover illustration

Butterhead lettuce and the most important soil-borne pathogens in lettuce in Belgium (from left to right: *Pratylenchus penetrans*, *Paratylenchus* sp., *Rhizoctonia solani*, *Botrytis cinerea*, *Pythium* spp., *Sclerotinia* spp., *Fusarium oxysporum* f. sp. *lactucae*)

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List of abbreviations

a	maximum multiplication rate
AG	anastomosis group
BS	bootstraps
cmda	calmodulin
cv.	cultivar
DD5	degree days using a base temperature of 5°C
DI	disease index
DIn	disease incidence
DNA	deoxyribonucleic acid
FRAC	Fungicide Resistance Action Committee
FUNSLA	Project funded by the 'Flanders Innovation & Entrepeneurship (VLAIO)' and focusses on the integrated management of soil-borne fungi and nematodes of glasshouse-grown lettuce
f. sp.	formae specialis
IGS	intergenic spacer region
ILVO	Flanders research institute for agriculture, fisheries and food
IPM	integrated pest management
IRAP	inter-retrotransposon amplified polymorphism
ITS	Internal transcribed spacer
LSD	least significant difference
m	relative minimum yield
M	maximum population density
ML	Maximum Likelihood
PCR	polymerase chain reaction
PDA	potato dextrose agar
P_i	initial population density
P_f	final population density
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphisms
SWOT	strengths, weaknesses, opportunities and threats
T	tolerance limit
TAE	tris-acetate EDTA
tef1	translation elongation factor 1-alpha
tub2	β-tubulin
Ymax	maximum yield
R	reproduction factor
rpb2	RNA polymerase II second largest subunit
R_s	Spearman's correlation coefficient
SNPs	single nucleotide polymorphisms

VCG	vegetative compatibility group
WA	water agar
y	yield

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Problem statement & thesis outline

Lettuce (*Lactuca sativa* L.) is ranked 9th in the top 10 of the economically most important products for the Association of Belgian Horticultural Cooperatives in 2018 (VBT, 2019). In Belgium, lettuce is mainly grown in an intensive production system in glasshouses in soil. The continuous production leads to a high incidence of soil-borne pathogens, such as nematodes, fungi and Oomycetes, which can cause serious economic losses. Before 2006, these soil-borne pathogens were managed by soil disinfestation with methyl bromide, a chemical that has been phased out in accordance with the Montreal protocol (UNEP, 2011) because of its ozon-depleting properties. Alternative soil fumigants have been used instead, such as dazomet, metam sodium or metam potassium, but they do not possess the same broad-spectrum activity. Due to European and Belgian phytosanitary regulations becoming more severe, the application of soil fumigants is more strict. In addition, lettuce production relies heavily on pesticides to ensure qualitative lettuce heads, but consumers and retail demand a limited amount of residues and a sustainably produced crop. Next to that, the European Directive 2009/128/EC demands a sustainable use of crop protection products and since 2014, Europe mandates the adoption of an integrated pest management (IPM). Therefore it is necessary that superfluous use of crop protection products is avoided and pesticides are only used when needed.

This doctoral thesis aimed to acquire the knowledge needed to reduce crop protection products in the production of butterhead lettuce in a glasshouse setting, both, in number as well as in timing. In this work, the focus lies on the root-lesion nematode *Pratylenchus penetrans* and the pin nematode *Paratylenchus* sp., and on soil-borne organisms causing basal rot, *viz. Rhizoctonia solani*, *Pythium* spp. *Sclerotinia* spp. and *Botrytis cinerea*. Special attention is given to Fusarium wilt, which has been causing serious losses in Belgian lettuce since 2015, and for which no adequate control measures are available.

In this study the following research questions were addressed:

RQ1: Which factors enable the populations of *P. penetrans* and *Paratylenchus* spp. to increase until the high numbers that cause damage? How can we avoid these high numbers and so reduce the frequency of chemical soil disinfestation?

RQ2: At which densities do *P. penetrans* and *Paratylenchus* sp. cause damage to lettuce? Based on this information, growers can be advised when to take actions to manage these nematodes.

RQ3: Can we reduce the amount of fungicide sprayings by only targeting the active basal rot pathogens?

RQ4: What is the diversity of *Fusarium oxysporum* f. sp. *lactucae* isolates in Belgium? And are there differences in their pathogenicity?

RQ5: Why do growers opt for intensive lettuce production in Belgium? Can this system be maintained with the current disease pressure and what are the alternatives?

A general literature overview is given in **chapter 1**.

The plant-parasitic nematodes *P. penetrans* and *Paratylenchus* sp. were found frequently in glasshouses where lettuce is grown. Very high densities are associated with economic damage. The population dynamics of these nematodes was monitored in the soil of several commercial glasshouses in order to understand the factors leading to a population build-up. In addition, a host status experiment was set up for the *Paratylenchus* species originating from lettuce glasshouses. This pin nematode was morphologically and molecularly characterized as it differed from *Paratylenchus* species described so far. Also damage thresholds were estimated for both nematodes in pot trials. The acquired information can contribute to management tools that prevent a population increase, avoiding unnecessary use of chemical soil disinfestation. The results for *P. penetrans* are reported in **chapter 2** and for *Paratylenchus* sp. in **chapter 3**.

In **chapter 4**, the activity of the basal rot pathogens, *R. solani*, *Pythium* spp., *Sclerotinia* spp. and *B. cinerea* were monitored during continuous cropping of butterhead lettuce at three different locations. Gathering this knowledge provided information on the appropriate timing of fungicide applications, possibly leading to a more specific use of narrow fungicides at appropriate croppings. In addition, the pathogenicity and the mycelial growth of different isolated *R. solani* anastomosis groups was investigated.

Fusarium wilt of lettuce was reported from Belgian glasshouses for the first time in 2015 (**Supplementary I**) and spread very fast. Differences in disease development between commercial glasshouses were observed. Therefore, several Fusarium isolates were collected and characterized in depth in **chapter 5**.

The production system of lettuce is analyzed in **chapter 6** using systems thinking. In addition, a SWOT-analysis and confrontation matrix were carried out to analyze the strengths, weaknesses, opportunities and threats of the application of pesticides and biocontrol agents to control lettuce pathogens.

In **chapter 7**, all findings are summarized and generally discussed. Subsequently, recommendations for future research are proposed.

CHAPTER

1

General introduction

1.1 Lettuce

Lettuce, belonging to the family of the Asteraceae, is an important leafy vegetable crop on the Belgian market and is generally produced in soil in glasshouses in a continuous system. Lettuce seeds are sown in cube peat blocks (3 to 5 cm side length) which are directly planted in the soil when the seedlings reach the 4-6 leaf stage, 30 cm apart. The growing period of a lettuce crop depends on the season and there can be up to five croppings per year. This type of lettuce production is mainly occurring in Flanders, where the production of qualitative heavy lettuce heads of 400-500 g is a specialty. The most important lettuce types grown are butterhead, crisphead, romaine and looseleaf (*e.g.* batavia, oakleaf) lettuce, with butterhead lettuce being the most important. In 2018, lettuce was produced in glasshouses in soil on 205 ha (235 growers), in hydroponics on 30 ha (13 growers) and outdoors on 83 ha (45 growers) (Vandevelde, personal communication). Butterhead lettuce was cultivated on 142 ha in glasshouses and on 42 ha in open air (StatBel, 2019). The production of butterhead lettuce decreased over the last few years and reached 67.0 million lettuce heads in 2018 (Table 1.1), of which 22,564 tonnes were exported (VLAM, 2019). The decrease in lettuce production since 2015 is mainly caused by the occurrence of *Fusarium oxysporum* f.sp. *lactucae* which spread very fast to almost the entire lettuce production and is causing severe damage.

Table 1.1 Production of butterhead lettuce in Belgium (VBT, 2015-2018)

Year	Lettuce heads (million)	Price (€) per lettuce head	Annual turnover (million €)
2014	91.1	0.284	25.9
2015	82.5	0.296	24.4
2016	76.8	0.315	24.2
2017	74.5	0.327	24.4
2018	67.0	0.405	27.1

1.2 Soil-borne pathogens

Several soil-borne pathogens can cause serious economic losses in Belgian lettuce. This thesis focusses on the most important nematodes, fungi and oomycetes. Their morphology and disease development is described in this chapter, their identification and characterization is discussed in greater detail in the following chapters.

1.2.1 Nematodes

Nematodes or roundworms (phylum Nematoda) are unsegmented worm-like animals that occur almost everywhere. There are more than 4100 plant-parasitic nematodes species described (Decraemer *et al.*, 2006). These can cause serious qualitative and quantitative yield losses. The top three of the economically and scientifically most important plant-parasitic nematodes, are root-knot nematodes (*Meloidogyne* spp.), cyst nematodes (*Heterodera* and *Globodera* spp.) and root-lesion nematodes (*Pratylenchus* spp.) (Jones *et al.*, 2013). Different plant-parasitic nematodes have been reported as associated with lettuce, such as *Paratrichodorus minor*, *Rotylenchulus reniformis*, *Nacobbus aberrans*, *Tylenchorhynchus clarus* and *Merlinius* spp. (Davis *et al.*, 1997), while some have been recorded to cause damage, like *Longidorus africanus, Pratylenchus penetrans, Rotylenchus robustus,*

Paratylenchus nanus, Merlinius microdorus and several *Meloidogyne* spp. (Blancard *et al.*, 2006; Davis *et al.*, 1997; Szczygiel, 1981; Winfield, 1985). Plant-parasitic nematodes can reduce the growth of lettuce due to the root damage caused by their feeding habit, which can be endo- or ecto-parasitic, depending on the nematode type. During a survey in 2014, the soil of 38 Belgian glasshouses where lettuce was grown in monoculture or in rotation with tomato, endive or celery was investigated for nematode damage and the presence of plant-parasitic nematode. In half of the samples, *Tylenchorhynchus* spp. were observed, while *Paratylenchus* spp. were found in 47% of the samples, followed by *P. penetrans* (18.4%), *Meloidogyne* spp. (10.5%), *Pratylenchus crenatus* (9.2%) and *R. robustus* (2.6%). In this thesis we focused on *Paratylenchus* sp. and *P. penetrans* because of their high occurrence in Belgian glasshouses and the damage often associated with these nematodes.

1.2.1.1 The root-lesion nematode *Pratylenchus penetrans*

The root-lesion nematode, *P. penetrans* (Cobb, 1917, Filipjev & Schuurmans Stekhoven, 1941), is a migratory endoparasite. The females are 0.31 to 0.81 mm long with a short stylet of 15-17 µm (Figure 1.1). Males are very common and are slightly smaller than females. They have a length of 0.31 to 0.57 mm. *Pratylenchus penetrans* is widely distributed, mainly in temperate regions and has been reported on more than 350 hosts. The nematode causes damage to different economically important crops such as maize, potato, cereals and different vegetables (Castillo & Vovlas, 2007). This nematode is also known to cause damage on lettuce. At an initial population density of 6000 nematodes per kg of soil, a yield loss of 18% was noticed (Olthof & Potter, 1973).

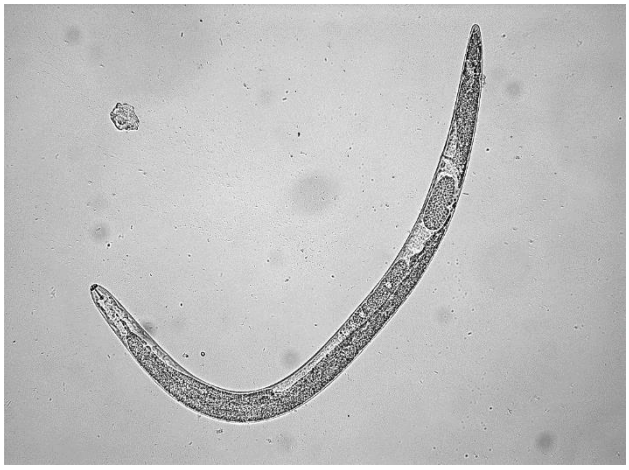

Figure 1.1 Female *Pratylenchus penetrans* (Picture: ILVO)

The disease cycle of *P. penetrans* is shown in Figure 1.2. Female root-lesion nematodes lay eggs nearby and inside the roots. *Pratylenchus penetrans* lays 1 to 2 eggs per day (Mamiya, 1971; Mizukubo & Adachi, 1997). The first molt occurs in the egg and it is the second-stage juvenile that hatches (Castillo & Vovlas, 2007). Hatching can be stimulated by root exudates and temperature (Pudasaini *et al.*, 2008). The second-stage juvenile molts to a third-stage juvenile, followed by a molting to the fourth-stage juvenile and finally to the adult stage. All the juvenile stages, except the first, can penetrate the roots and move inside the cortex. Damaged root tissue turns red to brown, and exhibits the typical lesions. During winter, and between two crops, *P. penetrans* is protected from desiccation inside the roots that

remain in the soil (Agrios, 2005; Blancard *et al.*, 2006; Castillo & Vovlas, 2007). This nematode is favored by sandy soils and its reproduction is stimulated by a pH of 5.2-6.4 (Willis, 1972), is optimal on lettuce at 21-22°C and reduced below 15°C and above 30°C (Moretti *et al.*, 1981). The duration of the life cycle can be influenced by different environmental conditions, such as soil temperature and moisture (Castillo & Vovlas, 2007). Depending on the host, differences in life cycle duration can be found (Table 1.2). Furthermore, one female could lay up to 68 eggs on *in vitro Cryptomeria* seedlings (Mamiya, 1971).

Table 1.2 Life cycle duration at different temperatures on different hosts

	Life cycle duration on different hosts (days)		
Temperature (°C)	*Cryptomera* seedlings[1]	*Trifolium repens*[2]	Carrot callus[3]
15	86	-	-
17	-	46	-
20	42-44	38	-
24	35	-	34-35
25	-	28	-
27	-	26	-
30	30-31	22	-

[1] Mamiya, 1971
[2] Mizukuba & Adachi, 1997
[3] Wu *et al.*, 2002

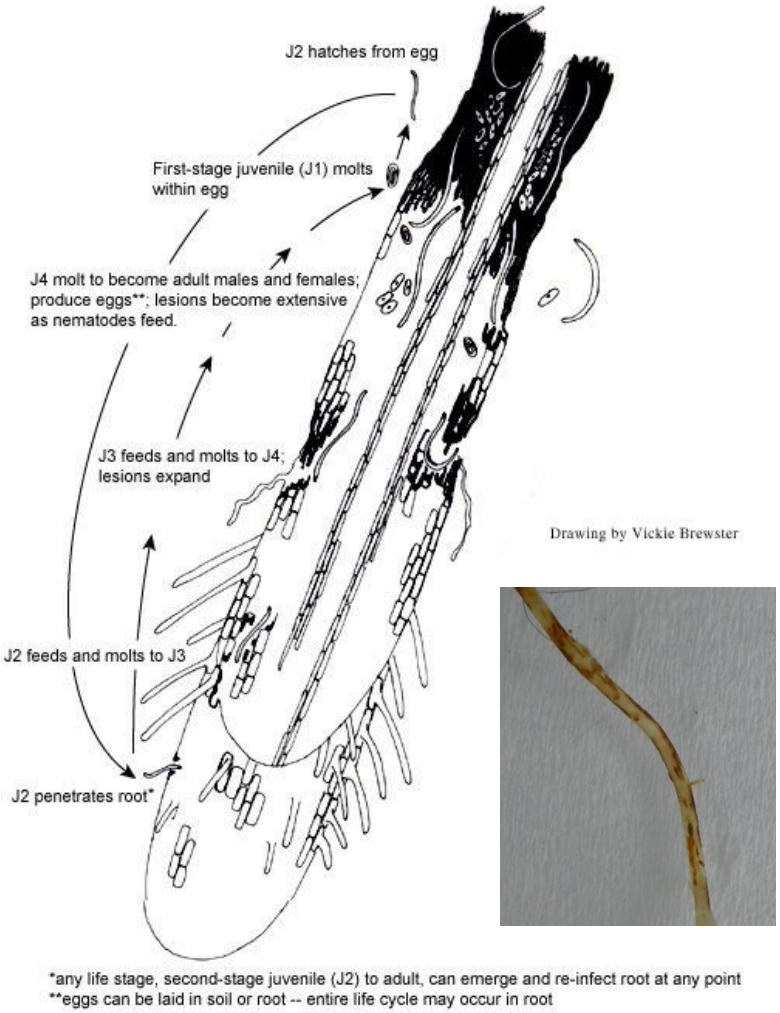

Figure 1.2 Disease cycle of *Pratylenchus* spp. (https://www.apsnet.org/edcenter/disandpath/nematode/pdlessons/Pages/LesionNematode.aspx) and damage symptoms on lettuce roots (Picture: Claerbout).

1.2.1.2 The pin nematode *Paratylenchus* spp.

Pin nematodes, *Paratylenchus* spp., are migratory ectoparasites that feed on the epidermal cells of roots (Rhoades & Linford, 1961; Wood, 1973). The females are smaller than 0.7 mm and have a long stylet of 10-120 µm long (Figure 1.3). Fourth-stage juveniles often have no stylet, but the other juvenile stages do. Furthermore, males are not always recorded and have no or a degenerated stylet (Ghaderi *et al.*, 2014). Pin nematodes are widely distributed across the world and associated with many plant species, such as fruit trees, ornamentals, grasses, and vegetables (Ghaderi *et* al., 2019; Van den Berg *et al.*, 2014). From the 120 nominal species known, only *P. projectus* and *P. nanus* are reported to be hosted by lettuce (Siddiqi, 2000; Goodey *et al.*, 1965; Winfield, 1985).

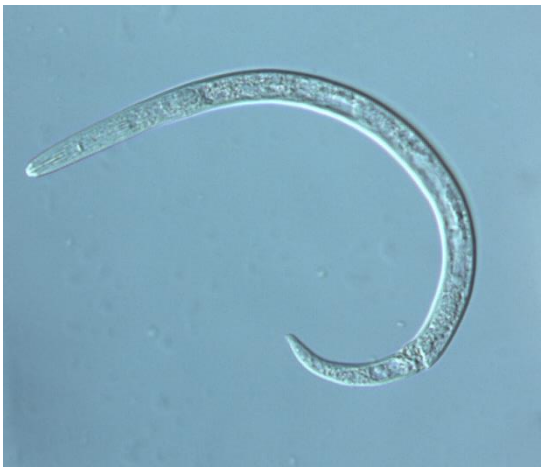

Figure 1.3 Female *Paratylenchus* spp. (Picture: Kigozi)

The life cycle of the pin nematodes, *P. projectus* and *P. dianthus*, has been studied *in vitro* on *Lolium perenne* L. (Wood, 1973) and *Trifolium pratense* L. (Rhoades & Linford, 1961b). Eggs are often laid in small groups, since females can feed up to 6 days on one feeding site and can lay 1 to 3 eggs per day. The first molting occurs in the egg. The second-stage juveniles hatch from the egg after 5 days at 25-28°C (Rhoades & Linford, 1961b) and 7-8 days at 18-20°C (Wood, 1973). The juveniles molt to a third-stage juvenile and later to a fourth-stage juvenile. The fourth-stage juvenile, also called a preadult, differs morphologically from the other stages due to the reduced stylet (Wood, 1973). A *Paratylenchus* spp. population can consist of very high proportions of preadults. This stage can survive for a long period in moist soil without feeding. In pot cultures of red clover, the proportion of preadults in the nematode population was 7.7% after 100 days and increased to 66% after 185 days. In many older pot cultures more than 80% of the population were preadults. Viable preadults and adults could still be observed in pot cultures after 4 years and 7 months of storage (Rhoades & Linford, 1959). Furthermore, this stage seems to be more tolerant to desiccation and sudden exposure to low temperatures (Rhoades & Linford, 1961b). The molting from a preadult to an adult is stimulated by root exudates (Rhoades & Linford, 1959). In the case of *Paratylenchus aciculus* only root exudates from host plants stimulated molting, while for *P. projectus* or *P. dianthus* host and non host plants stimulated molting (Ishibashi *et al.*, 1957; Rhoades & Linford, 1959). The duration of the life cycle is 36-38 days and 30-31 days at 18-20°C and 25-28°C *in vitro*, respectively (Rhoades & Linford 1961b, Wood 1973). The life cycle is reduced by 7-8

days in soil compared to the life cycle duration *in vitro* according to Rhoades & Linford (1961b) at 18-20°C. A similar reduction is hypothesized for the life cycle at 25-28°C (Wood, 1973).

1.2.2 Fungi and Oomycetes

Different soil-borne fungi and oomycetes are often associated with damage on lettuce. Rotting of the older leaves in contact with the soil is called basal rot. This disease is common in Belgium and occurs worldwide in open field and under shelter (Blancard *et al.*, 2006; Davis *et al.*, 1997). In Belgium, it is caused by four different pathogens: the fungi *Rhizoctonia solani*, *Botrytis cinerea*, and *Sclerotinia* spp. and the Oomycete *Pythium* spp. (Van Beneden *et al.*, 2009). Van Beneden *et al.* (2009) and Kooistra (1983) observed seasonal fluctuations in the appearance of these basal rot pathogens: *R. solani* in summer and *B. cinerea* in winter, while *Sclerotinia* spp. and *Pythium* spp. were observed in spring, summer and autumn. Since 2015, the Belgian lettuce production is threatened by another soil-borne pathogen which causes serious economic losses. This pathogen, *Fusarium oxysporum* f. sp. *lactucae,* is responsible for complete wilting of the lettuce heads (Claerbout *et al.*, 2018).

1.2.2.1 *Rhizoctonia solani*

Rhizoctonia solani (teleomorph *Thanatephorus cucumeris*) is a basidiomycete with a broad host range and worldwide distribution. This fungus is a species complex and is divided in 13 different anastomosis groups (AGs) based on hyphal anastomosis reaction (Carling *et al.*, 2002). These AGs can be further divided in subgroups that show high similarity in vitamin requirement, pathogenicity, cultural and molecular characteristics (Cubeta & Vilgalys, 1997; Kuninaga *et al.*, 1997; Ogoshi, 1987). Different AGs and subgroups have already been reported on lettuce: AG1-IB, AG1-IC, AG2-1, AG2-1 nt, AG3, AG4-HGI, AG5 and AG10 (Van Beneden *et al.*, 2009; Grosch *et al.*, 2004; Kooistra, 1983, Kuramae *et al.*, 2003; Wareing *et al.*, 1986). The basal rot symptoms on lettuce caused by *R. solani* are also called bottom rot.

Rhizoctonia solani survives as mycelium or sclerotia in the soil or on crops or plant debris (Figure 1.4). Mycelium is characterized by its T branches and colors yellow to brown (Agrios, 2005). Some AGs can produce sclerotia in unfavorable conditions. These melanin-rich structures can survive for several years in the soil (Naiki & Ui, 1978). The sclerotia germinate and form new young mycelium. This young mycelium or surviving mycelium will colonize the surface of the host and form infection cushions. Lettuce leaves are directly penetrated via the cuticle, stomata or injured tissue. Further colonization is stimulated by hydrolytic enzymes enhancing cell wall degradation, resulting in cell death (Agrios, 2005; Sneh *et al.*, 1996). The role of sexual reproduction is of minor importance (Blancard *et al.*, 2006; Sneh *et al.*, 1996).

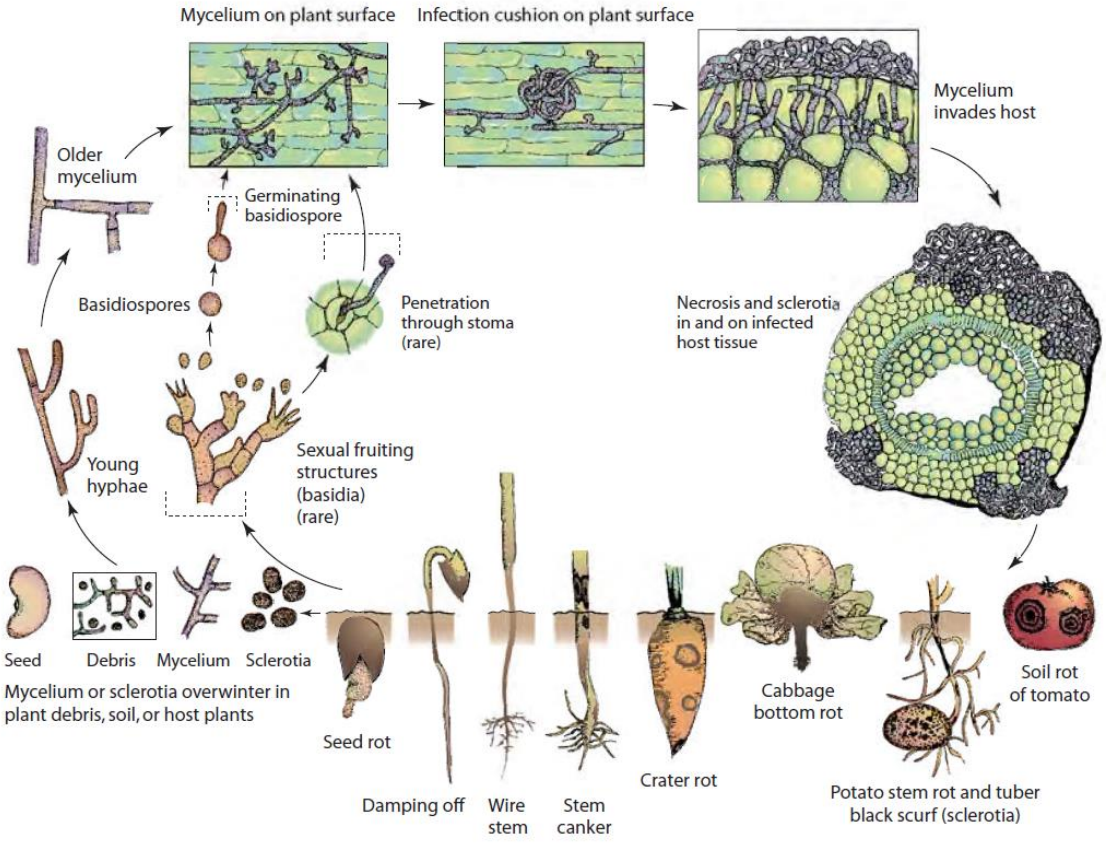

Figure 1.4 Disease cycle of *Rhizoctonia solani* (Agrios, 2005)

1.2.2.2 *Botrytis cinerea*

Botrytis cinerea (teleomorph *Botryotinia fuckeliana*) is an ascomycete with a broad host range and is also called grey mold. This pathogen appears very often in lettuce glasshouses and can cause serious losses, alone or in combination with other basal rot pathogens. In the UK losses of 50% to 80% were recorded due to this pathogen (O'Neill & McPherson, 1996). This fungus is characterized by its grey mycelium with long branched conidiophores (1-2 mm) with round apical cells carrying clusters of colorless to grey macroconida (6-18 x 4-11 µm). In unfavorable conditions, *B. cinerea* produces sclerotia, variable in size and shape (2-5 mm) (Agrios, 2005; Blancard et al, 2006; Davis, 1997).

Botrytis cinerea survives the winter in the soil as sclerotia or as mycelium on plant debris (Figure 1.5). Germinating sclerotia develop into mycelium and rarely in apothecia with ascospores. The mycelium produces condiophores with macroconidia that can be transported by the wind. The macroconidia penetrate the new host and colonize the plant tissue which will start to soften and rotten. *Botrytis cinerea* is favored by 17-23°C and a high relative humidity of 95%. At lower temperatures, the pathogen is still active and as a consequence can still damage lettuce heads in cold rooms (Agrios, 2005; Blancard *et al.*, 2006; Davis, 1997; Williamson *et al.*, 2007).

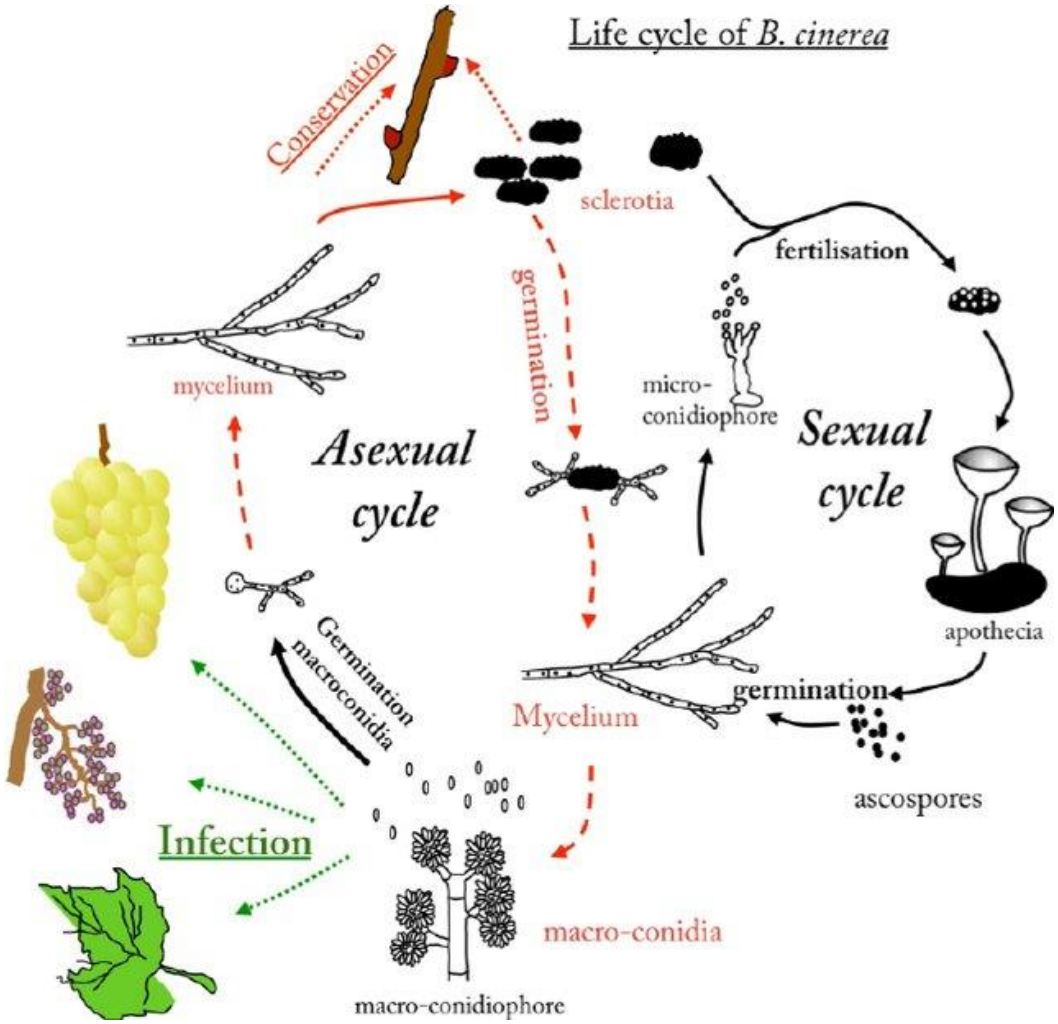

Figure 1.5 Life cycle of *Botrytis cinerea* on grape (Billard *et al.*, 2011)

1.2.2.3 *Sclerotinia* spp.

The ascomycetes *Sclerotinia minor* and *S. sclerotiorum* are reported worldwide, including Belgium, to cause lettuce drop or Sclerotiniose (Blancard *et al.*, 2006; Subbarao, 1998; Van Beneden *et al.*, 2009). *Sclerotinia nivalis* has only been reported in China (Li *et al.*, 2000) and *S. subarctica* in Norway (Nordskog *et al.*, 2014). Lettuce drop can cause losses up to 75%. These fungi are characterized by their white mycelium and black sclerotia that can survive for 8 to 10 years or as mycelium on plants (Figure 1.6). The sclerotia of *S. sclerotiorum* germinate carpogenically after two or more weeks at 4°C in soil moistened near the saturation level. Apothecia are formed which produce and release millions of ascospores during 2 to 3 weeks. These ascospores are transported by the wind to other lettuce heads. On senescing or dead leaves, ascospores germinate in the presence of water and produce hyphae that will infect the plant. The carpogenetic germination of sclerotia appears rarely for *S. minor*. The eruptive germination of sclerotia of *S. minor* is also affected by soil moisture and temperature. The hyphae will directly infect the roots and senescing leaves. This way of infection is rather uncommon in commercial glasshouses for *S. sclerotiorum* (Subbarao, 1998).

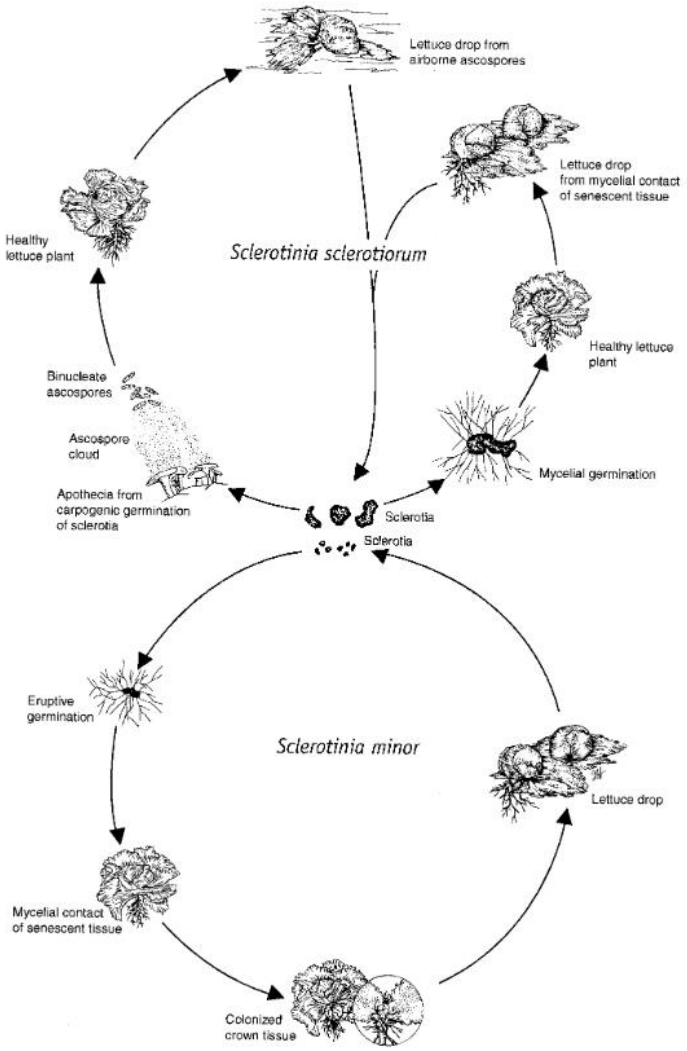

Figure 1.6 Disease cycle of *Sclerotinia sclerotiorum* and *S. minor* (Subbarao, 1998)

1.2.2.4 *Pythium* spp.

Different species of the oomycete *Pythium* were recorded to cause basal rot symptoms in lettuce. In Belgium, *P. sylvaticum*, *P. ultimum* and isolates closely related to *P. cylindrosporum*, *P. regulare* and *P. irregulare* were reported (Van Beneden *et al.*, 2009). While in the Netherlands, *P. sylvaticum, P. tracheiphilum* and *P. incinulatum* were recorded (Blok & Van der Plaats-Niterink, 1978).

Pythium spp. survive as saprophytes on plants or as survival structures, oospores. This fungus can reproduce asexually or sexually (Figure 1.7). Under favorable conditions, sporangia are produced that can germinate and directly infect the host at temperatures higher than 18°C. A secondary sporangium can be developed containing hundreds of zoospores in a vesicle. The zoospores spread easily via water in the soil. They will develop into a encysted zoospores that can infect their host again at optimal temperatures of 10 to 18°C. However, variance in pathogenicity can be observed between species at different temperatures. *Pythium ultimum* was highly pathogenic at 4, 12, 20, 28°C on soybean, while

pathogenicity decreased with increasing temperature for *P. irregulare* and *P. sylvaticum* (Wei *et al*., 2011). On soybean The sexual reproduction arises from an oogonium and antheridium that will melt together to a thick-walled oospore resistant to unfavorable conditions. In favorable conditions the oospores can infect the host directly via a germ tube or via zoospores (Agrios, 2005; Blancard *et al.*, 2006; Davis, 1997).

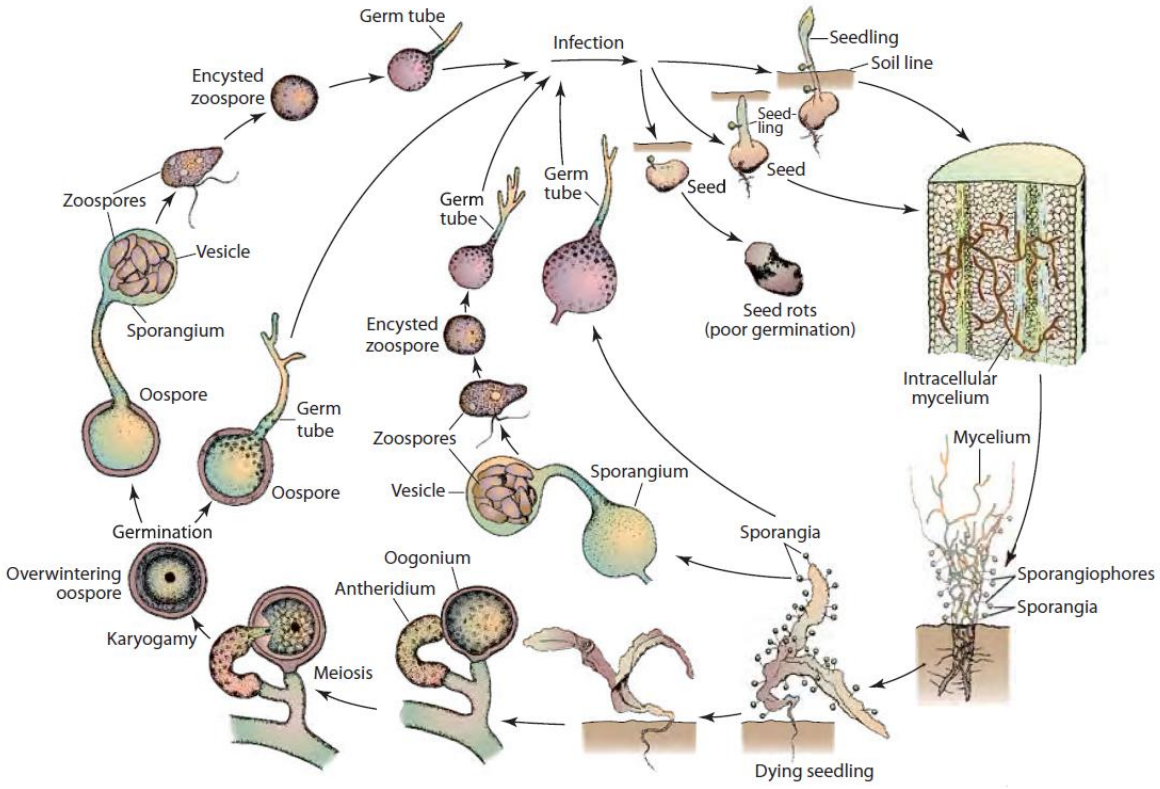

Figure 1.7 Disease cycle of *Pythium* spp. (Agrios, 2005)

1.2.2.5 *Fusarium oxysporum* f. sp. *lactucae*

The ascomycete *Fusarium oxysporum* occurs worldwide in almost every soil and harbors pathogenic and non-pathogenic isolates that cannot be distinguished based on their morphology. Their mycelium is white to pink colored. The pathogenic isolates are responsible for many important wilting diseases (Agrios, 2005). *Fusarium oxysporum* is a species complex that contains several cryptic species for which diverse classification systems are used, such as formae speciales with races and vegetative compatibility groups. Based on the host specificity more than 150 different formae speciales are described which are often divided in different races. These races are formed by the isolates' pathogenicity on different cultivars. Sequence analysis has shown polyphyletic relationships within the formae speciales which illustrates an independent evolution (O'Donnell *et al.*, 1998). Moreover, vegetative compatibility groups (VCGs) are used to classify *F. oxysporum* formae speciales and non-pathogenic strains. This classification is based on heterokaryon compatibility between two complementary auxotrophic nutritional mutants, which can not synthesize nitrate by themselves (Correl, 1991; Leslie, 1993). The diverse classification systems and lack of living ex-type material of *F.*

oxysporum limit the identification of the multiple cryptic species. Therefore, Lombard *et al.* (2019) proposed an epitype for *F. oxysporum* to enhance the taxonomic position of *F. oxysporum* and suggested 15 different cryptic taxa that were formerly identified as *F. oxysporum*. This is based on morphological features and a concatenated phylogenetic tree of partial sequences of four genes: calmodulin (*cmdA*), RNA polymerase II second largest subunit (*rpb2*), translation elongation factor 1-alpha (*tef1*) and β-tubulin (*tub2*). Some correlation related to the origin of strains was observed with this classification. For instance, most isolates from tomato (*Solanum lycopersicum*) clustered in *F. languescens* and few in *F. nirenbergiae.*

Fusarium oxysporum f. sp. *lactucae* causes serious wilting symptoms on lettuce. Typical symptoms are stunting of the plants, yellowing of the leaves and a brown to red colored vascular system. At high infection levels, the plants eventually die. These symptoms are more abundant at higher temperatures or when high temperatures occur at the beginning of the growing period (Scott *et al.*, 2010). Four different races are reported for *F. oxysporum* f. sp. *lactucae*. Race 2 only occurs in Japan and race 3 was reported from Taiwan and Japan (Fujinaga *et al.*, 2003; Lin *et al.*, 2014). Race 1 is widely spread and was reported from Japan, USA, Taiwan, Iran, Brazil, Argentina, Portugal, Italy, and France (Fujinaga *et al.*, 2003; Garibaldi *et al.*, 2002; Gilardi *et al.*, 2017; Hubbard & Gerik, 1993; Malbrán *et al.*, 2014; Millani *et al.*, 1999; Pasquali *et al.*, 2007; Ventura & Costa, 2008). The most recently discovered race, race 4, has only been reported from the Netherlands, Belgium, Ireland, England and Italy (Claerbout *et al.*, 2018; Gilardi *et al.*, 2016; Gilardi *et al.*, 2019; Taylor *et al.*, 2019). Race 1 and 4 are closely related based on their intergenic spacer region of the rDNA region (*IGS*) and their *tef1* (Gilardi *et al.*, 2016), while race 2 and 3 differ based on the *IGS* region only (Fujinaga *et al.*, 2005). The four races belong to different VCGs, which indicates that each race originates from another ancestor and evolved independently (Pintore *et al.*, 2017). The taxonomical position of *F. oxysporum* f. sp. *lactucae* within the classification of Lombard *et al.* (2019) has not been studied yet.

Fusarium oxysporum reproduces asexually and produces three types of spores, *i.e*. microconidia, macroconidia and chlamydospores (Figure 1.8). This fungus survives in the soil or on infected plant debris mainly as chlamydospores. Root exudates can trigger the germination of chlamydospores (Gordon, 2017). *Fusarium oxysporum* directly penetrates the roots via natural openings or wounded tissue. The mycelium grows inter- and intracellularly towards the vascular tissue where it produces microconidia which are transported with the water flow. These conidia germinate again and block the water transport which causes the wilting symptoms. Macroconidia are produced on senescing plant tissue, and thickwalled chlamydospores that can survive for prolonged periods are produced under unfavorable conditions, (Agrios, 2005). After 2.5 years of fallow, *F. oxysporum* f. sp. *lactucae* propagules were shown to be still viable (Gordon & Koike, 2015).

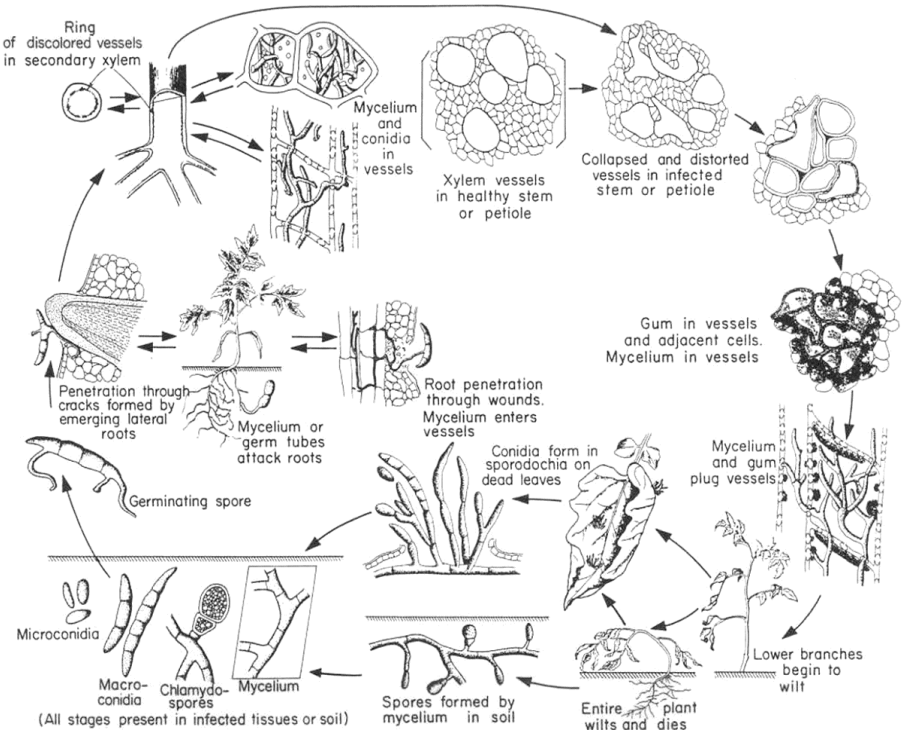

Figure 1.8 Disease cycle of *Fusarium oxysporum* f. sp. *lycopersici* in tomato (Agrios, 2005)

1.3 Lettuce disease control measures currently available in Belgium

The Belgian lettuce production relies heavily on chemical control. Chemical soil disinfestation used to be applied yearly or every other year, although often no disease problems were observed. The lettuce growers applied it as a prevention measure, to avoid problems with fungi, nematodes and weeds the coming year. Nowadays, only three soil fumigants are registered in Belgium for the control of soil-borne pathogens in glasshouse-grown lettuce (Table 1.3) and their use is restricted to certain periods during the year. Due to the stricter regulations and environmental concerns of chemical soil disinfestation, the application of chemical soil disinfestation is limited and soil steaming gains in popularity.

Table 1.3 Soil fumigants currently registered in Belgium with their activity against soil-borne pathogens (Fytoweb, 2019)

Active substance	Commercial name	Fungi	Nematodes
Metam sodium	Terresan, Solasan	x	x
Metam potassium	Tamifume	x	x
Dazomet	Basamid, Dazoclean	x	x

Soil steaming can eliminate practically all organisms. Different studies recommend a temperature of 70°C for at least 30 min to kill most plant pathogenic micro-organisms and weeds (Runia & Molendijk, 2010). There are different techniques of applying steam to heat soil, such as sheet steaming and steaming under negative pressure. Sheet steaming is currently the most commonly applied technique. Steam is blown under a sheet which lays on top of the soil while its borders are fixed. The pressure builds up under the sheet and drives the steam into the soil. For steaming under negative pressure, polypropene tubes are buried into the soil and a fan is connected to these tubes creating a negative pressure that pulls the steam through the soil. With the negative pressure method, higher temperatures can be reached in lower soil layers compared to sheet steaming. The advantages of steaming under negative pressure compared to sheet steaming are its higher efficacy and lower energy demand (Runia & Molendijk, 2010).

Next to soil disinfestation, fungicides are regularly applied to prevent symptoms of basal rot caused by *R. solani*, *B. cinerea*, *Pythium* spp. and *Sclerotinia* spp. Several active substances are registered to control *B. cinerea* and *Sclerotinia* spp., while only three products are registered against *R. solani* and solely propamocarb can be used against *Pythium* spp. (Table 1.4). No fungicides are registered for the control of *F. oxysporum* f. sp. *lactucae*. In addition, few biocontrol agents are registered for use in glasshouse-grown lettuce against basal rot pathogens and *Fusarium* (Table 1.5).

Table 1.4 Fungicides currently registered for the control of *Rhizoctonia solani* (Rs), *Botrytis cinerea* (Bc), *Pythium* spp. (P), *Sclerotinia* spp. (S), *Fusarium oxysporum* f. sp. *lactucae* (Fol) in glasshouse-grown lettuce (Fytoweb, 2019).

Active substance	FRAC code	Commercial name	Rs	Bc	P	S	Fol
Azoxystrobin	11	Amistar, Globaztar azt 250 sc, Mirador, Norios, Ortiva, Zakeo 250 SC, Zoxis 250 SC	x				
Boscalid and Pyraclostrobin	10 and	Bospy, Bospyrabel, Signum,	x	x		x	
Cyprodinil and Fludioxonil	9 and 12	Serenva, Switch, VSM Cyprodinil		x		x	
Fenhexamid	17	Teldor		x			
Fludioxonil	12	Geoxe, Safir		x		x	
Fluopyram	7	Luna privilege		x		x	
Fluopyram and Trifloxystrobine	7 and 11	Luna sensation, VSM Fluostrobine		x		x	
Isofetamide	7	Kenja		x		x	
Propamocarb	28	Proplant			x		
Tolclofos-methyl	14	Rizolex 500 SC	x				

Table 1.5 Biocontrol agents currently registered to control *Rhizoctonia solani* (Rs), *Botrytis cinerea* (Bc), *Pythium* spp. (P), *Sclerotinia* spp. (S), *Fusarium* (F) in glasshouse-grown lettuce (Fytoweb, 2019).

Active substance	Commercial name	Rs	Bc	P	S	F
Bacillus amyloliquefaciens strain QST 713	Serenada ASO	x	x		x	x
Bacillus amyloliquefaciens subsp. *plantarum* D747	Amylo-X WG		x		x	
Clonostachys rosea J1446	Prestop		x			x
Clonostachys rosea J1447	Prestop mix					x
Coniothyrium minitans	Contans				x	
Trichoderma asperellum T34	Asperello T34 Biocontrol			x		x
Trichoderma harzianum T22	Trianum	x		x	x	x

Lettuce is mainly grown in a continuous cropping system, although a good rotation could lower the inoculum density of soil-borne pathogens. Crop rotation can be rather difficult for some broad host spectrum pathogens *e.g. P. penetrans* or *R. solani*, although growing *Tagetes* spp. is known to reduce *Pratylenchus* population densities (Hooks *et al.*, 2010). Moreover, *R. solani* anastomosis groups have a certain host specificity, which makes crop rotation possible (Sneh, 1996). Black fallow was shown to reduce nematodes (Viaene *et al.*, 2006), but this can vary between species and this practice is less effective for fungi producing survival structures, such as sclerotia or chlamydospores. Inoculum of *F. oxysporum* f. sp. *lactucae* in naturally infested soil, for instance, has a half-life of six months and viable propagules were still found after 34 months (Gordon & Koike, 2015).

Other cultural approaches can also be implemented to control soil-borne pathogens. First of all pathogen-free plant material should be used. Moreover, lettuce should be planted in a well-drained and healthy soil. Planting the lettuce peat blocks only a few cm in the top soil layer, limits the contact between the soil and leaves and as a results prevents problems with basal rot pathogens. Subsequently, a low relative humidity and good ventilation are important. Removing infected plant material inhibits the spread of soil-borne pathogens (Agrios, 2005; Blancard *et al.*, 2006).

No resistant lettuce cultivars are known for the control of nematodes or pathogens causing basal rot. Only a few butterhead lettuce cultivars are on the market with partial resistance to *F. oxysporum* f. sp. *lactucae* race 4*.* In addition, some cultivars of other lettuce types, *e.g.* Lollo bionda and lollo rossa, are partial resistant to *F. oxysporum* f. sp. *lactucae* race 1 or 4 (Vandevelde *et al.*, 2019).

CHAPTER

2

Pratylenchus penetrans, a potential risk in glasshouse-grown lettuce: population dynamics and damage threshold

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2.1 Abstract

The root-lesion nematode, *Pratylenchus penetrans*, causes growth reduction in glasshouse-grown lettuce and is mainly controlled by chemical soil disinfestation. Integrated management strategies require more knowledge about the population dynamics and damage threshold densities. We monitored the population during 2.5 years in a commercial glasshouse by sampling soil in the same four 1 m^2 spots at 0-30 cm and 30-60 cm depth. The grower grew lettuce in rotation with leek, applied 1,3-dichloropropene in summer and left the field fallow during winter. Growing leek reduced the nematode population slightly, but chemical soil disinfestation lowered the numbers drastically, although 41% of the nematodes in the deeper layer survived. Black fallow resulted in a slight increase of the population, probably due to hatching. Two pot experiments with 10 densities of *P. penetrans* were conducted to estimate the damage threshold for a summer and autumn cultivar ('Cosmopolia' and 'Brighton', respectively). The thresholds for lettuce weight were 669 and 3834 *P. penetrans* (100 ml soil)$^{-1}$ in summer and autumn, respectively, but with considerable variability in estimated parameters. The thresholds for root damage were much lower: 204 and 48 *P. penetrans* (100 ml soil)$^{-1}$. Nematode numbers did not increase on lettuce in the pot tests (maximum multiplication rate was 0.40) but increased slightly in the commercial setting. These results show that populations of *P. penetrans* build up slowly when butterhead lettuce is rotated with leek and fallow, but chemical soil disinfestation is required to avoid numbers resulting in root damage.

2.2 Introduction

In Belgium, butterhead lettuce (*Lactuca sativa* L.) is mainly produced in soil in glasshouses as a monoculture with up to five harvests per year. In some cases, however, the production of lettuce is rotated with other crops, such as leek or celery. Occasionally, nematodes are associated with severe growth reduction of glasshouse-grown lettuce. In a Belgian survey in 2014, soil samples from 38 glasshouses were analysed in order to have more insight in the presence of plant-parasitic nematodes. The study revealed that the root-lesion nematode, *Pratylenchus penetrans* (Cobb, 1917) Filipjev & Schuurmans-Stekhoven, 1941, was present in 18% of the glasshouses (unpublished). *Pratylenchus penetrans* is spread worldwide and has a broad host range of nearly 400 plant species. This migratory endoparasitic nematode causes severe damage to economically important crops such as maize, potato and cereals, as well as several vegetables (Castillo & Vovlas, 2007). Damage on lettuce caused by *P. penetrans* was reported by Gracia *et al.* (1991), Kilpatrick *et al.* (1963), Moretti *et al.* (1981) and Olthof & Potter (1973).

Currently, growers mainly control nematodes in lettuce by chemical soil disinfestation, which is also applied to reduce several other soil-borne pathogens. Stricter phytosanitary regulations in Belgium, driven by increasing environmental concerns, demand a reduction in the use of chemical soil disinfestation. To set up integrated pest management strategies, knowledge about the population dynamics and the damage threshold of *P. penetrans* for lettuce is required. The damage threshold density of a nematode species for a certain crop can be estimated with the Seinhorst function, a mathematical model that describes the relationship between the initial nematode population density and the yield (Schomaker & Been, 2003). Although Olthof & Potter (1973) noticed damage by *P. penetrans* on field-grown lettuce at 6000 *P. penetrans* (kg soil)$^{-1}$, they did not define a threshold density. The knowledge of this threshold is important to provide good management advice for lettuce growers.

The objective of this study was to investigate the population dynamics of *P. penetrans* in glasshouse-grown butterhead lettuce under normal cropping practices. Therefore, one commercial glasshouse without other important plant-parasitic nematodes was selected from the Belgian survey conducted in 2014. The nematode population was monitored at the commercial glasshouse during 2.5 years to gain insight into the factors influencing nematode densities. In addition, the damage threshold was estimated by growing lettuce in pots in the glasshouse and recording lettuce growth parameters over a wide range of *P. penetrans* population densities. The obtained knowledge will contribute to the set-up of integrated pest management tools and the reduction of chemical soil disinfestation.

2.3 Materials and methods

2.3.1 Spatial and temporal dynamics of *P. penetrans* in glasshouse lettuce in rotation with leek

A glasshouse where *P. penetrans* was found in relatively high densities during a survey from 2014 (701 *P. penetrans* (100 ml soil)$^{-1}$ in the 0-30 cm soil layer and 143 *P. penetrans* (100 ml soil)$^{-1}$ in the 30-60 cm layer) was selected for this study since no other important plant-parasitic nematodes were observed. The lettuce grower followed a rotation pattern consisting of three months of black fallow from December

until March, cultivation of leek (*Allium porrum*) from March until June and two crops of butterhead lettuce. Every year or every other year, chemical soil disinfestation was applied in summer between the lettuce crops. During the survey chemical soil disinfestation was applied once in the summer of 2016. The soil texture in the glasshouse was a fine sand with 5.1% of carbon, a pH-KCl of 5.8, 38 kg $N0_3^-$ ha^{-1}, 12 kg NH_4^+ ha^{-1}, 2333 mg P_2O_5 and 480 mg K_2O (l soil)$^{-1}$, according to a soil analysis of January 2016. The nutrient status of the soil was adjusted regularly by applying fertilisers as recommended for each crop and was based on yearly soil analyses.

The study was carried out on a plot where reduced lettuce growth caused by *P. penetrans* had been observed previously. Eight composite soil samples were taken after each harvest of lettuce and leek, chemical soil disinfestation and black fallow. Samples were taken at four spots (= replications) at every sampling event. A grid of one square meter (0.75 m × 1.4 m) was positioned on the same spot at every sampling occasion and two samples of 10 cores each were collected per grid; one from 0-30 cm depth using a 2.5-cm diam. soil auger and one from 30-60 cm depth using a 3.5-cm diam. soil auger. This resulted in sample sizes of 1.47 and 2.88 l, respectively. In addition, eight randomly chosen leek plants with roots were harvested on each of the four grids, 6 days before the last soil sampling (21st of June 2018). At each sampling event, the grower was interviewed about the performance of the previous crop. Soil temperature was measured at 20 cm depth at sampling, except for the first sampling event. Samples were stored at 12°C and processed within 4 weeks.

The soil of each sample was thoroughly mixed and a subsample of 200 ml was taken. The organic fraction was separated from the mineral fraction by washing the soil through a 850 µm sieve (Chent *et al.*, 2000). The organic fraction, collected on top of the sieve and containing mainly small root fragments, was mixed in tap water for 1 min with a Waring blender at high speed (22,000 rpm) and added to the mineral fraction in a 1 l beaker. At the final sampling event, leek roots were sampled separately from soil samples. These leek roots with adhering soil were cut in 1 cm pieces and a 10 g subsample was mixed in tap water for 1 min, and washed over a 850 µm sieve into a 1 l beaker. The nematodes were extracted using the automated zonal centrifuge (Hendrickx, 1995) programmed for processing half of the volume, corresponding to 100 ml soil or 5 g roots. The obtained nematode suspension was collected in a glass beaker and subsequently transferred to a counting dish. Total numbers of *P. penetrans* and non-plant-parasitic nematodes were determined using a binocular microscope (50×).

Nematode counts taken before planting and at harvest of the different crops represented the initial (P_i) and final (P_f) population density, respectively. These counts were used to determine the influence of the crops on the nematode population in the soil. If the data were normally distributed and variances were equal, the initial and final population density were compared using the Student's *t*-test. If those assumptions were not fulfilled, the Wilcoxon test was applied. The Spearman's rank correlation test was used to find out to what extent the numbers of *P. penetrans* were related to those of non-plant-parasitic nematodes. The strength and direction of the correlation between nematode numbers in the two soil layers was also examined by calculating Spearman's coefficient, as was the influence of temperature on nematode numbers. The statistical tests were performed at a confidence level of $P = 0.05$.

2.3.2 Damage threshold for *P. penetrans* on butterhead lettuce

Two pot experiments were carried out during two different seasons. The first experiment was conducted in autumn with butterhead lettuce 'Brighton' and a second experiment in summer conditions with 'Cosmopolia'.

2.3.2.1 Multiplication of *P. penetrans*

Pratylenchus penetrans was multiplied on carrot discs (O'Bannon & Taylor, 1968) and in pots on maize (Teklu *et al.*, 2016) to ensure sufficient nematode numbers for the experiments. The population was originally collected from maize roots in Kinrooi (Belgium) and had been maintained on carrot discs since 2006. Culturing methods were slightly adapted from the original descriptions in the following ways. For the carrot disc method, carrots were washed with tap water, surface sterilised with ethanol, peeled and cut in slices of 5-10 mm with a sterile knife in a laminar flow. Vermiform stages of *P. penetrans* were sterilized in 2000 ppm streptomycin sulphate overnight and washed three times with sterile water before inoculating them on the carrot discs (20-40 nematodes $disc^{-1}$). The carrot discs were maintained in Petri dishes and incubated in the dark at 21°C for 3 months. After incubation, the discs were cut into pieces and placed on a Baermann funnel in the mist chamber to collect the nematodes.

Five maize seeds ('Koloris' and 'LG3220') were seeded in 6 l pots filled with 5610 g silver sand on a layer of 390 g hydro grains. After 2 weeks, pots were infested at a rate of 8 *P. penetrans* $(g\ dry\ soil)^{-1}$ in four holes between the plants. Maize was grown for 3 months at 20°C day/16°C night and 16 h light. Plants were watered and fertilised three times a week. At harvest, roots were carefully washed with tap water, cut in 1 cm pieces and placed on a Baermann funnel in a mist chamber. Nematodes were collected regularly from carrot discs and maize roots until sufficient inoculum of *P. penetrans* was obtained. The nematodes were stored at 4°C until being used in the experiments.

2.3.2.2 Pot experiments

Pots of 17 cm diam. and 16 cm height were filled with 1.6 l heat-sterilised field soil (6% clay, 20% loam, 74% sand and 1.4% organic carbon) in both experiments. Lettuce was sown in peat blocks (1 seed per block of 5 cm - 5 cm - 5 cm), provided by the applied research stations PCG and Inagro, and were transferred to the pots when plants reached the 4-6 leaf stage (BBCH=14-16). The peat blocks with seedlings were in a 2 cm deep hole made in the sandy soil. This took place in autumn (5th of October 2016) and spring (21st of March 2018) for 'Brighton' (ENZA Zaden) and 'Cosmopolia' (Rijk Zwaan), a popular winter and summer cultivar, respectively.

One day after lettuce planting, the soil in the pots was inoculated with nematode densities of 0, 50, 100, 200, 400, 800, 1600, 3200, 6400 and 12800 nematodes $(100\ ml\ soil)^{-1}$. Separate suspensions were made for every nematode density, so that an identical volume (80 ml) could be added to every pot. The inoculum was pipetted in four holes around each plant, each 5 cm deep. Immediately after applying the nematode suspension to the soil, the holes were carefully closed and plants were watered. Five replications for every density were used, except for the highest density where a shortage of inoculum only allowed four and three replicates with 'Brighton' and 'Cosmopolia', respectively.

Pots were organised in the glasshouse according to a randomised block design. Plants were watered and fertilised as needed and no pesticides were applied. The experiment with 'Brighton' was carried out during autumn (October-November 2016) at PCG, Kruishoutem, where pots were kept in a commercial glasshouse setting. Hence, the temperature fluctuated according to the season and was on average 15-20°C in the first part of the experiment and 10-15°C in the second part. Plants were harvested 8 weeks after planting. The experiment with the summer cv. 'Cosmopolia' was performed in a glasshouse at ILVO, Merelbeke, under controlled conditions simulating summer: day and night temperatures were 20 and 16°C, respectively, with 16h light. These plants were harvested 6 weeks after planting (BBCH = 47-49).

Nematode damage was evaluated by measuring lettuce head and root weight, and scoring head colour, head development and root quality on a scale from 1 to 9. The head colour scale ranged from score 1, very dark, indicating a stressed plant, to score 9, normal light green colour, for a healthy crop. The head development refers to the head shape, a very important parameter for the quality of the produce. When the head is open and hollow, score 1 is given, while for a qualitative closed and filled head score 9 is given. Root quality was also scored on a scale from 1 (very bad root quality, roots break easily, several root lesions) to 9 (very good root quality, no root lesions) (Figure 2.1).

Figure 2.1 Illustration of scores 3 to 9 of the scale used to score root damage. Scores 1 and 2 are not shown.

The final nematode population was determined for the soil fraction and roots in each pot. The entire root system was carefully removed from the soil, rinsed with water and cut into 1 cm pieces. The soil from the pot was mixed and a 200-ml subsample was taken for nematode extraction. Root and soil samples were processed as described above. Adults and juveniles of *P. penetrans* were counted in the total nematode suspension, except where there were high numbers when a subsample of at least 200 nematodes was counted. Eggs of *P. penetrans* were not counted as they cannot be distinguished from eggs of other nematodes.

2.3.2.3 Statistical analysis and modelling

Lettuce quality as well as nematode reproduction was described as a function of the applied initial nematode densities (P_i), using the models developed by Seinhorst (1966, 1986, 1998).

Lettuce quality

Measurements, directly or indirectly related to the quality of the lettuce heads (head development, head colour, head weight, root quality and root weight) were averaged for every P_i. The Seinhorst model with the constant Z and $Z^T = 0.95$ was fitted to the averaged data (Seinhorst, 1986, 1998):

$y = m + (1 - m) \times 0.95^{((Pi/T)-1)}$ for $P_i > T$ (Equation 1)

$y = 1$ for $P_i \leq T$

The parameter Z is a constant <1, representing the fraction of unaffected roots. As Z^T equals 0.95 for most plant and nematode combinations (Schomaker and Been, 2013), this value was used for fitting the lettuce quality data. The parameters tolerance limit (T) and relative minimum yield (m) were estimated for all measurements (y). The tolerance limit (T), also called the damage threshold, is the nematode density below which there is no yield reduction due to the nematodes. The minimum yield is the expected yield at very high nematode densities. This yield is expressed relative to the highest yield (when Pi < T), hence it is called the relative minimum yield and is always < 1.

Population dynamics

The final population density (P_f) for every P_i was log transformed, averaged over all replications and then back transformed. The maximum multiplication rate (a) and the maximum population density (M) for each experiment were estimated by modelling the relationship between P_i and P_f. The maximum multiplication rate (a) is the highest possible multiplication rate. At very low initial densities, $P_f = a.P_i$. While the maximum population density (M) is the highest nematode density the root system can bear, which makes $P_f = M$ at very high initial densities. The equation for migratory nematodes with more than one generation per growing period was used (Seinhorst, 1966; Schomaker *et al.*, 2013):

$P_f = M * P_i/(P_i + M/a)$ (Equation 2)

All datasets were analysed using R studio and run in R-version 3.5.0. The goodness-of-fit of models was expressed as the coefficient of determination (R^2). Statistical differences between the estimated parameters of the two experiments were analysed using the least significant difference (LSD) test at $P = 0.05$.

2.4 Results

2.4.1 The occurrence of *P. penetrans* in glasshouse lettuce in rotation with leek

Different cultivars of butterhead lettuce were grown in rotation with leek in a commercial glasshouse during the two and half year of the study (Figure 2.2). No above-ground symptoms indicating nematode damage were observed in any of the crops. The first soil samples were taken early in December 2015 after lettuce 'Gardia' (Figure 2.2). They contained only few *P. penetrans*, most residing in the 30-60 cm soil layer (10 (100 ml soil)$^{-1}$) and about 500 non-plant-parasitic nematodes (100 ml soil)$^{-1}$ (Figure 2.3A,

B). Chemical soil disinfestation with 1,3-dichloropropene had been applied in June 2015, before the start of the observations and was applied again in August 2016. Immediately after removal of the plastic, 3 weeks after the nematicide treatment, *P. penetrans* was absent in the 0-30 cm layer and only 7 *P. penetrans* (100 ml soil)$^{-1}$ were found in the 30-60 cm layer; this is a population decrease of 59% compared to the sampling before soil disinfestation in 2016. In addition, 93 and 90% of the non-plant-parasitic nematodes were killed due to this application in the higher and lower soil layer, respectively. However, the number of non-plant-parasitic nematodes recovered better after one cropping of butterhead lettuce compared with the numbers of *P. penetrans* (Figure 2.3B).

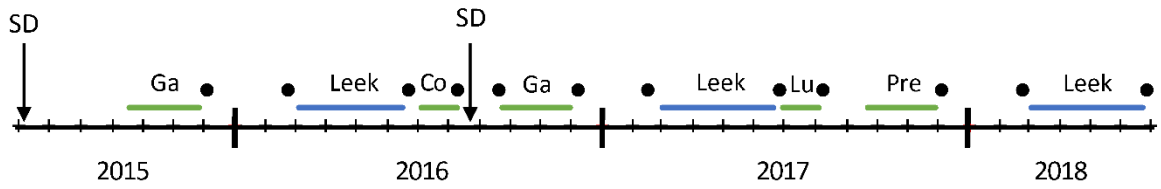

Figure 2.2 Timeline of the crops grown in the commercial glasshouse starting in June 2015. The black dots represent the sampling time points. The green line represents the cultivation of butterhead lettuce with different cultivars (Ga = 'Gardia', Co = 'Cosmopolia', Lu = 'Lucrecia' and Pre = 'Presteria'). The blue line represents the cultivation of leek cv. 'Krypton'. SD = chemical soil disinfestation.

Every winter there was a 3 month period of black fallow during which the population of *P. penetrans* remained more or less the same or increased slightly (Figure 2.3). In 2016, the increase was mainly observed in the lower layer (from 10 to 85 *P. penetrans* (100 ml soil)$^{-1}$), while in 2018 the numbers of *P. penetrans* increased in the upper soil layer (from 27 to 66 *P. penetrans* (100 ml soil)$^{-1}$). Many juveniles were observed in the samples after each period of black fallow.

Leek 'Krypton' was grown every year from March until the end of June. Its influence on the soil population density of *P. penetrans* was investigated by comparing the population densities in the soil before planting (P_i) and immediately after harvest (P_f) in both soil layers (Figure 2.4A). In 2016, the population density decreased significantly in the lower soil layer. The densities of *P. penetrans* were too low to notice any changes in 2017 (Figures 2.2 and 2.3A). In 2018, an increase was noticed in both soil layers but was not statistically significant. In 2018, roots were also investigated for the presence of *P. penetrans*. Elongated brown necrotic root lesions of 0.5 cm could be observed and 5 g of roots contained 1741 (± 608) vermiform stages of *P. penetrans* and 2188 (± 700) eggs (n = 4).

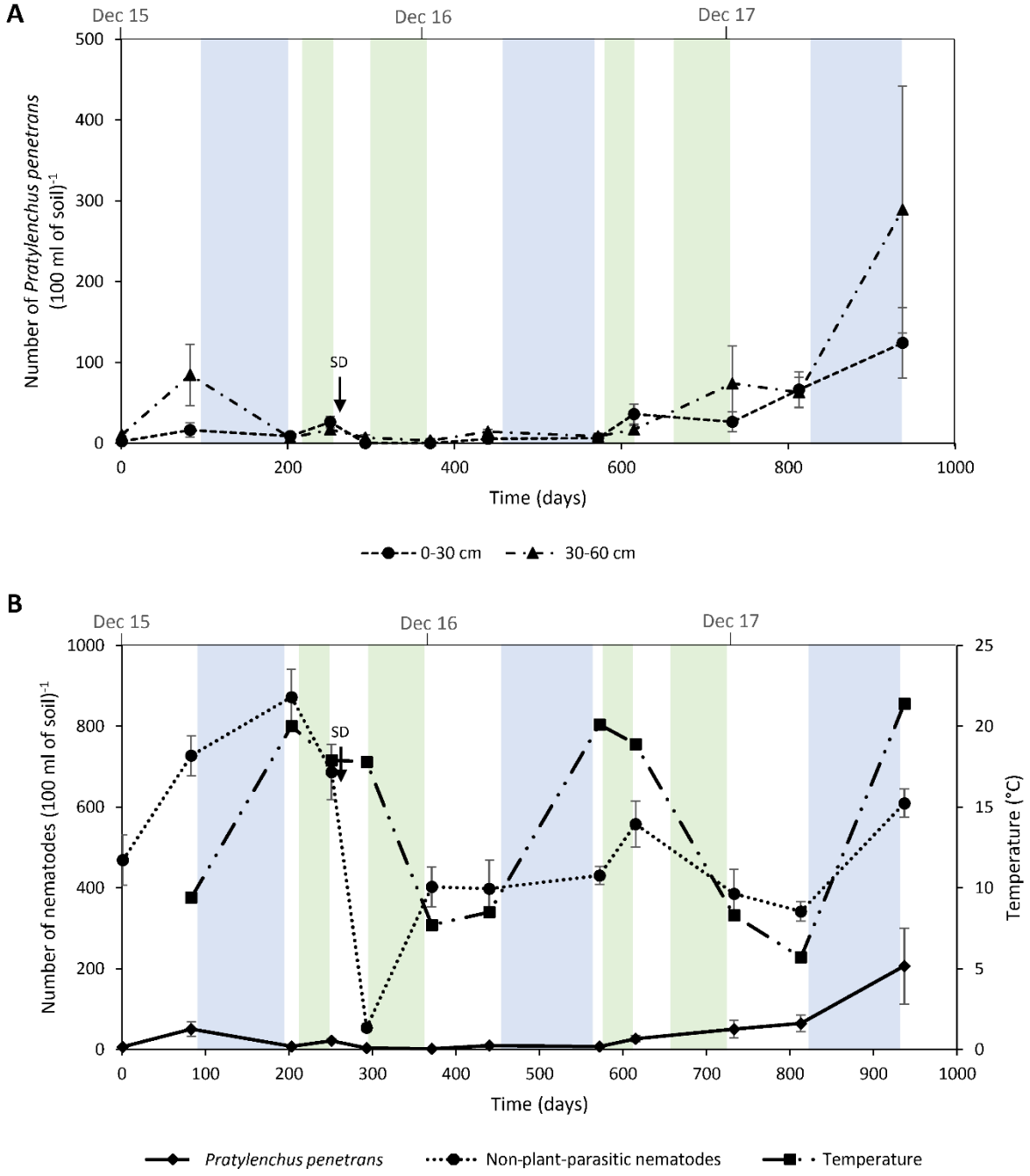

Figure 2.3 A: Number of *Pratylenchus penetrans* (100 ml soil)$^{-1}$ in the 0-30 and 30-60 cm soil layer. **B**: Number of *P. penetrans* and non-plant-parasitic nematodes (100 ml soil)$^{-1}$ averaged over the 0-60 cm soil layer and the ambient soil temperature over a period of 2.5 years. Day 1, the first sampling date, is the 4th of December 2015. Growing lettuce and leek is presented by the colors green and blue, respectively, white strips indicate fallow. SD = chemical soil disinfestation. Error bars represent the standard error (n = 4).

In general, growing butterhead lettuce resulted in no or just a slight increase in the numbers of *P. penetrans* in the soil (Figure 2.4B). The only significant increase in nematode population density was observed in the 30-60 cm layer after growing butterhead lettuce 'Cosmopolia' in the summer of 2016. As 'Gardia' was grown after chemical soil disinfestation, not enough *P. penetrans* were left in the soil to

notice differences in root-lesion nematode population densities. The numbers of *P. penetrans* increased, although not significantly, when growing 'Lucrecia' in the summer of 2017, and 'Presteria' in autumn 2017.

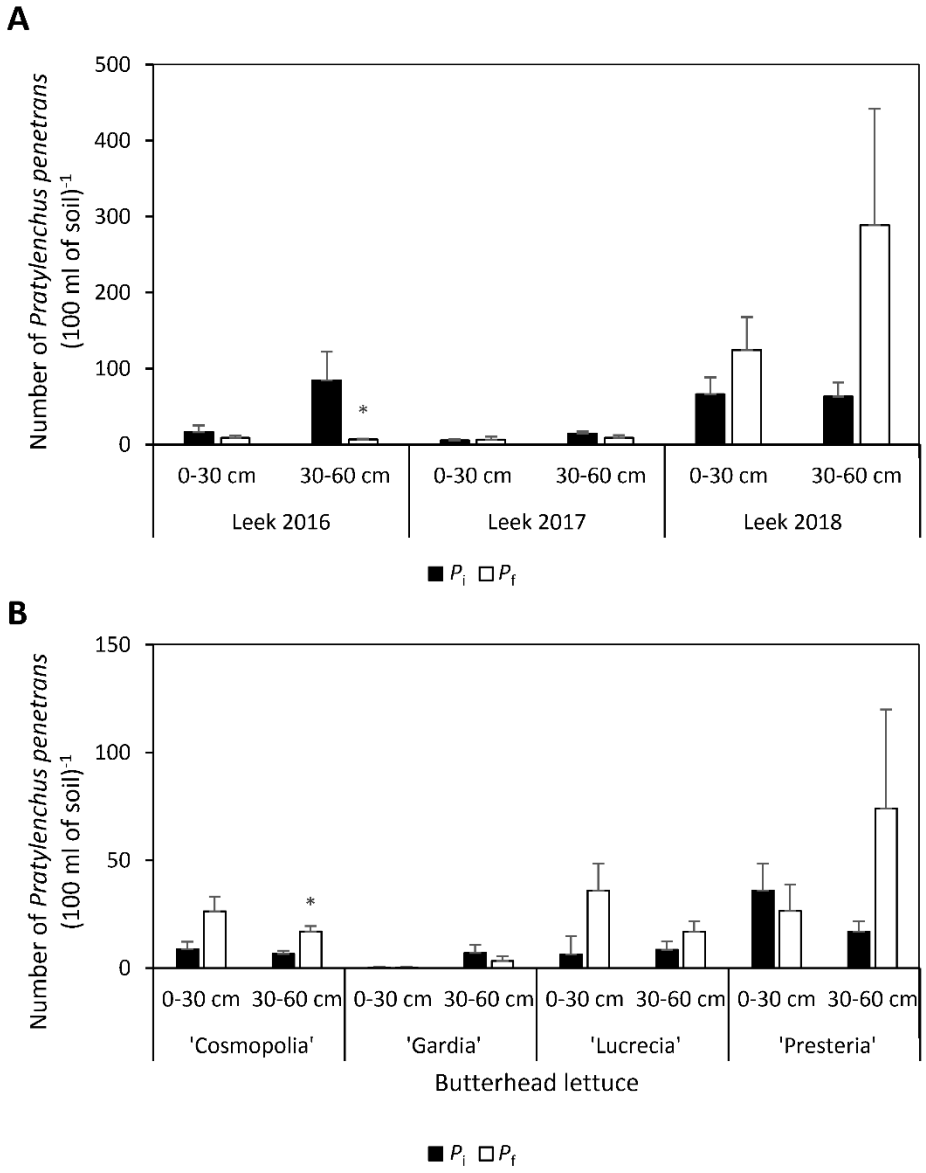

Figure 2.4 The initial (P_i) and final (P_f) population density of *P. penetrans* before and after growing **A**: Leek 'Krypton', **B**: different butterhead lettuce cultivars in the 0-30 and 30-60 cm soil layer. Error bars represent the standard error (n = 4).* shows significant differences between P_i and P_f (P = 0.05).

Overall, a weak linear correlation was found between the numbers of *P. penetrans* in the 0-30 cm layer and those in the 30-60 cm layer (r_s = 0.62, P < 0.01). This was also the case for the numbers of non-plant-parasitic nematodes (r_s = 0.64, P < 0.01). No correlation was found between the densities of *P. penetrans* and the densities of non-plant-parasitic nematodes (r_s = 0.28, P > 0.05), averaged over the 0-60 cm layer. Furthermore, there was no correlation between the population density of *P. penetrans* in the 0-60 cm layer and the ambient temperature (r_s = 0.01, P > 0.05) (data not shown).

2.4.2 Damage threshold for *P. penetrans* on butterhead lettuce

Two pot experiments were carried out to determine the damage threshold for *P. penetrans* on lettuce. A first experiment was set up in autumn with 'Brighton' and a second in summer conditions with 'Cosmopolia'. The lettuce plants grew for 8 and 6 weeks in the first and second experiment, respectively. The calculated degree-days (DD5 with base temperature 5°C) were 545 and 696, respectively.

2.4.2.1 Weight and quality of lettuce heads

The lettuce head weight ranged between 191 and 266 g and 95 and 147 g for 'Brighton' and 'Cosmopolia', respectively. Using the Seinhorst equation (Equation 1), a relative minimum weight (m_W) of 0.12 and 0.85, and a maximum yield ($Y_{max\,(W)}$) of 249 g and 132 g were calculated for the two cultivars (Table 2.1). The tolerance limit (T_w) was 3834 *P. penetrans* (100 ml soil)$^{-1}$ for 'Brighton' in autumn and 669 *P. penetrans* (100 ml soil)$^{-1}$ for 'Cosmopolia' in summer conditions. No significant differences could be detected for the relative minimum weight and the tolerance limit between both cultivars.

Table 2.1 Parameter values for the Seinhorst equations for the relation between initial population density (P_i) of *Pratylenchus penetrans* and measurements of lettuce. P_i and the tolerance limit (T) are expressed in *P. penetrans* (100 ml soil)$^{-1}$, while yield (y) and relative minimum yield (m) are proportions and the maximum yield (Y_{max}) is expressed in g for W = weight lettuce head and RW = root weight, or without unit (scores 1-9 for D = lettuce head development and Q = root quality). Parameter values of 'Brighton' grown in autumn were compared with those of 'Cosmopolia' grown in summer conditions using the LSD test (P = 0.05). SE = standard error, df = degrees of freedom.

Weight lettuce head (g)										
According to the equation: $y_W=m_W+(1-m_W)\ 0.95^{Pi/T_W-1}$ for $P_i>T_W$ and $y_W=1$ for $P_i \leq T_W$										
Cultivar	T_W	m_W	$Y_{max\,(W)}$	$SE_{T\,(W)}$	$SE_{m\,(W)}$	$SE_{Ymax\,(W)}$	R^2	df	$LSD_{T\,(W)}$	$LSD_{m\,(W)}$
'Brighton'	3834	0.12	248.69	2517	0.83	1.78	0.69	7	-	-
'Cosmopolia'	669	0.85	131.83	1178	0.19	1.73	0.46	7	5960	1.83
Lettuce head development										
According to the equation: $y_D=m_D+(1-m_D)\ 0.95^{Pi/T_D-1}$ for $P_i>T_D$ and $y_D=1$ for $P_i \leq T_D$										
Cultivar	T_D	m_D	$Y_{max\,(D)}$	$SE_{T\,(D)}$	$SE_{m\,(D)}$	$SE_{Ymax\,(D)}$	R^2	df	$LSD_{T\,(D)}$	$LSD_{m\,(D)}$
'Brighton'	185	0.79	6.23	202	0.09	0.25	0.36	7	-	-
'Cosmopolia'	1314	0.39	7.64	1741	0.76	0.22	0.43	7	3759	1.65
Root weight (g)										
According to the equation: $y_{RW}=m_{RW}+(1-m_{RW})\ 0.95^{Pi/T_{RW}-1}$ for $P_i>T_{RW}$ and $y_{RW}=1$ for $P_i \leq T_{RW}$										
Cultivar	T_{RW}	m_{RW}	$Y_{max\,(RW)}$	$SE_{T\,(RW)}$	$SE_{m\,(RW)}$	$SE_{Ymax\,(RW)}$	R^2	df	$LSD_{T\,(RW)}$	$LSD_{m\,(RW)}$
'Brighton'	262	0.76	6.25	312	0.14	0.26	0.30	7	-	-
'Cosmopolia'	400	0.51	11.14	173	0.15	0.43	0.62	7	765	0.44
Root quality										
According to the equation: $y_Q=m_Q+(1-m_Q)\ 0.95^{Pi/T_Q-1}$ for $P_i>T_Q$ and $y_Q=1$ for $P_i \leq T_Q$										
Cultivar	T_Q	m_Q	$Y_{max\,(Q)}$	$SE_{T\,(Q)}$	$SE_{m\,(Q)}$	$SE_{Ymax\,(Q)}$	R^2	df	$LSD_{T\,(Q)}$	$LSD_{m\,(Q)}$
'Brighton'	48	0.66	8.41	21	0.03	0.26	0.90	7	-	-
'Cosmopolia'	204	0.60	8.01	50	0.04	0.12	0.95	7	115 *	0.11

* Significantly different at P = 0.05

Lettuce head development is of importance for the quality of the crop. A decline in quality could be observed with increasing numbers of *P. penetrans* in the soil (Figure 2.5). The tolerance limit (T_D) for the head development was 185 and 1314 *P. penetrans* (100 ml soil)$^{-1}$ for 'Brighton' and 'Cosmopolia', respectively. The relative minimum score for head development (m_D) were 0.79 and 0.39; the maximum scores for head development ($Y_{max(D)}$) were 6.23 and 7.64. No differences in colour of the lettuce heads could be noticed in pots with different densities of *P. penetrans*, for both experiments (data not shown).

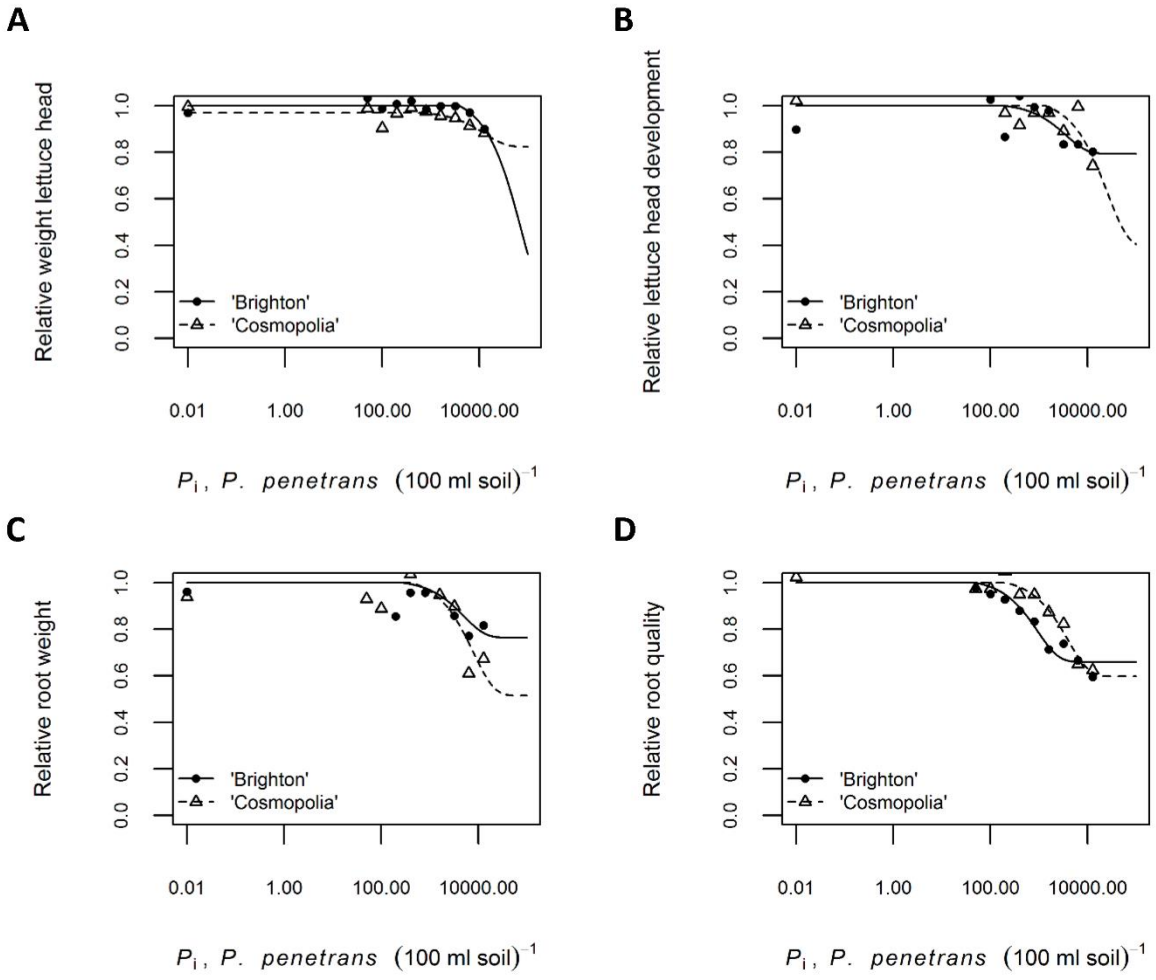

Figure 2.5 The relation between the initial population density (P_i) of *P. penetrans* and **A**: the relative weight of the lettuce head, **B**: the relative lettuce head development, **C**: the relative root weight and **D**: relative root quality for cv. 'Brighton' grown in autumn and cv. 'Cosmopolia' grown in summer conditions. A line was fitted according to the Seinhorst equation: $y = m+(1-m)0.95^{Pi/T-1}$ for $P_i > T_y$ and $y = 1$ for $P_i \leq T_y$.

2.4.2.2 Root weight and quality

Root weight and quality were measured as they affect head weight and development. The root weight ranged from 3.4 to 9.5 g and from 3.6 to 19.2 g for 'Brighton' grown in autumn and 'Cosmopolia' grown in summer conditions, respectively. An inverse relation was observed between root weight and nematode population density (Figure 2.5). The relative minimum root weight (m_{RW}) was 0.76 and 0.51 g and the tolerance limit (T_{RW}) was 262 and 400 *P. penetrans* (100 ml soil)$^{-1}$ for 'Brighton' and 'Cosmopolia', respectively. The maximum root weight ($Y_{max(RW)}$) was 6.25 g for 'Brighton' and 11.14 g for 'Cosmopolia'. Additionally, root quality was affected by *P. penetrans* population density. The maximum root quality scores ($Y_{max(Q)}$) and the relative minimum root quality scores (m_Q) were similar for 'Brighton' and 'Cosmopolia': around 8 for $Y_{max(Q)}$ and 0.6 for m_Q. (Table 2.1). However, there was a significant difference between the tolerance limits in both experiments; T_Q was 48 and 204 *P. penetrans* (100 ml soil)$^{-1}$ for 'Brighton' and 'Cosmopolia', respectively.

2.4.2.3 Population dynamics

The relation between P_i and P_f was very well described with the model for population dynamics (Equation 2) in both experiments (R^2 = 0.96 and 0.99; Table 2.2). 'Brighton' and 'Cosmopolia' are both poor host for *P. penetrans,* because a decrease of the population was observed for every initial population density tested (Figure 2.6). The maximum multiplication rate (a) of the *P. penetrans* population was 0.31 for 'Brighton' and 0.40 for 'Cosmopolia' (Table 2.2). The maximum population density (M) was 3609 and 13090 *P. penetrans* (100 ml soil)$^{-1}$ for 'Brighton' and 'Cosmopolia', respectively.

Table 2.2 Parameter values of the population dynamics model for the relation between initial (P_i) and final population density (P_f) of *Pratylenchus penetrans* (100 ml soil)$^{-1}$ for 'Brighton' grown in autumn and 'Cosmopolia' grown in summer conditions, according to the model $P_f = M \times P_i/(P_i+M/a)$. Multiplication rate (a) is dimensionless while maximum population (M) is measured as *P. penetrans* (100 ml soil)$^{-1}$. Parameter values of 'Brighton' were compared with those of 'Cosmopolia' using the LSD-test (P = 0.05). SE = standard error, df = degrees of freedom.

Parameter values								
Cultivar	M	a	SE_M	SE_a	R^2	df	LSD_M	LSD_a
'Brighton'	3609	0.31	1247	0.05	0.96	7	-	-
'Cosmopolia'	13090	0.40	8180	0.03	0.99	7	1.43	0.35

* Significantly different at P = 0.05

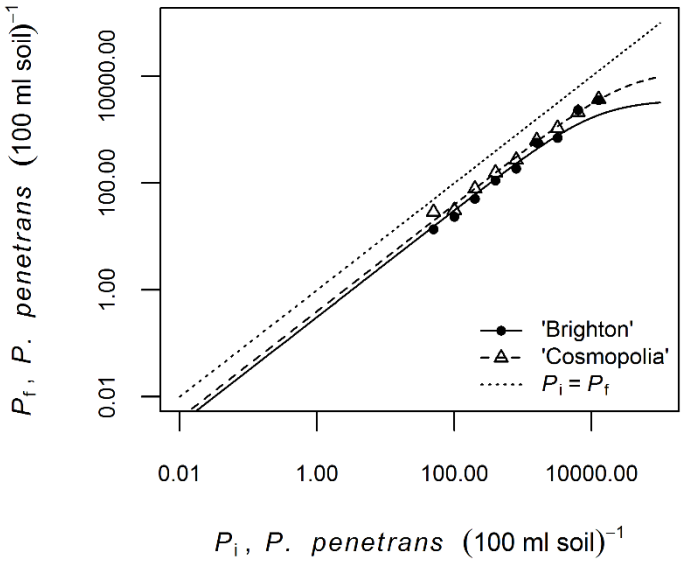

Figure 2.6 The relation between the initial (P_i) and final (P_f) population density of *P. penetrans* (roots and soil) for 'Brighton' grown in autumn and 'Cosmopolia' grown in summer conditions. A line was fitted according to the equation: $P_f = M*Pi/(P_i+M/a)$ for population dynamics. The diagonal dashed line represents the population equilibrium line ($P_f = P_i$).

2.5 Discussion

To obtain more insight into the factors influencing the dynamics of a *P. penetrans* population in a commercial lettuce glasshouse, nematode population dynamics were monitored during a 2.5 year period. In addition, we set up pot tests to determine the damage threshold densities of the root-lesion nematode for butterhead lettuce in summer and autumn croppings. Overall, numbers of *P. penetrans*

were low in this study, which makes it hard to gain a deeper insight into factors influencing *P. penetrans* dynamics. Moreover, nematode numbers in the four replicated spots were highly variable. The annual use of the soil fumigant 1,3-dichloropropene most likely contributed to the low occurrence of *P. penetrans* (Zasada *et al.*, 2010). Although nematode numbers declined considerably, nearly half (41%) of the population in the 30-60 cm soil layer survived the application. This is not unexpected as it is known that a nematicide application cannot be 100% effective (Haydock *et al.*, 2006). The surviving nematodes can migrate to the upper soil layers when a new crop is planted and infect roots growing downwards, so they are a risk for future crops. Indeed, a slow return of the population in the 0-30 cm soil layer was noticed in the commercial glasshouse when sampling 1 year after the disinfestation. The mobility of *P. penetrans* depends on several factors, such as soil texture and water availability (Castillo & Vovlas, 2007). The sandy soil in the glasshouse we monitored and the regular water supply were conducive to the upwards migration of remaining *P. penetrans.*

It is known that black fallow in general has a negative effect on the population of *P. penetrans* (Pudasaini *et al.*, 2006; Viaene *et al.*, 2006). However, it was reported that at low population densities, the number of nematodes can remain the same (Grabau *et al.*, 2017). Although low densities were observed in our study, the population surprisingly increased sometimes after 3 months of black fallow in winter. This contradictory result can probably be explained by hatching, since a lot of juveniles were observed at the end of fallow period. Hatching of *P. penetrans* is influenced by the host species, temperature (Pudasaini *et al.*, 2008) and soil moisture (Gaur & Haque, 1987). During winter the soil temperature in non-heated glasshouses is around 4-6°C at 10-15 cm depth, which still allows the development of *P. penetrans.* Mokrini *et al.* (2016) showed that the reproduction factor of different *P. penetrans* populations was 1.0-1.6 on carrot discs incubated for 8 weeks and even 2.7-3.7 after 12 weeks at 10°C. Dunn (1972) showed that *P. penetrans* can hatch even at 0-4°C. The lettuce grower only watered the soil at the end of black fallow. We assume that the change of moisture content stimulated hatching of *P. penetrans.*

Eggs in the nematode suspensions after extraction of the soil samples were not counted as those from *P. penetrans* cannot be distinguished from eggs of other nematode species. The number of eggs before and after leek, fallow and lettuce could have explained some of the observations made in this study, especially if the observed increase number was due to hatching from eggs already present, or due to reproduction of *P. penetrans* on the plants.

The population of *P. penetrans* decreased or remained the same after growing leeks. This is in contrast to Koot & Kroonenbackbier (1999), who showed that leek is a good host for *P. penetrans.* At the last cropping cycle, besides taking soil samples leek plants were also harvested and the roots were analysed for *P. penetrans*. Overall, 5 g roots contained 1741 (± 608) *P. penetrans*, which corresponds with 16 *P. penetrans* (100 ml soil)$^{-1}$ in the upper layer or 11% of the total soil population. Leek can be considered as a trap crop as the roots are removed from the soil at harvest. A growth reduction of leek was never observed, probably due to the low numbers in the glasshouse. Koot & Kroonenbackbier (1999) observed a growth reduction of leek at 545 *P. penetrans* (100 ml soil)$^{-1}$. Moreover, the host status or influence of different leek cultivars on *P. penetrans* damage is not known, but can be expected.

Growing lettuce had a minor impact on the population of *P. penetrans* in the commercial glasshouse, even though lettuce is known as a good or maintenance host (Oostenbrink *et al.*, 1957; Moretti *et al.*, 1981). Moretti *et al.* (1981) showed that both temperature and cultivar play a role in the reproduction of *P. penetrans.* They observed a maximum population increase of 2.1 at 21-22°C with an initial population density of 250 *P. penetrans* (100 ml soil)$^{-1}$, whilst at 14-15°C the reproduction was reduced but not inhibited. In addition to reproduction, the length of the life cycle of *P. penetrans* also depends on temperature and can take between 3 and 8 weeks. According to Mizukubo & Adachi (1997) the life cycle of *P. penetrans* was 46, 38, 28 and 26 days at 17, 20, 25 and 27°C, respectively. In the current study the temperature measured in the glasshouse at sampling before and after the first lettuce crop in summer was around 20°C, while it was lower at the end of the second crop, indicating a life cycle duration of at least 38 days. Comparing soil temperatures with other Belgian glasshouses, the average soil temperature is estimated to be around 20°C and 13-14°C during the first and second lettuce crop, respectively. The first lettuce crop in the commercial glasshouse, 'Cosmopolia' grown in 2016, lasted 35 days and resulted in a small population increase. The other lettuce cultivars in the commercial glasshouse with growing periods of 48 days for the first crop and 91-95 days for the second crop did not affect the population density. In our pot experiments, summer conditions were simulated with a temperature of 16-20°C for 'Cosmopolia', but a decrease in *P. penetrans* numbers was observed with a maximum multiplication rate of 0.41. The plants were grown for 42 days, corresponding with a total of 696 DD5, which is enough to complete a life cycle. The population in the pot experiment during autumn with 'Brighton', lasting 56 days at a maximum of 20°C and accumulating 574 DD5, had a maximum multiplication rate of 0.31. The short periods that lettuce plants remain in the field and not taking into account the number of eggs could explain the small or lack of increase in *P. penetrans*. Lettuce grown outside in the field resulted in an increase of the population of *P. penetrans* in summer and even in autumn (Olthof & Potter, 1973, 1974). This lettuce was planted in infested soil and grown for 57 days in summer (around 23°C) and 75 days in autumn, respectively, which allowed the completion of a life cycle. In addition, crisphead lettuce was used instead of butterhead lettuce, which can also influence the reproduction.

Another reason for the lack of reproduction in the pot experiments could be the selected *P. penetrans* population. Pot tests were conducted with the Kinrooi population, originating from maize and cultured on carrot discs for more than 13 years. Tests with another population (PSKW), originating from lettuce and cultured on carrot discs and maize for only 2 years, did not result in different lettuce weights compared to Kinrooi (unpublished data). However, the final populations of PSKW on lettuce 'Cosmopolia' were the same as the initial population, *i.e.*, higher than those obtained with the Kinrooi population. The populations used in the studies of Olthof & Potter (1973, 1974) and Moretti *et al.* (1981) were cultured on different hosts, including lettuce. Differences in reproduction and pathogenicity between *P. penetrans* populations have already been reported (Olthof, 1968; France & Brodie, 1995; Castillo & Vovlas, 2007) and could also explain the failure of *P. penetrans* to reproduce in our pot experiments. In addition, the extraction efficiency of the automated zonal centrifugation machine is not 100% and eggs are not counted, so the real population might be 10% higher than assumed.

Often the whole nematode community is used as a bio-indicator to evaluate cultivation strategies or crop types (Neher & Olson, 1999; van Diepeningen *et al.,* 2006; Berkelmans *et al.,* 2003; Quist *et al.*, 2016). Large fluctuations in the number of non-plant-parasitic nematodes in the samples were observed during the monitoring but no link with the *P. penetrans* population was found. We hypothesised that soil fumigation would result in a significant reduction of beneficial nematodes and this could have been an explanation of the expected increase of *P. penetrans* (Timper *et al.*, 2012). However, we observed a relatively fast rebound of the non-plant-parasitic nematodes after an initial decline immediately following the fumigation but no considerable increase in *P. penetrans*. Furthermore, 1,3-dichloroporopropene is a nematicide that does not affect the overall microbial community structure (Zeng *et al.*, 2019), so microorganisms that would control *P. penetrans* are not affected by the application. This could lead to the conclusion that an assumed competition between non-plant-parasitic nematodes or microorganisms and *P. penetrans* remained the same before and after chemical disinfestation, and that *P. penetrans* is suppressed by certain micro-organisms or other types of nematodes. We did not identify the composition of the nematode or the microbial community, so we were unable to check this hypothesis. However the explanation can also be found in the simple fact that *P. penetrans* needs time to build up its population, unlike the bacterial feeding nematodes, and that regular application of nematicides in the commercial glasshouse we monitored does not allow this. In addition, lettuce and leek are slowing down the population build-up in the soil. Similar observations were made in a field study after application of 1,3-dichloropropene where it took years for the plant-parasitic nematodes *Rotylenchus robustus, Trichodurus primitivus* and *Paratrichodorus pachydermus* to reach their pre-fumigation densities (Boag & Alphey, 1988). A more thorough investigation of the nematode community could be informative to work out relations between crop management practices and *P. penetrans*.

There are no reports of the damage threshold for *P. penetrans* in butterhead lettuce determined with the Seinhorst model. For crisphead lettuce, the damage threshold for *Meloidogyne hapla* was estimated to be 100-200 eggs (100 ml soil)$^{-1}$ in microplots, and 700-800 eggs (100 ml soil)$^{-1}$ in pots (Viaene & Abawi, 1996). In our study, the damage threshold for the crop weight was 669 and 3834 *P. penetrans* (100 ml soil)$^{-1}$ in pots in summer and autumn conditions, respectively. Outside-grown transplanted crisphead lettuce showed losses in lettuce yield of 36 and 27% at initial population densities equivalent to 840 *P. penetrans* (100 ml soil)$^{-1}$ in summer and autumn, respectively (Olthof & Potter, 1973, 1974). In our study the damage threshold for the autumn-grown lettuce was much higher than the other reported thresholds but it was quite similar for the summer crop. Our pot experiments were conducted in heat sterilized soil by which secondary infections, that could have been present in the field in the autumn experiment, were avoided. This might explain the fact that higher nematode populations were necessary to obtain yield losses in our pot test, next to the possible influence of cultivar, soil and climatic conditions. Furthermore, the standard errors for the calculated parameter values were very high and therefore the estimated damage thresholds should be interpreted with caution. This high variability is due to errors estimating P_i and yield; therefore, more repetitions should be included. Next to that, pot test are indicative and field test should be carried out to validate these results. For both pot tests, the damage thresholds for root weight and root quality were lower than those for crop weight, which means that damage on roots does not immediately result in lower crop weight.

No damage was observed on lettuce during the monitoring of the commercial glasshouse, probably due to the low densities of *P. penetrans* (≤ 124 *P. penetrans* (100 ml soil)$^{-1}$). However, in the summer of 2018, the grower observed growth reduction in another glasshouse, where 220 *P. penetrans* (100 ml soil)$^{-1}$ and 323 *P. penetrans* (5 g roots)$^{-1}$ were counted in a soil sample taken after damage was observed. Furthermore, in the preliminary survey on lettuce, damage was found at a final population density of 701 *P. penetrans* (100 ml soil)$^{-1}$. In commercial lettuce production, heads should reach 450 g to be marketable. Plants in the pot experiments had maximum weights of 266 g and 147 g in the autumn and summer crop, respectively. Therefore, it is strongly recommended to consider the damage thresholds calculated for the root quality when making management decisions, because healthy roots will ensure a healthy lettuce head.

The damage threshold for *P. penetrans* can vary a lot between vegetables. The damage thresholds for *P. penetrans* for faba bean and carrot were 6.2 (g soil)$^{-1}$ and 1.37-1.88 (g dry soil)$^{-1}$, respectively (Seinhorst, 1998; Teklu *et al.*, 2016). These numbers are equivalent to 868 and 192-263 *P. penetrans* (100 ml soil)$^{-1}$. Other studies showed damage at 50 *P. penetrans* (100 ml soil)$^{-1}$ for bean; 0.45 *P. penetrans* (g soil)$^{-1}$ (equivalent to *ca* 63 (100 ml soil)$^{-1}$) for Brussels sprouts, cucumber, eggplant and tomato; 666 *P. penetrans* (kg soil)$^{-1}$ (equivalent to *ca* 93 (100 ml soil)$^{-1}$) for onion; 6000 *P. penetrans* (kg soil)$^{-1}$ (equivalent to *ca* 840 (100 ml soil)$^{-1}$) for cabbage, cauliflower, potato and 18000 *P. penetrans* (kg soil)$^{-1}$ (equivalent to *ca* 2520 (100 ml soil)$^{-1}$) for beet and spinach (Olthof & Potter, 1973, 1974; Miller, 1978; Elliott & Bird, 1985). Our calculated damage thresholds are comparable with those for cabbage, cauliflower, potato, beet and spinach.

In conclusion, this study revealed that *P. penetrans* is a minor problem in glasshouse-grown lettuce but one that should not be overlooked. The population densities must be kept below 48-204 *P. penetrans* (100 ml soil)$^{-1}$ to secure a good root quality, necessary to obtain a marketable crop. However, other plant pathogens can also influence lettuce yield, so regular monitoring for pathogens as well as nematodes is highly recommended. Combining black fallow with a rotation of leek and lettuce seems to be a good practice to keep the nematode population low for at least 21 months. However, critical nematodes densities might be achieved over time, requiring measures that reduce numbers effectively, such as chemical soil disinfestation. Lettuce growers should be aware that this management strategy does not kill all the nematodes and that the population of *P. penetrans* will recover slowly.

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CHAPTER 3

A thorough study of a *Paratylenchus* sp. in glasshouse-grown lettuce: characterisation, population dynamics, host plants and damage threshold as keys to its integrated management

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3.1 Abstract

In glasshouses practicing monoculture of butterhead lettuce, high densities of pin nematodes (*Paratylenchus* spp.) are frequently associated with reduced plant growth. Growers currently apply chemical soil disinfestation measures to manage this problem, although stricter phytosanitary regulations are forcing a shift towards integrated management. Efficient implementation of such management requires knowledge about the factors influencing nematode population dynamics, and the damage threshold for lettuce. The nematode populations in five Belgian glasshouses were monitored for at least one year by frequently sampling the same four 1-m² spots at 0-30 cm and 30-60 cm depth. An undescribed species of *Paratylenchus* was identified in all glasshouses based on morphological and molecular features. High nematode densities (> 20,000 (100 ml soil)$^{-1}$) occurred in winter and spring. Chemical soil disinfestation lowered these populations spectacularly, although up to 14% survived in the deeper soil layer. After soil steaming under negative pressure, no pin nematodes were found. Two months of black fallow resulted in 50 to 76% fewer pin nematodes. Cultivation of lamb's lettuce could be an alternative to soil disinfestation, as numbers of *Paratylenchus* sp. did not increase when this crop was grown. Parsley and wild rocket were found to be poor hosts in a pot experiment, while reproduction factors (P_f/P_i) on lettuce cultivars varied between 1 to 2. In three experiments with lettuce cv. 'Cosmopolia' in pots with a series of 9 or 10 densities of *Paratylenchus* sp. (up to 35,000 (100 ml soil)$^{-1}$), no damage to lettuce heads was observed. However, root weight and root quality were reduced, and the corresponding damage thresholds were rather low (1,754 and 362 *Paratylenchus* sp. (100 ml soil)$^{-1}$, respectively). Management strategies such as crop rotation, soil disinfestation or fallow are recommended to avoid population build-up.

3.2 Introduction

A survey, carried out in 2014 and comprising 38 glasshouses, revealed that the pin nematode *Paratylenchus* Micoletzky, 1922, was present in 47% of these glasshouses, in densities ranging from 1 to 5,100 nematodes (100 ml soil)$^{-1}$ (unpublished). Although this nematode appears to be common in Belgian glasshouses, the species has never been formally identified. Densities of 17,000 *P. nanus* (liter soil)$^{-1}$ were associated with growth reduction in glasshouse-grown lettuce in England (Winfield, 1985). *Paratylenchus projectus* has also been reported to parasitize lettuce (Coursen *et al.*, 1958).

Currently, growers mainly control nematodes in lettuce by chemical soil disinfestation, which is also applied to reduce several other soil-borne pathogens. Boag & Alphey (1988) observed that the population of *P. nanus* increased drastically after the application of the fumigant 1,3-dichloropropene in a forest nursery field. They concluded that this nematode has an r survival strategy, which means that its numbers increase rapidly in the absence of competition and show seasonal fluctuations. In contrast, K strategists are influenced by intraspecific competition and have a slow multiplication rate. This might also be the case in the intensive lettuce production system, where high densities of pin nematodes can be reached. Stricter phytosanitary regulations, driven by increasing environmental concerns, demand a reduction in the use of chemical soil disinfestation. In order to implement effective integrated pest management strategies, and to provide sound practical advice for lettuce growers, thorough knowledge about the population dynamics and the damage threshold of *Paratylenchus* spp. for lettuce is essential.

The overall goal of this study was to obtain knowledge that will contribute to the set-up of integrated pest management tools for pin nematodes in Belgian glasshouses. The first objective was a molecular and morphological characterisation of *Paratylenchus* populations occurring in the glasshouses, to find out which species we are dealing with. Secondly, the population dynamics of *Paratylenchus* sp. were studied in several glasshouses with butterhead lettuce production to gain insights into the factors influencing nematode densities. A third objective was to investigate the host status of different lettuce cultivars and other plants to find crops potentially useful in rotation schemes. Finally, the damage threshold of the *Paratylenchus* sp. for lettuce was estimated by growing butterhead lettuce in pots in the glasshouse and recording crop parameters over a wide range of nematode population densities.

3.3 Materials and methods

3.3.1 Morphological and molecular characterisation

Paratylenchus specimen were extracted from soil using the automated zonal centrifuge (Hendrickx, 1995). The soil samples originated from the five glasshouses that were sampled to investigate the population dynamics (see below) and each of the corresponding five populations were characterised morphologically and molecularly (Table 3.1). For the morphological characterisation, nematodes were killed and fixed in hot 4% fresh paraformaldehyde fixative (PFA) buffered in phosphate buffer saline (PBS). After fixation, the nematodes were transferred to glycerin-alcohol according to Seinhorst (1959) modified by De Grisse (1969), dehydrated in a desiccator and mounted in anhydrous glycerin using the wax ring technique. Nematodes were examined and photographed using an Olympus BX51 DIC Microscope (Olympus Optical), equipped with an Olympus C5060Wz camera and a drawing tube.

Measurements of 50 females (10 for each glasshouse) were made using Image Pro-plus software. A comparison with similar species was made using the keys of Andrássy (2007), Brezski (1998) and Ghaderi *et al.* (2014, 2016), relevant original descriptions and data published by Van den Berg *et al.* (2014).

Temporary slides with fresh female nematodes were used to take digital light microscope pictures and measurements before genomic DNA was extracted for molecular analysis. Individual nematodes (one nematode for each glasshouse) were transferred in 8 µl worm-lysis buffer (50 mM KCl, 10 mM Tris-Cl pH 8.3, 1.5 mM $MgCl_2$, 1 mM DTT, 0.45% Tween-20) and 1 µl of proteinase K (1.2 mg ml^{-1}). The mixture was incubated for 1 h at 65°C and 10 min at 95°C followed by a centrifugation step for 1 min at 16000 g. The following gene fragments were amplified: the internal transcribed spacer (*ITS*) rDNA region with primers TW81b (5'-GTAGGTGAACCTGCAGCTG-3', adapted TW81 primer by ILVO, unpublished) and AB28 (5'-ATATGCTTAAGTTCAGCGGGT-3', Joyce *et al.,* 1994) and part of the *28S* rDNA gene with primers D2A (5'-ACAAGTACCGTGAGGGAAAGTTG-3', De Ley *et al.,* 1999) and D3B (5'-TCGGAAGGAACCAGCTACTA-3', De Ley *et al.* 1999). A 50 µl PCR reaction volume contained 21.4 µl distilled water, 2 µl $MgCl_2$, 25 µl 2X BIO-X-ACT (Bioline, Germany), 0.3 µl of each forward and reverse primer (50 µM) and 1 µl DNA. The thermal cycling profile was as follows: initial denaturation step at 96°C for 3 min; 35 cycles of 96°C for 30 s, X°C for 30 s, 72°C for 1 min with X being the annealing temperature of 49°C for the ITS rDNA region and 55°C for D2-D3 of 28S rDNA gene; final extension step at 72°C for 10 min. The PCR products were visualized after electrophoresis (100V, 30 min) on agarose gels (1.5%) with Gel red using a UV_transilumminator. Purification was done following the protocol accompanying the SmartPure Gel DNA purification kit (a Eurogentec, Belgium). The amplified fragments were sent for sequencing to Macrogen (the Netherlands). All fragments were sequenced in forward and reverse directions. The consensus sequences were obtained by assembling forward and backward sequences using BioEdit version 7 (Hall, 1999). The BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to check for closely related sequences of other species on GenBank. Multiple alignments were made from 40 and 54 *Paratylenchus* sequences and 2 and 3 outgroup taxa for the *ITS* rDNA region and the *D2-D3 region of the 28S* rDNA gene, respectively, by using MUSCLE in MEGA 7 (Kumar *et al.*, 2016) (Table 3.1). The best-fit models were selected by using MEGA 7 based on BIC criterion. K2+G model was chosen for the *ITS* rDNA dataset and Tamura-Nei+G for the *D2-D3 region of the 28S* rDNA dataset. Maximum Likelihood (ML) was performed using MEGA 7 and included 1000 bootstrap (BS) replicates.

Table 3.1 Information, derived from GenBank, on the *Paratylenchus* spp. and outgroup taxa included in the phylogenetic analysis for comparison.

Species	Isolate	Location	Associated plant	Accession number		Reference
				D2-D3 of 28S	***ITS***	
Aglenchus agricola	-	Belgium: Merelbeke	-	AY780979	-	Subbotin *et al.*, 2005
Basiria gracilis	CA1	USA	-	DQ328717	-	Subbotin *et al.*, 2006
Coslenchus costatus	-	Germany	-	DQ328719	-	Subbotin *et al.*, 2006
Paratylenchus aculentus	-	Taiwan	-	-	EU247526	Chen *et al.*, 2008
Paratylenchus aquaticus	CD619	USA: HI, Waimanalo	Bromelid	-	KF242277, KF242278	Van den Berg *et al.*, 2014
Paratylenchus bukowinensis	-	Italy: Monopoli	-	AY780943	-	Subbotin *et al.*, 2005
Paratylenchus dianthus	CD552	South Africa: Tarlton, Gauteng	Chrysanthemum	KF242227 - KF242229	KF242271, KF242272	Van den Berg *et al.*, 2014
Paratylenchus hamatus	CD1319	USA: AZ, Sedona	-	KF242219	-	Van den Berg *et al.*, 2014
Paratylenchus hamatus	CD17	USA: CA, Live Oak, Sutter county	Peach	KF242218	KF242244	Van den Berg *et al.*, 2014
Paratylenchus hamatus	CD19	USA: CA, Patterson, Stanislaus county	Apricot	-	KF242258	Van den Berg *et al.*, 2014
Paratylenchus hamatus	CD315	USA: CA, Wasco, Kern county	Rose	KF242213, KF242215	-	Van den Berg *et al.*, 2014
Paratylenchus hamatus	CD454	USA: CA, Maricopa, Kern county	Apricot	KF242216, KF242217	KF242247	Van den Berg *et al.*, 2014
Paratylenchus hamatus	CD454	USA: CA, Maricopa, Kern County	Plum	-	KF242256	Van den Berg *et al.*, 2014
Paratylenchus hamatus	CD455	USA: CA, Maricopa, Kern county	Plum	-	KF242248	Van den Berg *et al.*, 2014
Paratylenchus hamatus	CD480	USA: CA, Westley, Stanislaus county	Peach	KF242214	KF242246, KF242257	Van den Berg *et al.*, 2014
Paratylenchus hamatus	CD489	USA: CA, Kingsburg, Kings county	Peach	KF242204	KF242245	Van den Berg *et al.*, 2014
Paratylenchus labiosus	ARK26	USA: Arkansas	-	-	JQ708154	Cordero Lopez *et al.*, 2013
Paratylenchus lepidus	-	Taiwan	-	-	EF126178	Chen *et al.*, 2007
Paratylenchus minutus	-	Taiwan	-	-	EF126180	Chen *et al.*, 2009
Paratylenchus nanus	D4-1	South Korea	-	KY468899	KY468904	Kim *et al.*, 2019
Paratylenchus nanus	D4-2	South Korea	-	KY468900	KY468905	Kim *et al.*, 2019
Paratylenchus nanus	D4-3	South Korea	-	KY468901	KY468906	Kim *et al.*, 2019
Paratylenchus nanus	D4-5	South Korea	-	KY468902	KY468907	Kim *et al.*, 2019
Paratylenchus nanus	D8-1	South Korea	-	-	KY468908	Kim *et al.*, 2019
Paratylenchus nanus	D8-3	South Korea	-	-	KY468909	Kim *et al.*, 2019

Paratylenchus nanus	D8-4	South Korea	-	KY468903	KY468910	Kim *et al.*, 2019
Paratylenchus nanus	MS-2	USA: North Dakota	Field pea	MH237651	MH236098	Unpublished
Paratylenchus nanus type A	-	Germany: Niebuell	-	AY780946	-	Subbotin *et al.*, 2005
Paratylenchus nanus type A	869	Germany: Niebuell	-	-	KF242269, KF242270	Van den Berg *et al.*, 2014
Paratylenchus nanus type A	C850	USA: CA, Marin county	*Festuca* sp.	KF242192, KF242193	-	Van den Berg *et al.*, 2014
Paratylenchus nanus type A	CD728	USA: CA, Riverside	Grasses	KF242197	KF242267	Van den Berg *et al.*, 2014
Paratylenchus nanus type A	CD860	USA: CA, Marin county	Grasses	KF242191, KF242195	-	Van den Berg *et al.*, 2014
Paratylenchus nanus type A	CD883	USA: CA, Marin county	Grasses	KF242196	-	Van den Berg *et al.*, 2014
Paratylenchus nanus type B	CD137	USA: CA, Gridley, Butte county	Walnut	KF242199	KF242266	Van den Berg *et al.*, 2014
Paratylenchus nanus type B	CD186	USA: CA, Roosevelt, Los Angeles county	Alfalfa	KF242201	-	Van den Berg *et al.*, 2014
Paratylenchus nanus type B	CD587	South Africa: George, Western Cape	Bent grass	KF242198, KF242200	KF242264, KF242263	Van den Berg *et al.*, 2014
Paratylenchus neoamblicephalus	-	USA: Ohio	Soybean	KY584086	-	Anrkom *et al.*, 2017
Paratylenchus sp.	T1	Belgium: Sint-Katelijne-Waver	Butterhead lettuce	MN535542	MN535547	This study
Paratylenchus sp.	T2	Belgium: Sint-Katelijne-Waver	Butterhead lettuce	MN535543	MN535548	This study
Paratylenchus sp.	T3	Belgium: Aarschot	Butterhead lettuce	MN535544	MN535549	This study
Paratylenchus sp.	T4	Belgium: Lint	Butterhead lettuce	MN535545	MN535550	This study
Paratylenchus sp.	T5	Belgium: Jabbeke	Lamb's lettuce	MN535546	MN535551	This study
Paratylenchus sp. 1	CD57	USA: CA, Orland, Glenn county	Prune	KF242223, KF242225	-	Van den Berg *et al.*, 2014
Paratylenchus sp. 1	CD61	USA: CA, Butte City, Glenn county	Prune	KF242224	KF242259, KF242260	Van den Berg *et al.*, 2014
Paratylenchus sp. 2	CD604	USA: CA, Davis, Yolo county	Grasses	KF242220, KF242221	KF242243	Van den Berg *et al.*, 2014
Paratylenchus sp. 3	CD1017	USA: AR, Mulberry	*Equisetum hyemale*	KF242230	-	Van den Berg *et al.*, 2014
Paratylenchus sp. 3	CD232	USA: AR, Mulberry	*Equisetum hyemale*	KF242231, KF242232	-	Van den Berg *et al.*, 2014
Paratylenchus sp. 3	CD232	USA: CA, Goleta, Santa Barbara county	Lemon	-	KF242274, KF242273	Van den Berg *et al.*, 2014
Paratylenchus sp. 4	CD1092	USA: MN, Saint Paul	-	KF242202	-	Van den Berg *et al.*, 2014
Paratylenchus sp. 4	CD986	USA: Oregon	-	KF242203	-	Van den Berg *et al.*, 2014
Paratylenchus sp. 5	CD106	USA: CA, Napa, Napa county	Grape	-	KF242275	Van den Berg *et al.*, 2014

Paratylenchus sp. 6	CD1223	USA: CA, Madera, Madera county	Grasses	KF242190	-	Van den Berg *et al.*, 2014
Paratylenchus sp. 6	CD1288	USA: CA, Lodi, San Joaquin county	Grasses	KF242189	-	Van den Berg *et al.*, 2014
Paratylenchus sp. 7	CD1004	USA: CA,Riverside, UCR Campus	-	KF242242	-	Van den Berg *et al.*, 2014
Paratylenchus sp. 8	CD1053	USA: CA, Strawberry canyon, Berkeley	Grasses	KF242233, KF242234	-	Van den Berg *et al.*, 2014
Paratylenchus sp. Subbotin-UCR	-	USA: CA, Fresno	*Salix* sp.	AY780945	-	Subbotin *et al.*, 2005
Paratylenchus straeleni	CD786	USA: CA, Mendocino county	-	KF242235	-	Van den Berg *et al.*, 2014
Paratylenchus straeleni	KAB3	Turkey: Black Sea region, Ordu	*Corylus avellana* L.	KM875547	-	Akyazi *et al.*, 2015
Paratylenchus tenuicaudatus	irN2	Iran	-	KU291239	-	Esmaeili *et al.*, 2016
Sphaeronema alni	714_TW	Germany: Muenster	*Alnus glutinosa*	-	GU253921	Palomares-Rius *et al.*, 2010
Tylenchorhynchus leviterminalis	-	Taiwan	-	-	EF030984	Chen *et al.*, 2006

3.3.2 Spatial and temporal dynamics of *Paratylenchus* spp. in glasshouse lettuce and lamb's lettuce

Five glasshouses were selected from the survey in 2014 to study their population of *Paratylenchus* spp. (Table 3.2). No other important plant-parasitic nematodes were found. Lettuce and lamb's lettuce were either grown alone or in rotation. The nutrient status of the soil was adjusted regularly by applying fertilizers as recommended for each crop and was based on annual soil analyses.

Table 3.2 Characteristics of the glasshouses where the population of *Paratylenchus* spp. was monitored.

Characteristics	**Glasshouse**				
	1	**2**	**3**	**4**	**5**
Crop	lettuce	lettuce	lettuce	lettuce/lamb's lettuce	lamb's lettuce
Soil texture	sandy loam	sandy loam	fine sand	sand	coarse sand
Soil disinfestation prior to monitoring	June 2015	never	July 2015	August 2015	? 2011
Product	1,3-dichloropropene	-	metam potassium	1,3-dichloropropene	unknown
pH[1]	7.1	6.9	5.9	6.2	5.8
% C[1]	2.2	2.1	5.2	-	2.1
HWC[2]	1329	1174	1531	1828	1608
HWP[2]	14.1	16.0	53.8	17.0	39.3

HWC = hot water extractable carbon (mg per kg dry soil), HWP = hot water extractable phosphor (mg per kg dry soil)
[1]pH and % C in September 2015, November 2015, October 2016, September 2016 and November 2016 for glasshouse 1, 2, 3, 4 and 5, respectively
[2]HWC and HWP in November 2016 for glasshouse 1, 2 and 3, February 2016 and December 2016 for glasshouse 4 and 5, respectively

The study in glasshouses 1 to 4 was carried out on a plot where reduced lettuce growth caused by *Paratylenchus* spp. had been observed previously. In glasshouse 5, a plot with high numbers of *Paratylenchus* spp. was selected based on a preliminary sampling of a spot where reduced growth of chrysanthemum had been observed. The glasshouse temperature was measured every hour in glasshouse 1 and 2 only. Soil samples were collected and analyzed as described in Chapter 2.

Spearman's rank order correlation was used to analyze the numbers of *Paratylenchus* spp. and non-plant-parasitic nematodes in both layers, since the data were not normally distributed (Shapiro-Wilk test) and homogeneity of variances (Levene's test) were not met at the 5% significance level. ARTool (Aligned Rank Transfrom, Wobbrock *et al.*, 2011) package in R-version 3.5.0 was used to conduct a multifactorial ANOVA for non-parametric data to investigate the effects of season, glasshouse and their interaction.

3.3.3 Host plants

The host status for *Paratylenchus* sp. of lettuce (*Lactuca sativa* L.) type butterhead cvs. 'Brighton' and 'Cosmopolia', type lollo rossa cv. 'Tuksa' and type oakleaf cv. 'Prunai' was investigated in a pot experiment in the glasshouse. At the same time, the host suitability of lamb's lettuce (*Valerianella locusta*) cvs. 'Audace' and 'Pulsar', parsley (*Petroselinum crispum*) cv. 'Frise vert Fonce-rina', chrysanthemum (*Chrysanthemum morifolium*) cv. 'Medonia' and wild rocket (*Diplotaxis tenuifolia*) cv.

'Grazia' were examined. Naturally infested sandy loam soil originating from a glasshouse in Sint-Katelijne-Waver was homogenized by coning before filling two-liter pots (17 cm diameter, 16 cm height) with 1.6 l soil. Three soil samples were analyzed to determine the initial nematode population density. Similar to commercial practice, seeds of lamb's lettuce and wild rocket were directly seeded (10 seeds per pot), while for butterhead lettuce, parsley or chrysanthemum a 4-week-old plantlet in a peat block was added to each pot. Prior to this, butterhead lettuce and parsley had been sown in peat blocks, while cuttings of chrysanthemum had been used to establish planting material for the test. There were five replicates for each cultivar and the pots were arranged in a randomized block design. The plants were grown for 7 weeks at an average temperature of 23.2°C (min 15.6 and max 25.5°C) with 16 h light and 8 h dark. The pots were watered three times a week to ensure a humidity of about 23% vol. The initial and final nematode densities were analyzed as described above, with the difference that developmental stages of *Paratylenchus* spp. were counted separately. However, second and third-stage juveniles were not distinguished and no eggs were counted, as these are not distinguishable from other nematode eggs. The normality and homogeneity of variances were tested with the Shapiro-Wilk test and Modified Levene's test. Since the assumptions were not fulfilled, non parametric statistics were used to analyze the countings (Kruskal-Wallis and Wilcoxon tests at the 5% significance level).

3.3.4 Damage threshold of *Paratylenchus* spp. on butterhead lettuce

Three pot experiments with butterhead lettuce cv. 'Cosmopolia' were carried out to determine the damage threshold for the *Paratylenchus* sp. present in the glasshouses. A series of nematode densities was obtained by diluting a naturally infested soil, originating from Sin-Katelijne-Waver, with non-infested soil in a 1:1 ratio. The obtained mixtures were homogenized by coning three times. Three soil samples of each dilution were collected to verify the initial population densities. Ten densities were obtained for experiment 1 (3 to 19,250 *Paratylenchus* sp. (100 ml soil)$^{-1}$) and 9 for experiments 2 and 3 (0 to 11,591 and 0 to 34,951 *Paratylenchus* sp. (100 ml soil)$^{-1}$, respectively). The whole range of densities and the air temperature for each of the three experiments can be found in Supplementary data (Table S3.1 and Figure S3.1). The nitrogen levels (NO_3-N and NH_4-N) in the infested and non-infested soils were determined. Differences between the soils were corrected with Floranid Permanent (N-P-K (MgO)) fertilizer (16-7-15 (+2)) in each pot at planting. Pots of 17 cm diam. and 16 cm height were filled with 1.6 l soil. Lettuce seedlings in peat blocks (1 seed per block of 5 cm - 5 cm - 5 cm), provided by the Research station for vegetable production in Sint-Katelijne-Waver (PSKW), were transferred to the pots when plants reached the 4 to 6 leaf stage. The peat blocks with seedlings were planted in a 2-cm deep hole. This took place in spring (April 2016 and May 2017) and winter (December 2017) for experiments 1, 2 and 3, respectively. Eight replications were used per nematode density in the first and second experiment, while 12 replications for the third experiment.

Pots were organized in the glasshouse according to a randomized block design. Plants were watered as needed and no pesticides were applied. The first and second experiment were carried out at PSKW, where pots were kept in a commercial glasshouse setting. Hence, the temperature fluctuated. The third experiment was carried out in a glasshouse at ILVO, Merelbeke, under controlled conditions simulating summer (20°C day and 16°C night). Plants were harvested 42, 50 and 58 days after planting in experiments 1, 2 and 3, respectively.

Nematode damage was evaluated as described in Chapter 2. The final nematode population was determined for the mineral and organic fraction together in each pot. The soil from the pot was mixed and a 200-ml subsample was processed as described above using the automated zonal centrifuge. Adults and juveniles of *Paratylenchus* spp. were counted in the total nematode suspension, except in cases of high numbers when a subsample of at least 200 nematodes was counted.

3.3.4.1 Statistical analysis and modelling

A similar procedure was followed as described in Chapter 2.

3.4 Results

3.4.1 Morphological and molecular characterisation

Morphological and molecular analysis revealed that the populations of the five different glasshouses belong to the same *Paratylenchus* species. Female specimens had four lateral lines, a round spermatheca, distinct vulval flaps, a pronounced annulation on the dorsal side of the tail and a round to blunt tail. The morphometric characters of female *Paratylenchus* sp. are presented in Table 3.3. Males were rarely observed and had no stylet.

Table 3.3 Morphometric measurements of five different *Paratylenchus* populations (T1-T5) based on 10 females per population. All lengths are in µm, data are presented as mean ± standard deviation (range).

Character	T1	T2	T3	T4	T5
Body length L	365±40 (308-465)	335±20 (302-360)	365±39 (313-422)	358±43 (300-411)	328±31 (293-368)
a	24.2±3.8 (14.9-27.6)	24.3±3.4 (19.3-27.2)	26.7±2.3 (22.0-29.0)	23.7±2.6 (18.5-27.5)	23.2±3.3 (18.1-28.1)
b	3.7±0.7 (2.7-4.6)	4.1±1.5 (3.1-4.1)	3.4±0.7 (2.5-4.9)	3.2±0.5 (2.8-4.2)	2.8±0.5 (2.3-2.7)
c	15.0±1.5 (12.3-17.2)	14.9±1.5 (13.2-17.0)	14.9±1.9 (12.7-17.8)	14.8±2.3 (13.7-19.8)	13.0±1.5 (10.1-15.7)
V (%)	83.2±2.1 (80.4-87.8)	83.2±2.1 (80.0-87.0)	83.0±1.5 (80.0-84.0)	83.5±0.9 (82.8-84.9)	83.1±2.1 (80.1-88.0)
Stylet length	27.3±1.3 (23.5-28.4)	25.5±1.6 (22.3-26.5)	26.6±1.5 (25.2-30.5)	26.8±1.3 (24.6-27.9)	27.0±1.5 (24.6-28.6)
s/L (%)	7.5±0.9 (6.0-8.8)	7.6±0.7 (7.2-8.8)	7.3±0.7 (6.2-7.9)	7.6±0.8 (6.6-8.4)	8.3±0.5 (7.3-8.9)
Pharynx length	100.7±19.7 (75.2-137.7)	88.0±23.3 (42.9-105.8)	109.9±16.9 (83.3-123.5)	114.7±18.4 (84.6-125.7)	120.4±14.6 (95.0-144.0)
Tail length	24.4±3.1 (21.7-30.8)	22.6±1.6 (20.3-26.2)	24.6±1.8 (21.0-26.1)	24.5±3.3 (21.2-32.7)	25.4±2.6 (22.0-30.0)

a = body length/maximum body diameter, b = body length/distance from anterior to esophago-intestinal valve, c = body length/tail length, V = percentage distance of vulva from anterior and s/L = stylet length/ body length expressed in percentage

The obtained sequences of the *ITS* rDNA and the *D2-D3 of the 28S* rDNA regions of one nematode of each of the five populations were 707-735 bp and 1003-1022 bp long, respectively. The *ITS* rDNA alignment included 40 sequences and 2 outgroup taxa and was 1123 bp in length. The *D2-D3* alignment included 54 sequences and 3 outgroup taxa and was 600 bp in length. Pairwise comparison of the five sequences of the *ITS* rDNA region revealed two 100% identical groups differing only in one nucleotide, *i.e.* T1 and T4 *vs.* T2, T3 and T5. The *D2-D3 of 28S* rDNA was 100% identical for all sequences. The molecular phylogenetic analyses based on the *D2-D3 of the 28S* rDNA and the *ITS* rDNA indicated *Paratylenchus* sp. as a well-supported clade clearly different from all other species (Figures 3.1 and 3.2). In the ITS-based tree, *Paratylenchus* sp. is sister to a poorly supported clade (bootstrap value of 26%) of *P. nanus* type A, *P. dianthus*, *Paratylenchus* sp. 3, *P. lepidus* and *P. minutus* and the sequences were most similar to *P. nanus* (accession numbers MH236098 and KY468907, 90%). In the *D2-D3 of the 28S* rDNA tree, *Paratylenchus* sp. is sister to a poorly supported clade (bootstrap value of 44%) of *P. hamatus* and *Paratylenchus* sp. 2 and the sequences were most similar to *P. tenuicaudatus* (accession number KU291239, 95%) and *P. nanus* (accession number MH237651, 93%).

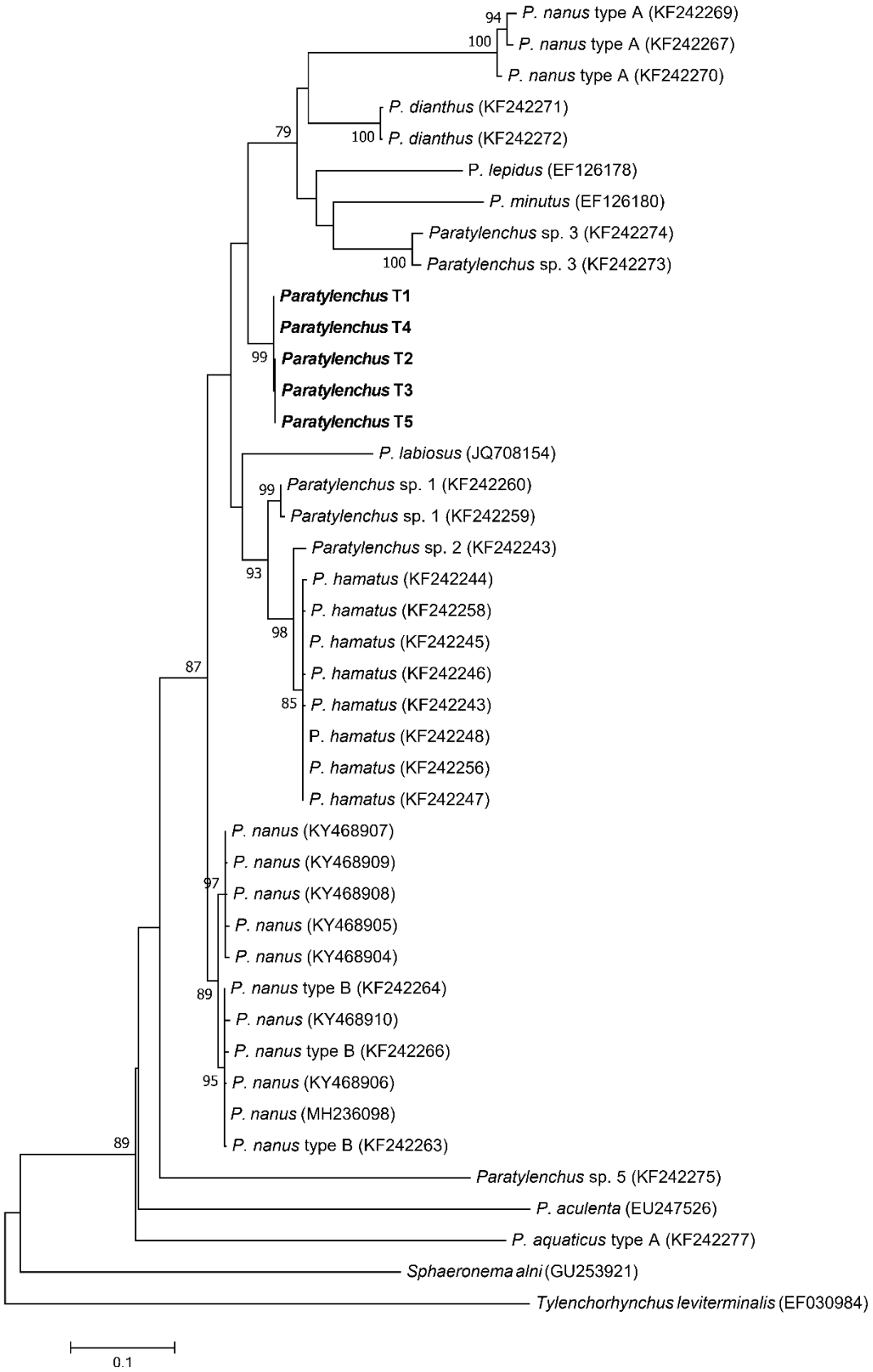

Figure 3.1 Maximum likelihood phylogenetic tree based on the *ITS* rDNA region. Bootstraps indicated on the branched nodes are only given for those branches with a value higher than 70. Belgian *Paratylenchus* sp. are indicated in bold.

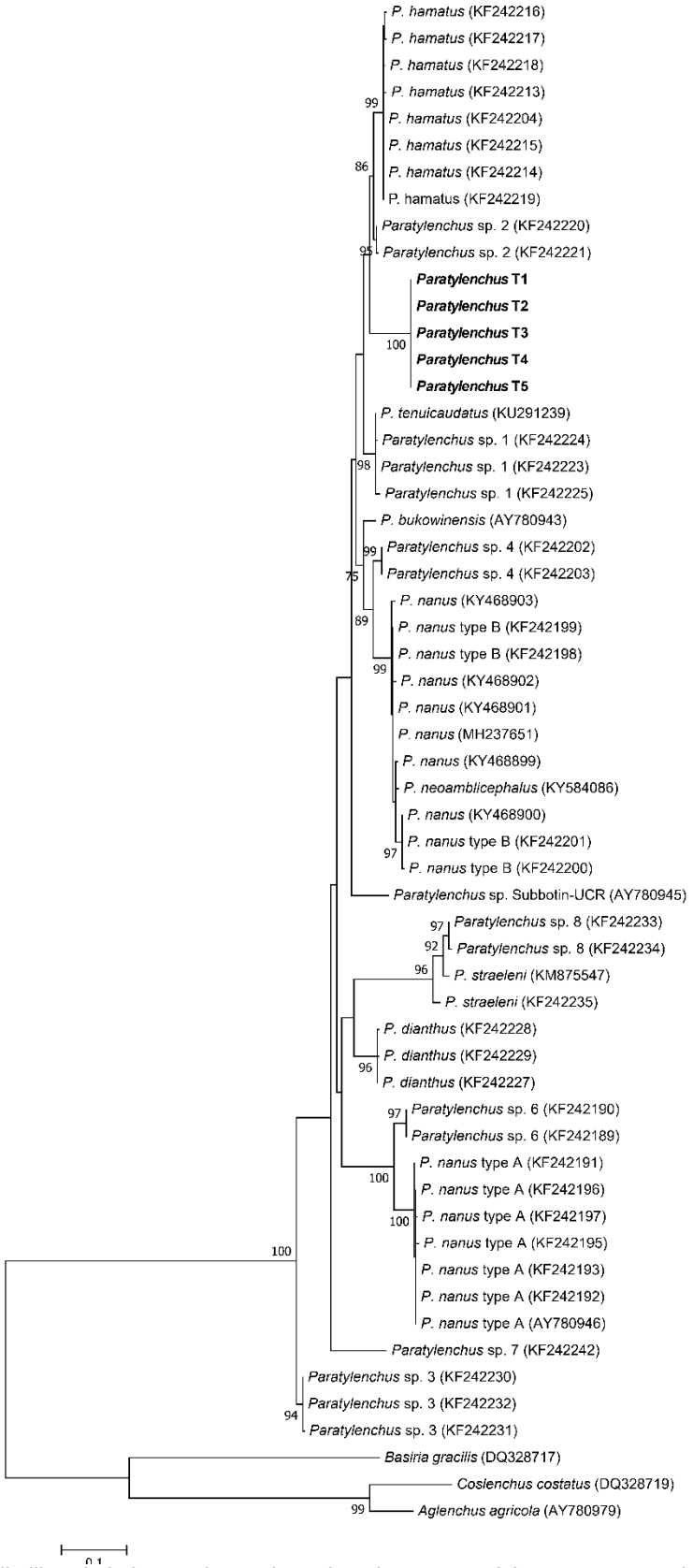

Figure 3.2 Maximum likelihood phylogenetic tree based on the *D2-D3 of the 28S* rDNA region. Bootstraps indicated on the branched nodes are only given for those branches with a value higher than 70. Belgian *Paratylenchus* sp. are indicated in bold.

3.4.2 Spatial and temporal dynamics of *Paratylenchus* sp. in glasshouse lettuce and lamb's lettuce

Populations of *Paratylenchus* sp. and non-plant-parasitic nematodes were monitored in the 0-30 cm and 30-60 cm soil layer in five glasshouses from 2015 until 2017. The periods of lettuce cropping, fallow and soil disinfestation are depicted for every glasshouse in Figure 3.3, together with the cultivars of butterhead and lamb's lettuce planted. The air temperature in glasshouse 1 and 2 is shown in Supplementary data (Figure S3.2). Very high densities of *Paratylenchus* sp. were observed frequently, with a maximum of 23,701 and 19,848 nematodes (100 ml soil)$^{-1}$ in the higher and lower soil layer, respectively (Figure 3.4). These high numbers were mainly observed in glasshouses 2 and 3 during winter and spring. There was a significant effect of the factors glasshouse and astronomical season on the nematode counts, and this for both soil layers. In addition, there was an interaction between glasshouse and season in the lower soil layer (Table 3.4). The numbers of *Paratylenchus* sp. in the top and lower soil layers were strongly correlated (r_s = 0.78, P < 0.05). This was also the case for non-plant-parasitic nematodes (r_s = 0.70, P < 0.05). The numbers of *Paratylenchus* sp. and non-plant-parasitic nematodes were correlated at 30-60 cm depth (r_s = 0.36, P < 0.05), while this was not the case at 0-30 cm depth (r_s = 0.15, P > 0.05).

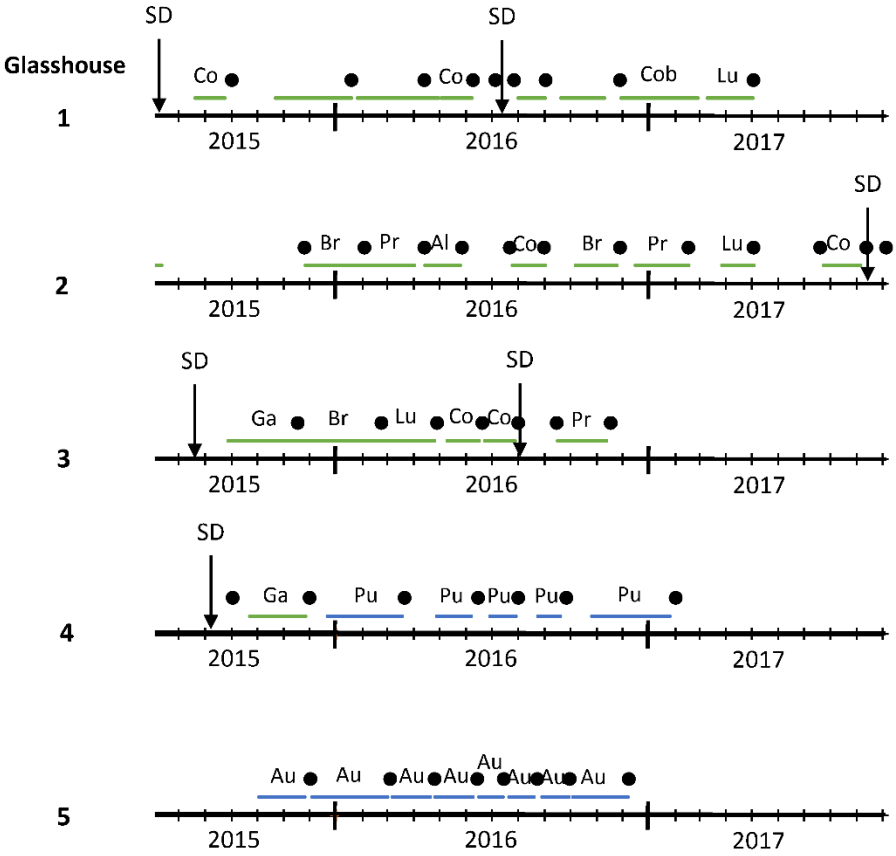

Figure 3.3 Timeline of the crops grown in the five commercial glasshouses starting from June 2015. The black dots represent the sampling time points. The green line represents the cultivation of lettuce with different cultivars (Co = 'Cosmopolia', Cob = 'Coby', Lu = 'Lucrecia', Br = 'Brighton', Al = 'Alexandria', Pr = 'Presteria' and Ga = 'Gardia'). The blue line represents the cultivation of lamb's lettuce with different cultivars (Pu = 'Pulsar' and Au = 'Audace'). SD = soil disinfestation.

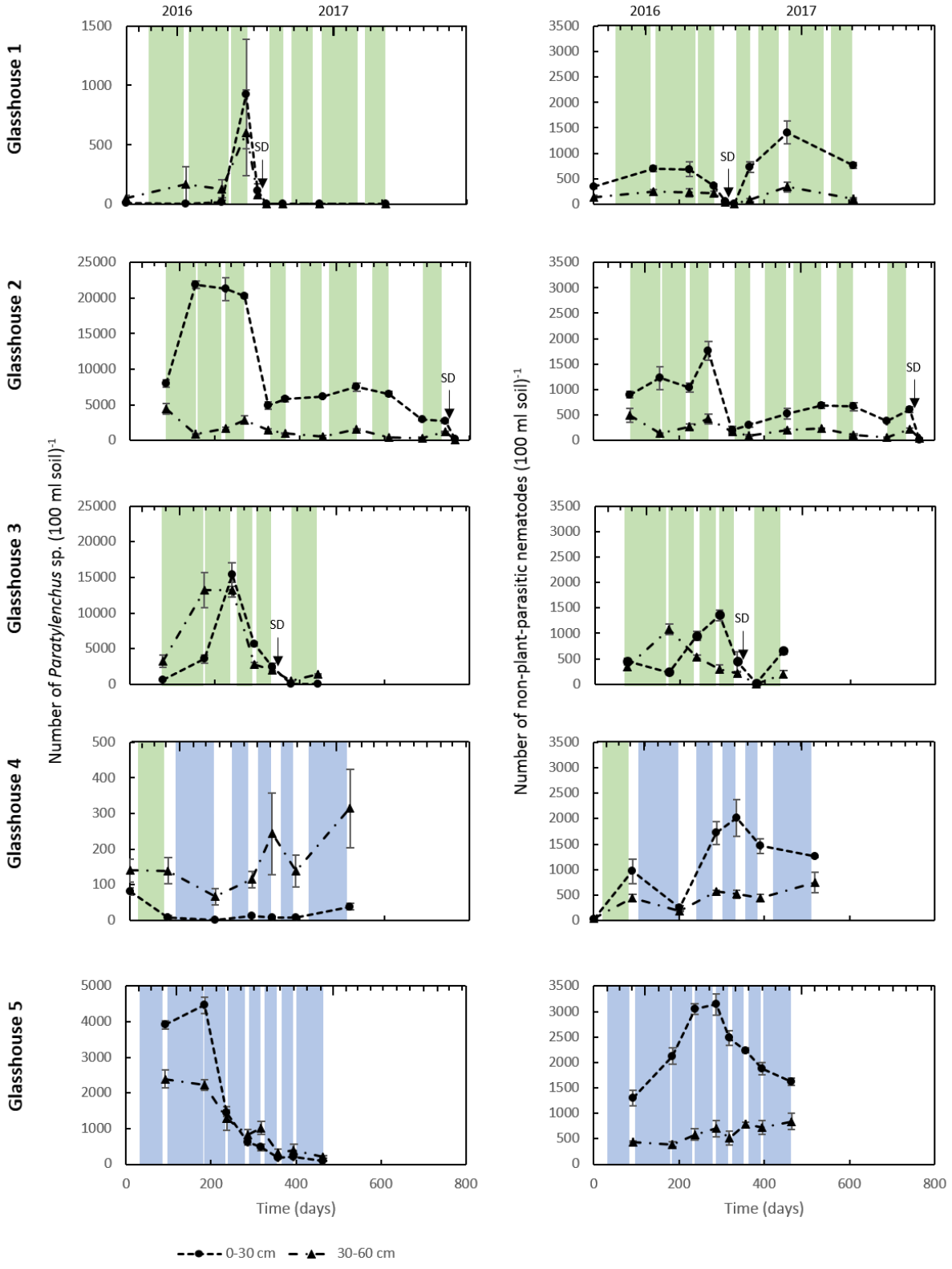

Figure 3.4 Number of *Paratylenchus* sp. (left) and non-plant-parasitic nematodes (right) (100 ml soil)$^{-1}$ in the 0-30 cm and 30-60 cm soil layer in five glasshouses. Day 1 is September 1, 2015. Growing lettuce and lamb's lettuce is represented by the colors green and blue, respectively, white stripes indicate fallow. SD = soil disinfestation. Error bars represent the standard error (n = 4).

Table 3.4 Two-way ANOVA of the numbers of *Paratylenchus* sp. in the 0-30 and 30-60 cm soil layer.

Factor	F	Df	Df residuals	*P*-value
0-30 cm layer				
Glasshouse	10.2522	4	23	$6.5 .10^{-5}$ *
Season	5.4971	3	23	0.005 *
Glasshouse:Season	2.1796	12	23	0.052
30-60 cm layer				
Glasshouse	8.5177	4	23	0.0002 *
Season	6.3559	3	23	0.002 *
Glasshouse:Season	2.4497	12	23	0.031 *

In glasshouses 1, 3 and 4, chemical soil disinfestation was applied in the summer of 2015. This resulted in low nematode numbers in the 0-30 cm soil layer at the start of the study: only 12, 627 and 81 *Paratylenchus* sp. (100 ml soil)$^{-1}$ were counted. Similarly, summer applications of 1,3-dichloropropene in glasshouse 3 in 2016, and of metam sodium in 2017 in glasshouse 2, resulted in very low nematode densities. For example, a 100% and 76% decrease of *Paratylenchus* sp. in the 0-30 cm and 30-60 cm soil layer, and 96 and 95% reduction in non-plant-parasitic nematodes were noted in glasshouse 3. The numbers of non-plant-parasitic nematodes recovered faster than numbers of *Paratylenchus* sp. in both glasshouses 2 and 3.

In glasshouse 1, soil steaming with negative pressure was applied in summer 2016. Soil movement for installing the drainage pipes at 65-70 cm depth every 2.7 m resulted in a 88% decrease of the nematode population in both layers. Subsequent steaming during 9 h resulted in a temperature of 70°C during 7 h at 40-45 cm depth. The whole operation killed all *Paratylenchus* sp., as well as most non-plant-parasitic nematodes, in the 0-60 cm soil layer. The non-plant-parasitic nematodes recovered rather quickly, while still no *Paratylenchus* sp. were observed during the next 9 months.

Next to soil disinfestation, black fallow resulted in a considerable population decrease of *Paratylenchus* sp. in glasshouse 2. Two to three months of black fallow (a bare soil without weeds or plants), from May until June, decreased the population by 76 and 50% in 2016, and by 55 and 15% in 2017, in the higher and lower soil layer, respectively. The same was true for non-plant-parasitic nematodes; this population decreased by 88 and 59% in 2016, and by 44 and 42% in 2017, in the higher and lower soil layer, respectively.

In general, low numbers of *Paratylenchus* sp. (< 2,000 nematodes (100 ml soil)$^{-1}$) were observed when lamb's lettuce was grown (Figure 3.4). High densities were only counted at the beginning of the monitoring in glasshouse 5: up to 4,462 *Paratylenchus* sp. (100 ml soil)$^{-1}$ in the upper layer and 2,391 in the lower layer. There was no clear effect of lamb's lettuce on non-plant-parasitic nematodes.

Growing butterhead lettuce cv. 'Brighton' from November 2015 until February 2016 resulted in a huge increase in *Paratylenchus* numbers in glasshouses 2 and 3. In the 0-30 cm layer, the population increased from 7,994 to 21,815 and from 627 to 3,568 *Paratylenchus* sp. (100 ml soil)$^{-1}$, while in the 30-60 cm the population decreased from 4,425 to 919 *Paratylenchus* sp. (100 ml soil)$^{-1}$ in glasshouse 2, but increased from 3,237 to 13,230 *Paratylenchus* sp. (100 ml soil)$^{-1}$ in glasshouse 3. However, when

cv. 'Brighton' was grown again in the fall of 2016 in glasshouse 2, the population density did not change much (5,792 to 6,176 in the 0-30 cm layer, and 957 to 568 in the 30-60 cm layer). In glasshouse 3, a large population increase was observed (from 3,568 to 15,391 *Paratylenchus* sp. (100 ml soil)$^{-1}$ in the upper soil layer), after growing butterhead lettuce cv. 'Lucrecia' in spring 2016 (February until April). However, the high numbers of *Paratylenchus* sp. in both layers in April 2016 (>13,000 *Paratylenchus* sp. (100 ml soil)$^{-1}$) were much lower at the next sampling event in June 2016 after growing butterhead lettuce cv. 'Cosmopolia'. Between the two samplings, heavy rainfall had caused inundations in the area and a risen ground water table in the glasshouse.

3.4.3 Host status

Nine different crops and cultivars were grown in soil naturally infested with *Paratylenchus* sp. (5,542 ± 71 nematodes (100 ml soil)$^{-1}$) during seven weeks (Table 3.5). The final population was the highest after lettuce, although the difference with the final populations on other crops was not significant for two cultivars, *viz*. butterhead lettuce cv. 'Cosmopolia' and lollo rossa cv. 'Tuska'. All lettuce cultivars were shown to be a host for *Paratylenchus* sp. as their reproduction factors (R) varied between 1.01 and 2.26. Lamb's lettuce cultivars, parsley and wild rocket were poor hosts, reducing the initial populations *Paratylenchus* sp. by about 50% (R-values 0.45 to 0.56). Chrysanthemum hardly maintained the nematode population (R = 0.90) (Table 3.5).

Table 3.5 Final population density of *Paratylenchus* sp. in soil after growing different leafy vegetables and chrysanthemum. The reproduction factor is calculated by dividing the final by the initial population (5,542 ± 71 nematodes (100 ml soil)$^{-1}$). Means ± the standard error in the same column followed by the same letter are not significantly different (Wilcoxon rank sum test, P = 0.05).

Crop/Type	Cultivar	Final population (number of *Paratylenchus* sp. (100 ml soil)$^{-1}$)			Reproduction facor (R)		
Lettuce							
Butterhead	Cosmopolia	5,609	± 1,581	ab	1.01	± 0.29	ab
	Brighton	12,537	± 2,448	a	2.26	± 0.44	a
Lollo rossa	Tuska	5,897	± 1,885	ab	1.07	± 0.34	ab
Oakleaf	Prunaï	10,178	± 192	a	1.84	± 0.03	a
Lamb's lettuce							
	Audace	2,471	± 162	b	0.45	± 0.03	b
	Pulsar	2,708	± 111	b	0.49	± 0.02	b
Other plants							
Parsley	Frise vert Foncerina	3,121	± 94	b	0.56	± 0.02	b
Chrysanthemum	Medonia	5,008	± 1,394	b	0.90	± 0.25	b
Wild rocket	Grazia	2,718	± 298	b	0.49	± 0.05	b

The initial population of *Paratylenchus* sp. consisted mainly of juveniles (89%), comprising 51% fourth-stage juveniles and 38% second- and third-stage juveniles. Only 10% were females and 1% were males. The final population density was dominated by fourth-stage juveniles for all crops (80-96%) (Figure 3.5). The largest proportions of females and males were found after growing chrysanthemum (5% females, 0.7% males) and wild rocket (7% females, 1.3% males). The highest proportions of second- and third-stage juveniles were observed when chrysanthemum, wild rocket and lettuce were grown (7-14%).

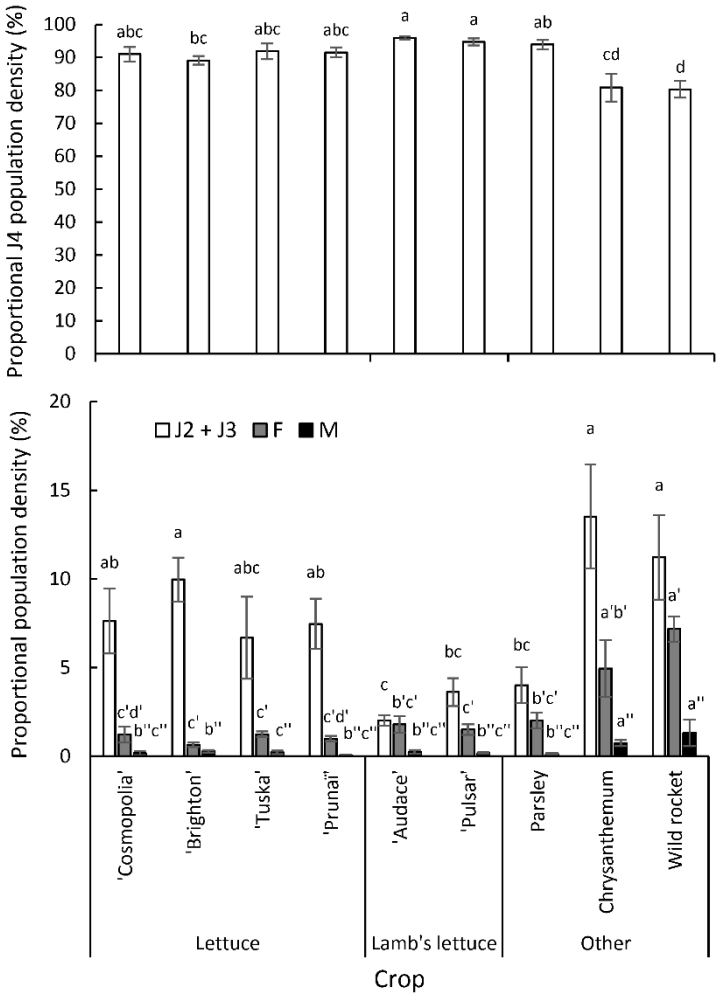

Figure 3.5 Composition of the *Paratylenchus* sp. population according to its developmental stages (excluding eggs) after growing different crops for 7 weeks in infested soil. J2 = second-stage juvenile, J3 = third-stage juvenile, J4 = fourth-stage juvenile, F = female, M = male. Same letters are not statistically different from each other (Wilcoxon rank sum test, $P < 0.05$). Error bars represent the standard error (n = 5).

3.4.4 Damage threshold for *Paratylenchus* sp. on butterhead lettuce

Three different pot experiments were set up to estimate the damage threshold density. The average temperatures were 18.1, 21.6 and 20.5°C and ranged between 10.2 and 26.4°C, 13.2 and 30.0°C, and 17.5 and 25.5°C for experiment 1, 2 and 3, respectively. There was a considerable difference in nutrient status (0.192 g N per pot) between the infested and non-infested soil in experiment 3. Therefore, a fertilizer (1 g of Floranid permanent, 16% N) was added to the pots with high densities of *Paratylenchus* sp. to make up for the shortage in nitrogen and lower densities of *Paratylenchus* sp. received a serial dilution of the fertilizer. Unfortunately, this resulted in unreliable parameters for the above-ground lettuce crop (head weight, head development and head color), so only data of experiment 1 and 2 are shown for these measurements.

3.4.4.1 Lettuce quality

The lettuce weight ranged from 103 to 220 g and from 71 to 175 g for experiments 1 and 2, respectively, but no differences in lettuce head weights were observed between the population densities (Figure 3.6).

An inverse relation between lettuce head development and initial population density was observed for experiment 1 only (Figure 3.7). The Seinhorst equation fitted well to the data in experiment 1 ($R^2 = 0.82$), but not in experiment 2 ($R^2 = 0.1$) (Figure 3.7, Table 3.6). A tolerance limit (T_D) of 279 *Paratylenchus* sp. (100 ml soil)$^{-1}$, a relative minimum for head development (m_D) of 0.6 and a maximum score ($Y_{max(D)}$) of 4.7 were calculated for experiment 1 (Table 3.6).

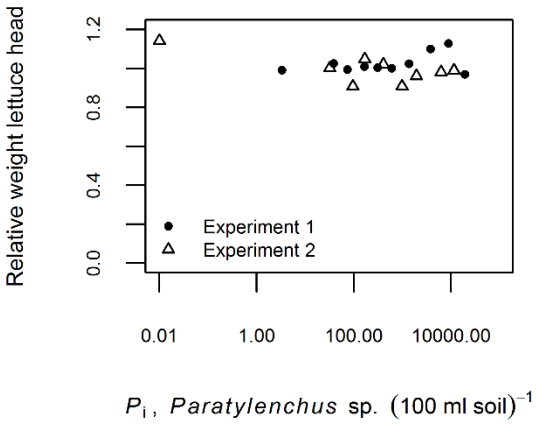

Figure 3.6 Relative weight of lettuce heads at different initial population densities (P_i) of *Paratylenchus* sp. in experiments 1 and 2.

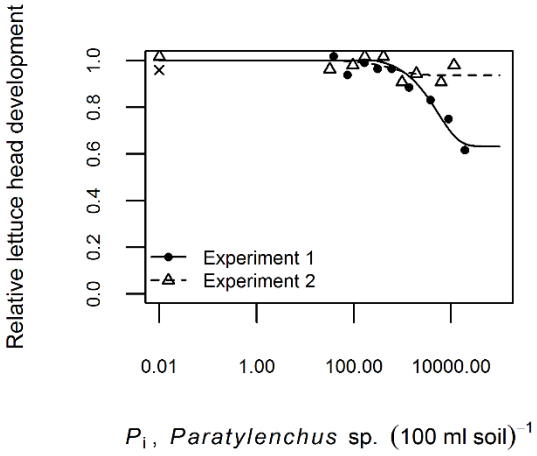

Figure 3.7 The relation between the initial population density (P_i) of *Paratylenchus* sp. and the relative lettuce head development for experiment 1 and 2. A line was fitted according to the Seinhorst equation: $y = m+(1-m)0.95^{Pi/T-1}$ for $P_i > T_y$ and $y = 1$ for $P_i \leq T_y$.

Furthermore, root weight and quality were measured as they affect head weight and development. The root weight ranged from 5.7 to 25.8 g, 23 to 57.0 g and 3.6 to 23.1 g for experiments 1, 2 and 3, respectively. The lettuce roots were strongly damaged due to the presence of *Paratylenchus* sp. (Figure 3.8). Hence, the root weight and root quality decreased with increasing nematode densities (Figure 3.9). The relation between the root weight and initial population density was very well described with the Seinhorst model for experiments 1 and 3 (R^2 was 0.74 and 0.93, respectively, Table 3.6). The tolerance limits (T_{RW}) were 2,000 and 1,754 *Paratylenchus* sp. (100 ml soil)$^{-1}$, the relative minimum root weights

(m_{RW}) were 0.10 and 0.00 and the maximum root weights ($Y_{max(RW)}$) were 14.60 and 14.73 for experiments 1 and 3, respectively. The values for the tolerance limit and maximum root weight for experiment 2 were much higher (T_{RW} = 6,334 *Paratylenchus* sp. (100 ml soil)$^{-1}$, m_{RW} = 38.01 g) as root weight was not affected, even at the highest density of *Paratylenchus* sp. (Figure 3.9, Table 3.6). No statistical differences were found between the tolerance limits and relative minimum root weights of the three experiments.

The quality of the roots was already affected at low nematode densities. The calculated tolerance limits (T_Q) were 438, 362 and 1,308 *Paratylenchus* sp. (100 ml soil)$^{-1}$ and the relative minimum scores for the root quality (m_Q) were 0.5, 0.7 and 0.2 for experiments 1, 2 and 3, respectively. No statistical differences were found between the experiments for these two parameters. Furthermore, the maximum root quality scores ($Y_{max(Q)}$) were 7.8, 8.2 and 8.0 for experiments 1, 2 and 3, respectively.

Table 3.6 Parameter values for the Seinhorst equations describing the relation between initial population densities (P_i) of *Paratylenchus* sp. and measurements of lettuce. P_i and the tolerance limit (T) are expressed in *Paratylenchus* sp. (100 ml soil)$^{-1}$, while yield (y) and relative minimum yield (m) are proportions and the maximum yield (Ymax) is expressed in gram for RW (root weight) or without unit (scores 1-9) for D (lettuce head development) and Q (root quality). Parameter values of experiments 2 and 3 were compared with those of experiment 1 using the LSD-test (P = 0.05). SE = standard error, df = degrees of freedom.

Lettuce head development										
According to the equation: $y_D = m_D+(1-m_D)\ 0.95^{P_i/T_D{}^{-1}}$ for $P_i>T_D$ and $y_D = 1$ for $P_i \leq T_D$										
Experiment	T_D	m_D	$Y_{max\ (D)}$	$SE_{T\ (D)}$	$SE_{m\ (D)}$	$SE_{Ymax\ (D)}$	R^2	df	$LSD_{T\ (D)}$	$LSD_{m\ (D)}$
1	279	0.6	4.7	212	0.10	0.15	0.82	8	-	-
2	38	0.9	6.9	46	0.04	0.18	0.19	7	462	0.22 *
3	-	-	-	-	-	-	-	-		
Root weight (g)										
According to the equation: $y_{RW} = m_{RW}+(1-m_{RW})\ 0.95^{P_i/T_{RW}{}^{-1}}$ for $P_i>T_{RW}$ and $y_{RW} = 1$ for $P_i \leq T_{RW}$										
Experiment	T_{RW}	m_{RW}	$Y_{max\ (RW)}$	$SE_{T\ (RW)}$	$SE_{m\ (RW)}$	$SE_{Ymax\ (RW)}$	R^2	df	$LSD_{T\ (RW)}$	$LSD_{m\ (RW)}$
1	2000	0.10	14.60	2133	0.94	0.40	0.74	8	-	-
2	6334	0.00	38.01	265311	215.87	1.40	0.11	7	565469	460
3	1754	0.00	14.73	1228	0.50	0.58	0.93	7	5246	2
Root quality										
According to the equation: $y_Q = m_Q+(1-m_Q)\ 0.95^{P_i/T_Q{}^{-1}}$ for $P_i>T_Q$ and $y_Q = 1$ for $P_i \leq T_Q$										
Experiment	T_Q	m_Q	$Y_{max\ (Q)}$	$SE_{T\ (Q)}$	$SE_{m\ (Q)}$	$SE_{Ymax\ (Q)}$	R^2	df	$LSD_{T\ (Q)}$	$LSD_{m\ (Q)}$
1	438	0.5	7.8	228	0.12	0.19	0.91	8	-	-
2	362	0.7	8.2	385	0.14	0.17	0.80	7	954	0.39
3	1308	0.2	8.0	683	0.25	0.29	0.94	7	1536	0.60

* Significantly different at p = 0.05

Figure 3.8 Lettuce roots grown in pots with increasing densities of *Paratylenchus* sp. (left to right: 3 to 19,250 *Paratylenchus* sp. (100 ml soil)$^{-1}$) (Experiment 1).

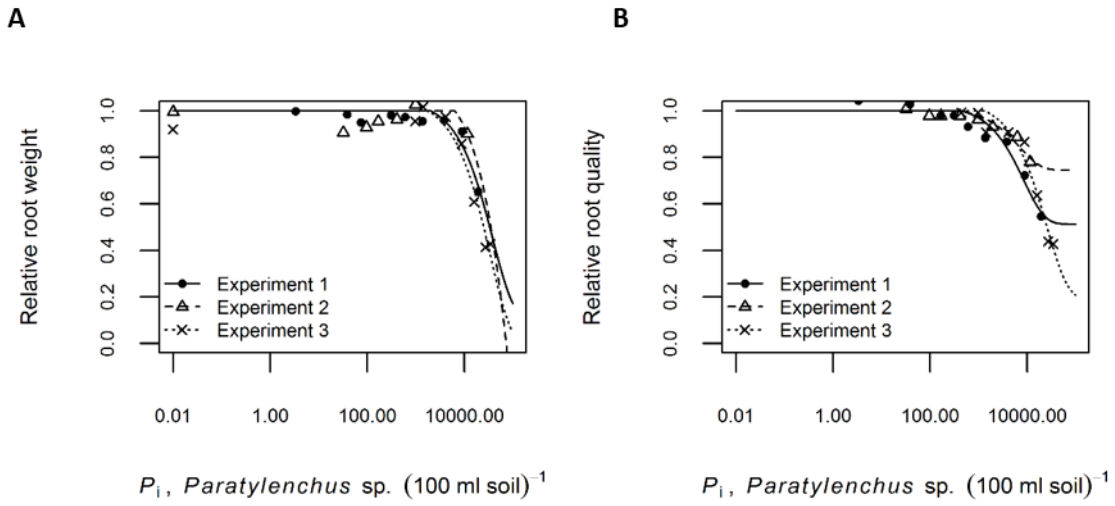

Figure 3.9 The relation between the initial population density (P_i) of *Paratylenchus* sp. and the relative root weight (**A)**, and relative root quality (**B)** in experiments 1, 2 and 3. A line was fitted according to the Seinhorst equation: $y = m+(1-m)0.95^{Pi/T-1}$ for $P_i > T_y$ and $y = 1$ for $P_i \leq T_y$.

3.4.4.2 Population dynamics in damage threshold experiment

The relation between P_i and P_f was very well described by the model for population dynamics (Eq. 2) in the three experiments (R^2 = 0.94, 0.99 and 0.95) (Table 3.7). Although the same cultivar 'Cosmopolia' was used in each experiment, the maximum multiplication rate (a) of *Paratylenchus* sp. differed considerably between experiments (0.31, 0.74 and 0.14 for experiments 1, 2 and 3, respectively). The low values of a (< 1) indicate that the nematode population was not maintained by this cultivar; indeed the population decreased at every P_i (Figure 3.10). Furthermore, the maximum population (M) of each experiment was 12,208, 6,474 and 4,214 *Paratylenchus* sp. (100 ml soil)$^{-1}$, but did not significantly differ.

Table 3.7 Parameter values of the population dynamics model for the relation between initial (P_i) and final population density (P_f) of *Paratylenchus* sp. (100 ml soil)$^{-1}$ for experiments 1, 2 and 3, according to the model $P_f = M*Pi/(Pi+M/a)$. Multiplication rate (a) is dimensionless while maximum population (M) is measured as *Paratylenchus* sp. (100 ml soil)$^{-1}$. Parameter values of experiments 2 and 3 were compared with those of experiment 1 using the LSD-test (P = 0.05). SE = standard error, df = degrees of freedom.

Parameter values								
Experiment	M	a	SE_M	SE_a	R^2	df	LSD_M	LSD_a
1	12208	0.31	7361	0.07	0.94	7	-	-
2	6474	0.74	1724	0.06	0.99	6	1.33	0.48 *
3	4214	0.14	1484	0.02	0.95	6	1.41	0.56 *

* Significantly different at P = 0.05

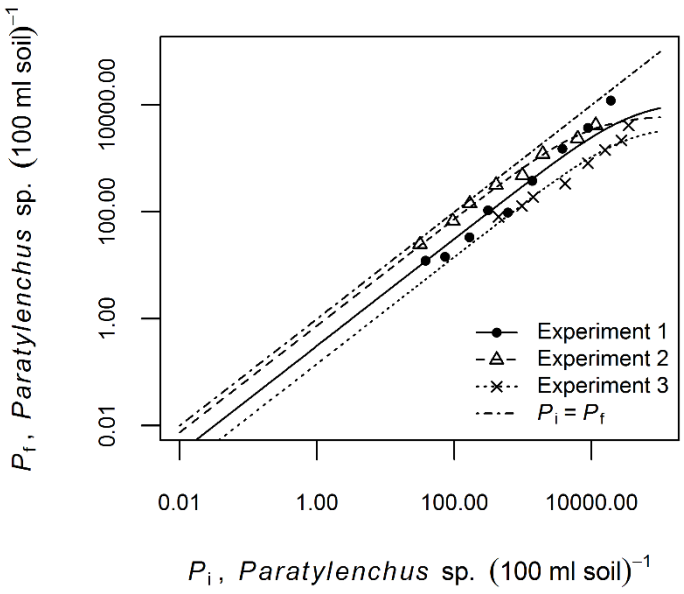

Figure 3.10 The relation between the initial (P_i) and final (P_f) population densities of *Paratylenchus* sp. for experiments 1, 2 and 3. A line was fitted according to the equation: $P_f = M*P_i/(P_i+M/a)$ for population dynamics. The diagonal line represents the population equilibrium line ($P_f = P_i$).

3.5 Discussion

Paratylenchus sp. was observed in 47% of the commercial Belgian glasshouses studied in 2014 (unpublished) and the current study confirmed the importance of this species. Both molecular analyses (*ITS* rDNA and the *D2-D3 of the 28S* rDNA region) and morphological analyses demonstrated that this species is different to all hitherto described species (Andrássy, 2007; Brzeskii, 1998; Ghaderi *et al.*, 2014, 2016; Van den Berg *et al.*, 2014).

The densities in the soil of five populations of *Paratylenchus* sp. were monitored for at least one year to gain insights into the factors influencing the population dynamics in each corresponding glasshouse. In samples gathered over that period, the numbers varied widely, ranging from 0 to 23,701 *Paratylenchus* sp. (100 ml soil)$^{-1}$. High numbers of pin nematodes were also observed in pea fields in North Dakota where densities of 7,114 (200 g soil)$^{-1}$ (Upadhaya *et al.*, 2019) were recorded, though this is less than what we observed in lettuce in the glasshouse. In general, we observed a seasonal variation in numbers of pin nematodes with maximum population densities in winter and spring. This is in contrast not only with the study by Bell & Watson (2001a), which revealed maximum population densities of *P. nanus* in grass during spring and summer, but also with that of Verschoor *et al.* (2001), who found maximum population densities to occur in summer and autumn, also in grass. Cropping lettuce differs with pasture in terms of food availability. The long growing periods of lettuce in winter and spring provide a sustained presence of actively growing roots without disturbance of the soil due to tillaging; these conditions encourage nematode population build-up. In addition, specific lettuce cultivars are used for every

season. The winter cultivar 'Brighton' was found to increase the nematode population, except for the cropping in fall in 2016 in glasshouse 2. This observation is due to an artefact because the sampling "prior to planting" was followed by a month of black fallow and not immediately by planting. This fallow period most probably caused a decline in pin nematodes which explains the minor changes on 'Brighton' for this lettuce planting. The host status experiment, carried out in summer conditions, showed again the highest reproduction on cultivar 'Brighton' (R = 2.26), compared with the summer cultivar 'Cosmopolia' (R = 1.01). Differences in nematode reproduction on different lettuce cultivars have already been shown for the root-lesion nematode *Pratylenchus penetrans* (Moretti *et al.*, 1981). The good host status of cultivar 'Brighton', together with the longer undisturbed growing conditions for lettuce roots , could explain the high *Paratylenchus* sp. population densities in winter.

Several management strategies are known to decrease nematode populations (Pudasaini *et al.*, 2006; Viaene *et al.*, 2006; Haydock *et al.*, 2006). In the current study, black fallow, chemical soil disinfestation and soil steaming resulted in a considerable reduction of the *Paratylenchus* sp. population. Reductions in pin nematode populations of up to 76% and 50% were recorded in the higher and lower soil layer, respectively, after applying fallow from May to July (61-82 days), while chemical soil disinfestation killed 100% and 90% of the populations at 0-30 cm and 30-60 cm depth, respectively. A proportion of the nematodes was always surviving in the lower layer. These remaining nematodes can pose a risk to the subsequent crops as they can migrate to the upper soil layers with new lettuce plantings. Nematodes can in fact move towards roots and are transported with water or soil movements. For soil steaming, a temperature of 70°C for 30 min is recommended to kill plant-parasitic nematodes, fungal and bacterial plant pathogens and soil insects, slugs, worms and centipedes (Runia & Molendijk, 2010). Indeed, steaming under negative pressure at 70°C for 7 h at 40-45 cm depth was the only procedure after which no pin nematodes were found at the following sampling, and this in both layers. Application of such high temperature during a sufficient amount of time and in pipes at 45 cm depth, explains the effective eradication of the pin nematodes in this case. For a glasshouse of 5000 m², the installation of drain pipes costs 3 euro/m², renting the steam boiler, pipes, sheets and chains costs around 0.45 euro/m². More or less 2 liter gasoil is needed for one square meter, which costs around 0.60 euro/m² (Vandevelde, personal communication).

Boag & Alphey (1988) observed that *P. nanus* populations increased rapidly a year after nematicide application of 1,3-dichloropropene in a tree nursery field. They explained this rapid increase by the absence of competition and fast reproduction. Our study did not support this statement for glasshouses 1 and 4, in which *Paratylenchus* sp. barely recovered after fumigation. However, in glasshouse 3, a high population increase was observed two crop cycles after the application of metam potassium, resulting in more than 3,500 and 13,000 pin nematodes (100 ml soil)$^{-1}$ in the higher and lower soil layers, respectively. This could be explained by reduced competition due to the soil disinfestation. The populations of non-plant-parasitic nematodes recovered after just one crop cycle.

Growing lamb's lettuce in glasshouses 4 and 5 kept *Paratylenchus* sp. densities low. The host status experiment confirmed the very poor reproduction of *Paratylenchus* sp. on lamb's lettuce (R = 0.45-0.49). The high densities of pin nematodes at the start of the study in glasshouse 5 could be explained by the

cropping of chrysanthemum (species unknown) in the spring before monitoring. Winfield (1985) reported that growing *Chrysanthemum multicaule* increased the population of *P. nanus* by more than 8 times. Our study showed that *C. morifolium* is a poor host for *Paratylenchus* sp. (R = 0.90).

The host status of different crops was investigated in a pot test that lasted 7 weeks at an average temperature of 23.2°C. As a life cycle takes 36-38 days at 18-20°C (Wood, 1973) or 30-31 days at 25-28°C (Rhoades & Linford, 1961b) nematodes could complete their life cycle in the host status experiment. The highest proportions of second- and third-stage juveniles were found in pots containing lettuce, chrysanthemum and wild rocket, while the lowest proportions were found alongside the lamb's lettuce and parsley. The presence of these two juvenile stages indicated that *Paratylenchus* sp. was still reproducing, albeit to a lesser extent on lamb's lettuce, parsley, chrysanthemum and wild rocket since the reproduction factor on these plants was less than 1. The final population densities revealed that lettuce is a good host for *Paratylenchus* sp. (R = 1.01-2.26), as it is for *P. nanus* (R = 2.1; Winfield, 1985). Lamb's lettuce (R = 0.45-0.49), parsley (R = 0.56) and wild rocket (R = 0.49) reduced the pin nematode population in our study; lettuce growers could rotate with these crops to lower the soil populations of pin nematodes found in the glasshouses we studied.

Nevertheless, the reproduction of *Paratylenchus* sp. on the lettuce cultivar 'Cosmopolia' in the damage threshold experiments was low (a = 0.31, 0.74 and 0.14) compared to that in the host status experiment (R = 1.01). As the growing period was similar (49 days for the host status experiment, and 42, 50 and 58 days for the damage threshold experiments), temperature most probably influenced the population development of *Paratylenchus* sp. Bell & Watson (2001b) reported that the population of *P. nanus* in pasture was positively correlated with temperature. The life cycle of *Paratylenchus* spp. is 36-38 days at 18-20°C (Wood, 1973) and 30-31 days at 25-28°C (Rhoades & Linford, 1961). Indeed, the host status experiment was conducted at an average temperature of 23.2°C and the damage threshold experiments with a = 0.74 at 21.6°C, while the other experiments were conducted at lower temperatures (18.1 and 20.5°C).

We used naturally-infested soil in our pot experiments, due to the difficulty in culturing these pin nematodes. We tried to culture this nematode on carrot discs, but reproduction was very low. Also, collecting ectoparasitic nematodes from cultures on plants is very difficult. In general, the final population densities were low in the pot experiments compared with the densities that can occur in the glasshouses. The overall proportion of fourth-stage juveniles increased from 51% at the beginning to 80-96% at the end of the host status experiment. This stage is non-feeding and can survive for prolonged periods before evolving further (Rhoades & Linford, 1961), which indicates that the pin nematodes had difficulties to establish in the pots following the disturbance of soil due to mixing.

Damage thresholds of nematodes on for lettuce were only calculated for *Meloidogyne hapla* and *Pratylenchus penetrans* (Viaene & Abawi, 1996; Claerbout *et al.*, in press) and hitherto never for *Paratylenchus* spp. Winfield (1985) observed poorly-grown lettuce plants at a final population density of 1,700 *P. nanus* (100 ml soil)$^{-1}$. In our study, no reduction in lettuce head weight was measured due to the nematode populations. The maximum weights were 220 and 175 g in experiments 1 and 2, respectively, while in commercial production the heads must reach a weight of 450 g to be economically

viable. It is likely that due to this lower weight, no discernible nematode damage was observed on the lettuce heads in this study. The highest density in experiment 2 was only 11,591 *Paratylenchus* sp. (100 ml soil)$^{-1}$, which resulted in poor model fitting for the Seinhorst equation, as no yield reduction was obtained. During the monitoring of the populations in the commercial glasshouses, no damage was observed due to nematodes. Interestingly, certain soil samples received in the Diagnostic Centre for Plants at ILVO from growers who observed reduced lettuce growth contained only 3,455 nematodes (100 ml soil)$^{-1}$. This indicates that *Paratylenchus* sp. is not always causing damage, although it is clear that in certain, still unknown, circumstances, the presence of this nematode population can pose a threat, as already mentioned for pin nematodes by Ghaderi (2019). The damage threshold for the root weight was 1,754 to 2,000 *Paratylenchus* sp. (100 ml soil)$^{-1}$, while the threshold for root quality was much less: 362 to 1,308 *Paratylenchus* sp. (100 ml soil)$^{-1}$. However, the standard errors for the calculated parameter values were very high and therefore the estimated damage thresholds should be interpreted with caution.

In conclusion, the *Paratylenchus* populations discovered by this study in Belgian commercial glasshouses were all found to belong to the same, hitherto undescribed species. This nematode can achieve high population numbers on lettuce, with more than 20,000 nematodes (100 ml soil)$^{-1}$ during winter and spring, which in some cases can cause reduced lettuce growth. A healthy, good quality root is vital to ensure a healthy crop and therefore nematode population densities should be kept under 362 to 1,308 *Paratylenchus* sp. (100 ml soil)$^{-1}$. Different management strategies can be applied to maintain or lower the population density. Chemical soil disinfestation, despite its proven effectiveness, is no longer to be recommended due to increasing environmental concerns. Soil steaming under negative pressure was shown to be more effective than chemical soil disinfestation, but demands a high energy input. An alternative to these methods is rotating lettuce with black fallow or leafy vegetables such as lamb's lettuce, wild rocket and parsley.

3.6 Acknowledgements

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3.7 Supplementary data

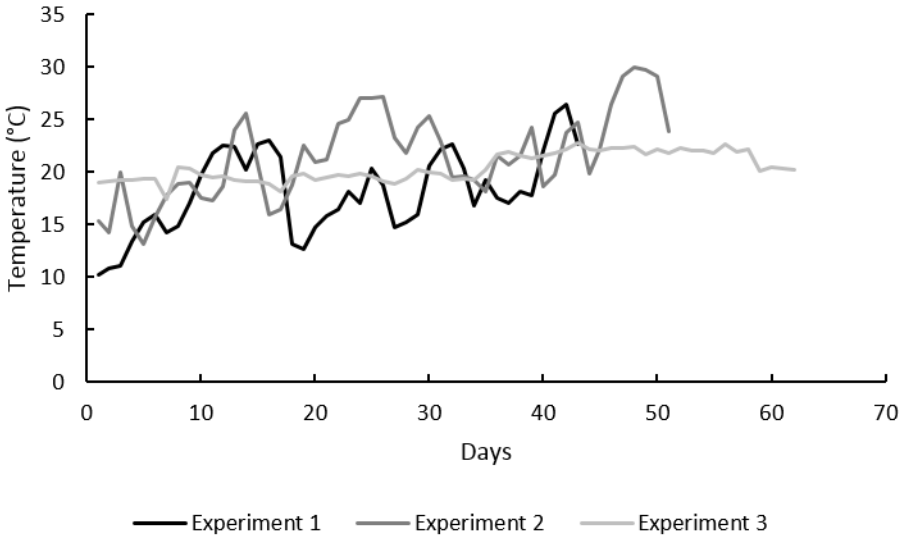

Figure S3.1 The air temperature in the glasshouses during the damage threshold experiments

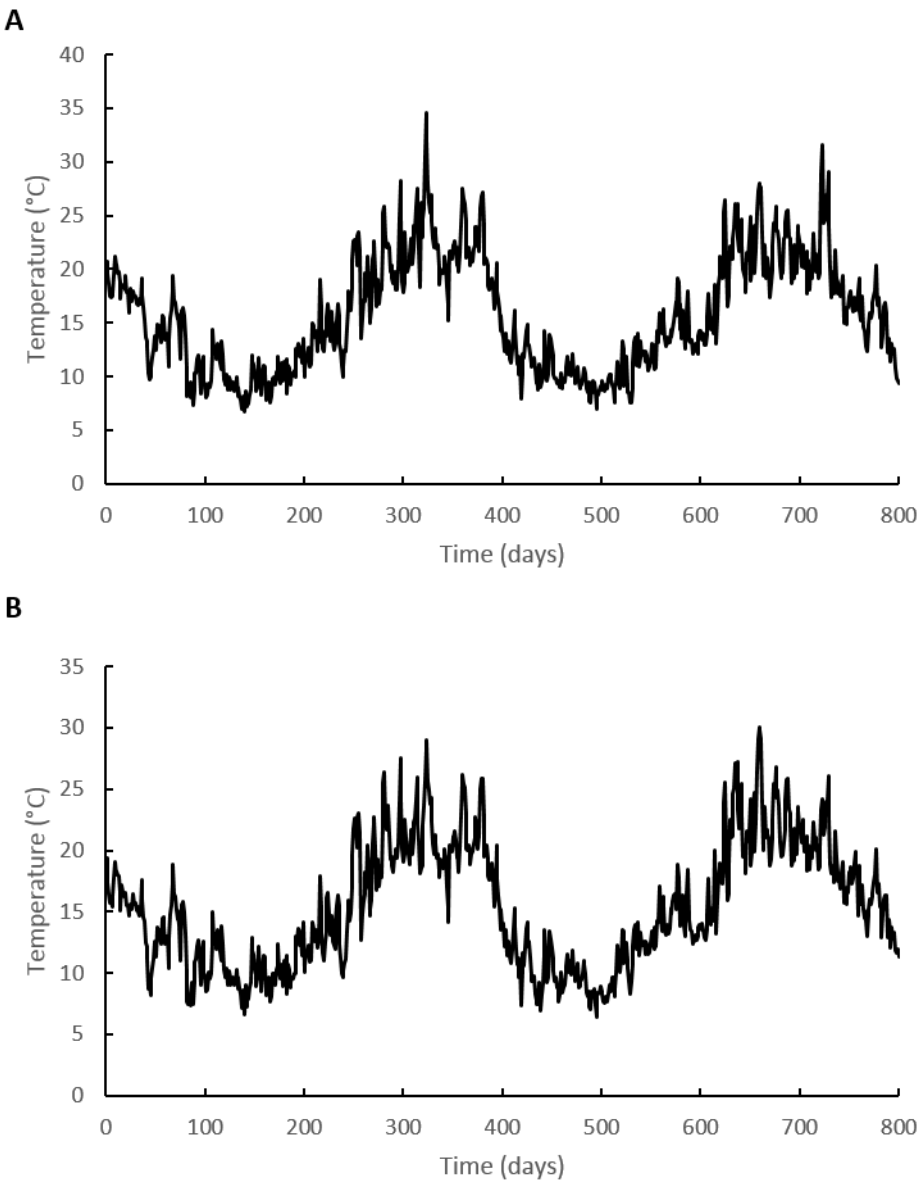

Figure S3.2 The air temperature in **A**: glasshouse 1 and **B**: glasshouse 2 during monitoring

Table S3.1 Different densities of *Paratylenchus* sp. (100 ml soil)$^{-1}$ used for the damage threshold experiments

Number	Experiment 1	Experiment 2	Experiment 3
1	3	0	0
2	38	32	444
3	74	96	976
4	166	169	1417
5	311	406	4125
6	607	987	8883
7	1371	1948	15907
8	3790	6301	27293
9	8889	11591	34951
10	19250	-	-

CHAPTER

4

Glasshouse-specific occurrence of basal rot pathogens and the seasonal shift of *Rhizoctonia solani* anastomosis groups in lettuce

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4.1 Abstract

Basal rot is a common disease in Belgian lettuce, which is mainly controlled by fungicides and chemical soil disinfestation. A seasonal appearance of the basal rot pathogens: *Rhizoctonia solani*, *Sclerotinia* spp., *Botrytis cinerea* and *Pythium* spp. has been reported, but lettuce growers use standard spraying schemes, irrespective of the occurrence of the pathogen. Due to stricter regulations and environmental concerns the superfluous use of fungicides should be omitted. We investigated if the use of fungicides could be reduced by only controlling the active pathogens. Therefore, lettuce was continuously grown in three glasshouses without any fungal disease control and the active pathogens causing basal rot were identified. The occurrence of basal rot pathogens appeared to be glasshouse specific and the different basal rot pathogens were active throughout the year. However, a seasonal appearance of *R. solani* anastomosis groups and *Pythium* spp. was observed with AG4-HGI and *Pythium ultimum* active at higher temperatures and AG2-1, AG-BI, AG1-IB and *Pythium sylvaticum* at lower temperatures. We report for the first time the isolation of AG-BI from infected plants. Each *R. solani* anastomosis group had its own optimal growth rate *in vitro.* Differences in pathogenicity between *R. solani* anastomosis groups were observed on detached leaves. AG1-IB and AG4-HGI were most pathogenic, followed by AG2-1 and AG-BI. These results show that the fungicide spraying scheme should be adapted to the occurring pathogens in the glasshouse. This information is of high importance in developing a sustainable control strategy for basal rot pathogens.

4.2 Introduction

Lettuce is mainly produced in soil in glasshouses as a monoculture with up to five harvests per year. This intensive production system has led to a high incidence of soil-borne pathogens which can cause basal rot, characterized by rotting of the older leaves. In Belgium, the causal agents of basal rot are *Sclerotinia* spp., *Botrytis cinerea, Pythium* spp. and different anastomosis groups of *Rhizoctonia solani* (Van Beneden *et al.*, 2009). Since the ban of methyl bromide in 2006, control strategies against basal rot rely on the intensive use of fungicides and chemical soil disinfestation, resulting in pesticide residues that can be present in soil and end product. Lettuce growers commonly use a standard fungicide spraying scheme with preventive applications. However, nowadays they try to limit the use of chemicals due to stricter regulations of pesticide residues in food and increased environmental concerns. Knowledge about the causal basal rot pathogens and their activity in different seasons is crucial when developing integrated control strategies.

Basal rot caused by *R. solani*, also called bottom rot, starts with an initial infection characterized by small rust-colored to chocolate brown spots, primarily on the underside of leaf midribs on lower leaves in direct contact with the soil (Davis *et al.*, 1997). Bottom rot lesions are capable of rapid expansion so that midribs and lettuce leaves can quickly rot (Blancard *et al.*, 2006). Sclerotia, brown in color due to the formation of melanin, can be observed at a later stage of infection (Naiki and Ui 1978).

Rhizoctonia solani is a species complex composed of 13 different anastomosis groups (AGs), based on hyphal anastomosis reactions (Carling *et al.*, 2002a, b). These anastomosis groups can be further divided in subgroups that show high similarity in vitamin requirement, pathogenicity, cultural and molecular characteristics (Cubeta and Vilgalys, 1997; Kuninaga *et al.*, 1997; Ogoshi, 1987). Different AGs and subgroups of *R. solani* were distinguished as causal agents of basal rot in Belgian lettuce; AG1-IB, AG4-HGI, AG10, AG2-1, AG2-1 Nt and AG3, with AG1-IB and AG4-HGI the most frequently observed (Van Beneden *et al.*, 2009). AG1, AG2 and AG4 were also found on lettuce in the Netherlands, the UK and the US (Kooistra, 1983; Wareing *et al.*, 1986). AG1-IB has also been reported from Brazil and Germany (Kuramae *et al.*, 2003) and AG2-1 from Germany, where AG1-IC was found as well, though prevalence of the last two AG groups was low (Grosch *et al.*, 2004). Additionally, AG5, was observed in the US (Herr, 1992).

Sclerotinia sclerotiorum and *S. minor* cause damage in lettuce production areas worldwide. *Sclerotinia nivalis*, however was only identified in China (Li *et al.*, 2000), while *S. subarctica* could only be observed in Norway (Nordskog *et al.*, 2014). Damping-off of the lower leaves and quick rotting of the leaves and nerves with a soft watery decay are typical symptoms of *Sclerotinia* spp. infection. Occasionally, collapsing of the whole plant in less than two days can be seen. In moist conditions, white mycelium is produced on the plant with large (2-20 mm long and 3-7 mm wide) or small (0.5-2 mm in diameter) black sclerotia, depending on the species (Blancard *et al.*, 2006; Davis *et al.*, 1997; Subbarao, 1998).

Botrytis cinerea appears wherever lettuce is grown, often together with *R. solani, Sclerotinia* spp. or *Pythium* spp. Soft rot of damaged or senescent leaves is a typical symptom, as is grey mycelium visible on the damaged tissues. In some cases, dark sclerotia are present, but in contrast with those of

Sclerotinia spp., they are closely attached to the infected plant tissue (Blancard *et al.*, 2006; Davis *et al.*, 1997).

Pythium sylvaticum and *P. ultimum* were recorded to cause basal rot symptoms in Belgian lettuce, as were three isolates closely related to *P. cylindrosporum*, *P. regulare* and *P. irregulare*, according to their *ITS* sequences (Van Beneden *et al.*, 2009). In the Netherlands, *P. sylvaticum* and also *P. tracheiphilum* and *P. incinulatum* were associated with basal rot (Blok & Van der Plaats-Niterink, 1978). *Pythium* spp. infection results in extensive dark spots and rot of the lower leaves. The lesions are slimier than those due to other basal rot pathogens.

Kooistra (1983) and Van Beneden *et al.* (2009) reported that lettuce was usually infected with *R. solani* in the summer, and with *B. cinerea* in winter, while *Sclerotinia* spp. and *Pythium* spp. were observed in spring, summer and autumn. Their observations indicate a correlation between the occurrence of the pathogen and the time of the year. We wanted to investigate whether it is possible to reduce the use of fungicides and to apply narrow spectrum fungicides at appropriate periods by only controlling the pathogens when they are active. Therefore, we grew continuously butterhead lettuce in the glasshouse without any fungal disease control and determined the pathogens causing basal rot whenever symptoms appeared. This was done at three different locations to obtain substantial information needed in the development of an integrated control strategy of basal rot in lettuce.

4.3 Materials and methods

4.3.1 Glasshouse trials

Butterhead lettuce was continuously cropped during one and a half year (2015 - 2017) in the glasshouse without any use of fungicides to observe the active pathogens causing basal rot. The study took place in three glasshouses simultaneously; at Kruishoutem, Sint-Katelijne-Waver and Rumbeke-Beitem (Belgium), on areas of 100 m² (5 × 20 m), 31.5 m² (1.8 × 17.5 m) and 53.8 m² (6.4 × 8.4 m), respectively. In the glasshouse in Kruishoutem and Sint-Katelijne-Waver lettuce had been grown regularly and the initial basal rot pressure was rather low. But in the glasshouse in Rumbeke-Beitem, no lettuce had been planted since a long time, so the initial pressure is unknown. After two crop cycles, the large plot (AB) in Kruishoutem was split in two plots (A and B, each 50 m²). Eight consecutive plantings were carried out on plot A and 7 on plot B. In Sint-Katelijne-Waver 7 consecutive plantings were conducted on plot C (0.9 × 17.5 m) and 1 on plot D (0.9 × 17.5 m). In Rumbeke-Beitem the large plot was split in four small plots (E, F, G and H, each 1.6 × 8.4 m). Seven plantings were conducted on plot E, 6 on plot F and G and 2 on plot H. The plots were split into smaller plots to conduct more field trials at different time points. A map of the different plots is shown in Figure 4.1. Soil characteristics were determined before the glasshouse trials took place (Table 4.1). The temperature in the glasshouse and in the soil at 15 cm depth was measured every five minutes during the trials in Kruishoutem and Rumbeke-Beitem and every hour in Sint-Katelijne-Waver. Butterhead lettuce in peat blocks at 5 – 6 leaf stage was planted 27 cm between and within the rows. Different cultivars were used according to the season and these cultivars had no resistance properties against basal rot. The crop was grown without any fungal disease control. The lettuce heads were checked regularly for basal rot symptoms. Twenty heads were cut off ad random when the first symptoms appeared. Based on the symptoms, the damage on each head was attributed

to *R. solani*, *B. cinerea*, *Pythium* spp. or *Sclerotinia* spp. Sometimes up to ten lettuce heads were sent to the laboratory to verify the identification and to identify up to species level or anastomosis group. One week after the first symptoms were observed, another twenty heads were cut off ad random and scored again for the presence of the pathogen. Thereafter, the whole plot was harvested and replanted after two weeks to two months with new young lettuce plants. The disease incidence (DIn) of basal rot was determined as follows: (number of heads with basal rot)/20×100. The disease incidence of *R. solani, Sclerotinia* spp., *Botrytis cinerea* and *Pythium* spp. was calculated as follows: (number of heads with *R. solani, Sclerotinia* spp., *Botrytis cinerea* or *Pythium* spp.)/(number of heads with basal rot symptoms)×100. The actual disease incidence due to each pathogen was calculated by multiplying the disease incidence of basal rot by the disease incidence of the pathogen, divided by 100.

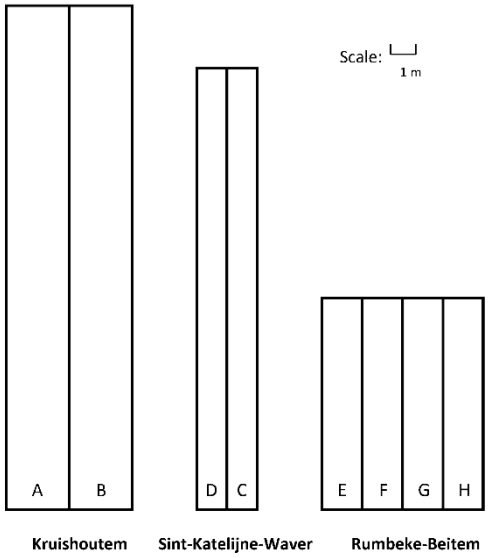

Figure 4.1 Plan of the plots A and B in Kruishoutem, C and D in Sint-Katelijne-Waver and E, F, G and H in Rumbeke-Beitem

Table 4.1 Soil characteristics at the three locations prior to lettuce planting of the glasshouse trials

	Glasshouse		
Soil characteristics	**Kruishoutem**	**Sint-Katelijne-Waver**	**Rumbeke-Beitem**
Soil type	sand	sandy loam	loam
C (%)	2.2	2.1	1.2
N (kg ha^{-1})	94	112	103
P_2O_5 (mg (liter soil)$^{-1}$)	1797	1402	1269
K_2O (mg (liter soil)$^{-1}$)	138	214	556
pH	6.7	6.9	7.3
Last soil disinfestation	June 2013	June 2014	June 2013
Soil disinfestation product	1,3-dichloropropene	1,3-dichloropropene and metam sodium	dazomet
Use of Contans	Regularly before trials	Regularly before trials	Never

4.3.2 *Rhizoctonia solani* anastomosis group determination and *Pythium* species identification

Isolation of *R. solani* and *Pythium* spp. was achieved by cutting eight small pieces (± 1 cm^2) of the affected lettuce heads from the glasshouse trials from the three locations. The plant pieces were surface

sterilized with 1% NaOCl during 30 s, washed three times with sterile demineralized water and dried in sterile air. The pieces were plated on potato dextrose agar (PDA; Becton, Dickinson and Company) and water agar (WA, Duchefa) both amended with streptomycin sulphate (100 mg liter^{-1}, Duchefa). After 48 h, *Rhizoctonia*-like and *Pythium*-like hyphal tips were transferred to fresh PDA. Each collected isolate originated from another lettuce head. When *R. solani* isolates with the same morphology grew out of different lettuce heads from the same planting, some isolates were selected to store at -80°C. In total, 26 *Rhizoctonia* isolates and seven *Pythium* isolates were stored and used for sequencing the rDNA-*ITS* fragment to identify the anastomosis group of the *R. solani* isolates and the species of *Pythium*.

For the extraction of genomic DNA, all isolates were transferred to potato dextrose broth and incubated for one week at 23°C. The mycelial mats were filtered and crushed into fine powder using liquid nitrogen. The genomic DNA was extracted using Invisorb Spin Plant Mini Kit (Stratec Molecular). The rDNA-*ITS* fragment was amplified using primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') for the *R. solani* isolates and primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 for the *Pythium* isolates (White *et al.*, 1990). The PCR amplification reactions were performed by adding 2 µL genomic DNA (5-10 ng µL^{-1}) to 23 µL of reaction mixture containing 5 µL PCR buffer (5x, Promega), 0.5 µL dNTPs (10 mM), 1.75 µL of each primer (10 µM), 0.15 µL Taq DNA polymerase (5 units µL^{-1}) and 13.85 µL ultrapure sterile water. Amplification was performed using a Flexcycler PCR Thermal Cycler (Analytik Jena) programmed with an initial denaturation at 94°C for 10 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were analyzed by electrophoresis (100V, 25 min) on 1% agarose gels in TAE buffer. Purification of PCR products with Exosap was carried out to remove excess of primers and dNTPs. The amplified fragment was sent for sequencing to LGC genomics (Berlin, Germany). Consensus sequences of the 26 *Rhizoctonia* isolates and seven *Pythium* isolates were obtained using Bioedit version 7 and were compared with those in GenBank using the BLASTn tool. Thereafter, a phylogenetic analysis for the *Rhizoctonia* isolates was conducted with 63 representative strains from Sharon *et al.* (2006), because inaccuracies in sequences of different *Rhizoctonia* isolates were indicated in Genbank (Sharon *et al.*, 2006). Multiple alignments were constructed using MUSCLE with Mega 7. The phylogenetic tree was constructed using 42 isolates from Sharon *et al.* (2006), resulting in an alignment of 843 bp.

4.3.3 Mycelial growth rate and temperature range of *Rhizoctonia solani*

Sixteen *R. solani* isolates, in particular, four isolates of each anastomosis group, were chosen to investigate their mycelial growth at different temperatures. A mycelium plug (6 mm diameter) of a one-week old culture, grown at room temperature (19-22°C), was put in the middle of a PDA plate. The plates, four repetitions for each isolate, were incubated at 7, 12, 18, 23, 28 and 33°C in the dark. Subsequently, the diameter of the fungal colony was measured frequently in orthogonal directions to calculate the mycelial radius growth rate per day in each plate. Pictures were taken after 10 days from plates incubated at 12, 18, 23, 28 and 33°C and after 30 days from plates incubated at 7°C.

4.3.4 *Rhizoctonia solani* pathogenicity assays

First, a small subset of five *R. solani* isolates (Rh2.01, Rh2.05, Rh2.10, Rh4.01 and Rh4.03) belonging to different anastomosis groups or origins, were chosen to investigate their pathogenicity towards lettuce. Later, this small subset was extended to the sixteen isolates used in the mycelial growth rate experiment. The pathogenicity was explored at 23°C using detached leaves (Thornton *et al.* 1999). In plastic containers (18 cm × 12 cm, 8 cm high) with moistened tissue paper at the bottom, a mycelium plug (6 mm diameter) of a one-week old *R. solani* culture was laid on top of a lettuce leaf. Sterile PDA plugs were used in the control treatment. There were four repetitions of each isolate for the experiments with the small subset, while the extended experiment was conducted with five repetitions. The lesion coverage was scored five days after inoculation on a scale from 0 to 4 with 0 = 0%, 1 = 1-25%, 2 = 26-50%, 3 = 51-75% and 4 = 76-100% of the leaf covered with symptoms caused by *R. solani.* The disease index (DIn) was calculated for every isolate as follows: [(0×number of plants within class 0)+(1×number of plants within class 1)+(2×number of plants within class 2)+(3×number of plants within class 3)+(4×number of plants within class 4)]/(total number of plants within treatment×4)×100. Two repetitions were conducted for the small subset, a first one with cultivar 'Lucrecia' and a second with cultivar 'Cosmopolia'. The extended experiment with sixteen isolates was conducted with cultivar 'Presteria' and once with cultivar 'Brighton'.

4.3.5 Statistical analysis

All data sets were analyzed using scripts written in R studio and run in R-version 3.5.0. The statistical tests were performed at a confidence level of $P = 0.05$. Correlations between the different factors in the glasshouse trials were revealed with the Spearman's rank correlation test, since the data were not-normal distributed. Repeated experiments were first statistically analyzed to determine if data could be pooled. The distribution of the data of the mycelial growth rate was analyzed for normality with QQ plots and the Shapiro-Wilk test for every isolate at every temperature. Homogeneity of variances was tested with the Levene's test. A factorial analysis of variance was carried out to determine the effects of temperature and anastomosis group, and their interaction, on mycelial growth rate. A one-way analysis of variance was also conducted on the mycelial growth rate between isolates at different temperatures and between temperatures of each isolate using Tukey's multiple comparison test. The ordinal data of the pathogenicity test were analyzed with non-parametric statistics; differences between isolates were detected based on the Wilcoxon rank sum test.

4.4 Results

4.4.1 Glasshouse trials and identification of *Rhizoctonia solani* and *Pythium* spp.

In three different glasshouses, butterhead lettuce was cropped continuously without any fungal disease control to determine the active pathogens causing basal rot through the year. One week after appearance of the first basal rot symptoms, disease symptoms were evaluated again before all lettuce heads were removed and the plot was replanted with young butterhead lettuce plants.

An overview of the course of the trials in each glasshouse is given in Figure 4.2, while detailed results are presented in Table 4.2. The air and soil temperature were strongly correlated ($r_s = 0.97$, $P < 0.01$,

Spearman). In all three glasshouses, symptoms appeared later when temperatures were low. We observed a monotonic power law relationship between the soil temperature and the growing period until the first basal rot symptoms appeared. The Spearman's correlation coefficient r_s was -0.85, -0.86 and -0.89 for the glasshouse in Kruishoutem, Sint-Katelijne-Waver and Rumbeke-Beitem, respectively ($P < 0.01$). It took at least 15 days to see the first symptoms of basal rot in the glasshouse in Kruishoutem, 30 days in Sint-Katelijne-Waver and 19 days in Rumbeke-Beitem (Figure 4.3 A). The lowest temperature at which disease symptoms were seen was 9°C for Kruishoutem (symptoms after 55 days), 10°C for Rumbeke-Beitem (symptoms after 61 days) and 11°C for Sint-Katelijne Waver (symptoms after 54 days). In general, *R. solani, B. cinerea* and *Pythium* spp. were most abundant and *Sclerotinia* spp. only seldom appeared. There was also a strong correlation between soil temperature and growing period needed to see the first symptoms for each pathogen, with Spearman's correlation coefficients of -0.67 for *R. solani,* -0.89 for *B. cinerea* and -0.96 for *Pythium* ($P < 0.01$). These observations also followed the power law, but a higher variability was observed for *R. solani* (Figure 4.3 B). At higher temperatures, the first symptoms of *Pythium* spp. and *B. cinerea* were observed after 26 and 19 days, while for *R. solani* they already appeared after 15 days. Symptoms of *R. solani* already occurred at 9°C, while *B. cinerea* and *Pythium* spp. needed a minimum temperature of 10 and 11°C, respectively. No correlation was found between the actual disease incidence of *R. solani, B. cinerea* or *Pythium* and the soil temperature ($r_s = 0.29$ with $P = 0.28$; $r_s = 0.17$ with $P = 0.51$; $r_s = -0.51$ with $P = 0.16$; respectively).

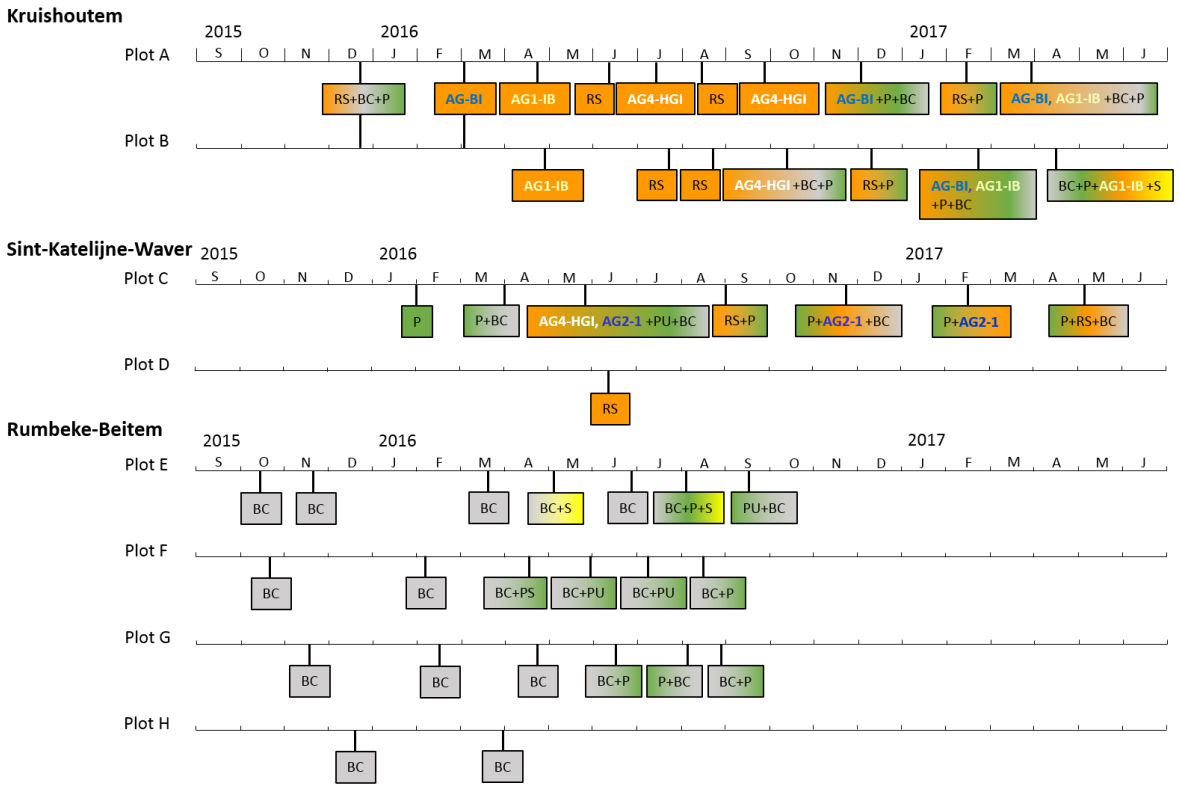

Figure 4.2 Timeline of the occurrence of basal rot pathogens at the end of each lettuce planting, one week after the first symptoms were noticed, in three glasshouse trials. Orange shows the occurrence of *R. solani* (RS), yellow the occurrence of *Sclerotinia* spp. (S), grey the occurrence of *B. cinerea* (BC) and green the occurrence of *Pythium* spp. (P, PS = *P. sylvaticum*, PU = *P. ultimum*).

In total 17, 8 and 21 plantings were carried out in Kruishoutem, Sint-Katelijne-Waver and Rumbeke-Beitem, respectively (Table 4.2). First, two plantings were carried out in the glasshouse in Kruishoutem on plots A and B simultaneously (Table 4.2, AB1 and AB2). Later, 8 consecutive plantings were carried out on plot A (Table 4.2, A1 to A8) and 7 consecutive plantings on plot B (Table 4.2, B1 to B7). Lettuce heads with *R. solani* symptoms were observed in all plantings, and their incidence was high (40-100%). *Botrytis cinerea* and *Pythium* spp. were often observed in autumn, winter and spring, but mostly with a low incidence. These pathogens were not observed in summer. *Sclerotinia* spp. was only found in planting B7 in April 2017 with an incidence of 3%, one week after the appearance of the first basal rot symptoms.

In the glasshouse in Sint-Katelijne-Waver, 7 consecutive plantings were conducted on plot C (Table 4.2, C1 to C7), while plot D was only planted once (Table 4.2, 4.D1). *Rhizoctonia solani* was observed in most plantings with a varying incidence (21-100%). The highest *R. solani* incidences were observed in spring and summer, when soil temperatures of 17°C or higher occurred (Table 4.2, C3, C4 and D1). The disease incidence of *Pythium* spp. was very high, when no or very little damage by *R. solani* was observed (Table 4.2, C1, C2, C5, C6 and C7). *Botrytis cinerea* could only be seen in spring and autumn with a very low incidence (8-19%) one week after noticing the first symptoms of basal rot (Table 4.2, C2, C3, C5 and C7).

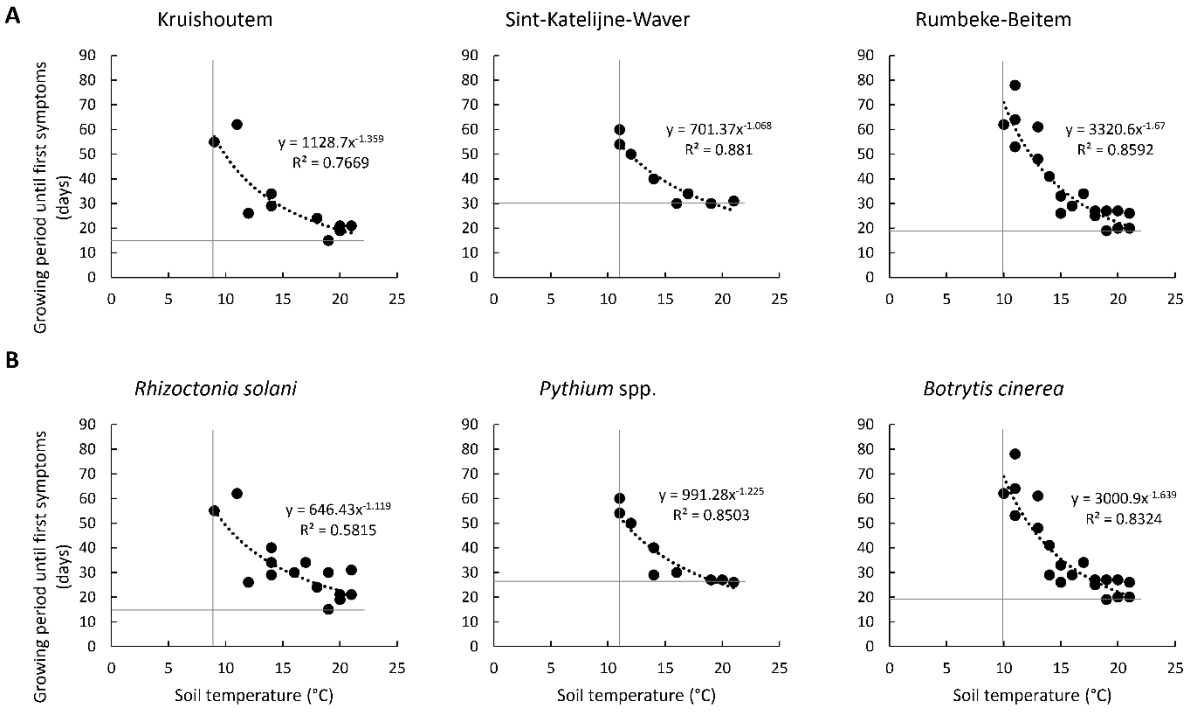

Figure 4.3 Relation between the soil temperature (°C) and the growing period until first symptoms appear for **A** basal rot in the three different glasshouses (Kruishoutem, Sint-Katelijne-Waver and Rumbeke-Beitem) and **B** the basal rot pathogens: *Rhizoctonia solani*, *Pythium* spp. and *Botrytis cinerea*.

In the glasshouse in Rumbeke-Beitem, four adjacent plots were used (plot E, F, G and H) for 7 consecutive plantings on plot E (Table 4.2, E1 to E7), 6 on plots F (Table 4.2, F1 to F6) and G (Table 4.2, G1 to G6) and 2 on plot H (Table 4.2, H1 and H2). *Rhizoctonia solani* was never observed, but high

incidences of *B. cinerea* were registered (56-100%). In planting E4 in May 2016, G5 in June 2016 and E6 in August 2016, a very low incidence (1 to 5%) of *Sclerotinia* spp. was noticed. *Pythium* spp. was often observed in spring and summer, at first also with a low incidence (17-44%) (Table 4.2, E7, F4 and G5), but one week after the first symptoms were observed, *Pythium* spp. were found on more lettuce heads, accounting for up to 95% of the basal rot symptoms (Table 4.2, E6, E7, F3, F4, F5, F6, G4, G5 and G6).

Table 4.2 Disease incidence of basal rot, *R. solani* (RS), *Sclerotinia* spp. (S), *B. cinerea* (BC) and *Pythium* spp. (P); air and soil temperature of the different trials in the glasshouses in Kruishoutem (plots A and B), Sint-Katelijne-Waver (plots C and D) and Rumbeke-Beitem (plots E, F, G and H)

						Disease incidence (%)					Temperature (°C)*	
Lettuce planting code	**Cultivar**	**Planting date**	**1st and 2nd observation**	**Growing period (days)**	**Weight (g)**	**Basal rot**	**RS**	**S**	**BC**	**P**	**Air**	**Soil**
Kruishoutem												
AB1	Coby	30/10/2015	15/12/2015	46	134	35	50	0	50	0	12	-
			22/12/2015	7	169	70	93	0	4	4	12	-
AB2	Presteria	24/12/2015	24/02/2016	62	185	18	100	0	0	0	8	11
			2/03/2016	7	276	28	100	0	0	0	8	11
A1	Zendria	17/03/2016	20/04/2016	34	192	85	100	0	0	0	11	14
A2	Cosmopolia	10/05/2016	3/06/2016	24	180	43	100	0	0	0	15	18
			8/06/2016	5	257	60	100	0	0	0	14	16
A3	Cosmopolia	15/06/2016	30/06/2016	15	26	13	100	0	0	0	17	19
			8/07/2016	8	96	28	100	0	0	0	16	20
A4	Cosmopolia	15/07/2016	5/08/2016	21	103	13	100	0	0	0	19	21
			11/08/2016	6	202	45	100	0	0	0	17	21
A5	Presteria	24/08/2016	23/09/2016	30	146	40	100	0	0	0	-	-
			27/09/2016	4	201	75	100	0	0	0	-	-
A6	Brighton	14/10/2016	24/11/2016	41	88	48	53	0	5	42	-	-
			2/12/2016	8	130	100	50	0	8	42	-	-
A7	Gardia	9/12/2016	7/02/2017	60	120	10	75	0	0	25	-	-
			13/02/2017	6	154	15	67	0	0	33	6	8
A8	Lucrecia	24/02/2017	22/03/2017	26	37	13	100	0	0	0	12	12
			30/03/2017	8	90	48	74	0	21	11	13	14
B1	Zendria	17/03/2016	20/04/2016	34	182	48	100	0	0	0	11	14
			27/04/2016	7	313	100	100	0	0	0	12	15
B2	Cosmopolia	22/06/2016	13/07/2016	21	131	20	100	0	0	0	18	20
			19/07/2016	6	268	68	100	0	0	0	18	21
B3	Cosmopolia	29/07/2016	17/08/2016	19	44	53	100	0	0	0	18	20
			22/08/2016	5	121	65	100	0	0	0	18	22
B4	Presteria	7/09/2016	27/09/2016	20	49	78	94	0	6	0	-	-
			10/10/2016	13	201	100	93	0	5	3	-	-
B5	Brighton	19/10/2016	2/12/2016	44	99	68	59	0	0	41	-	-
			9/12/2016	7	154	95	76	0	0	24	-	-
B6	Gardia	20/12/2016	13/02/2017	55	65	10	100	0	0	0	8	9
			20/02/2017	7	122	38	60	0	13	27	10	11
B7	Lucrecia	8/03/2017	6/04/2017	29	109	50	40	0	70	20	13	14
			13/04/2017	7	227	90	25	3	72	44	13	16
Sint-Katelijne-Waver												
C1	Brighton	26/11/2015	25/01/2016	60	-	45	0	0	0	100	10	11

			1/02/2016	7	267	95	0	0	0	100	11	11
C2	Presteria	3/02/2016	24/03/2016	50	-	35	0	0	0	100	11	12
			1/04/2016	8	432	90	0	0	10	100	13	14
C3	Alexandria	14/04/2016	18/05/2016	34	218	55	100	0	0	0	15	17
			27/05/2016	9	261	100	95	0	8	33	18	20
C4	Cosmopolia	25/07/2016	25/08/2016	31	245	70	100	0	0	0	20	21
			1/09/2016	7	357	100	100	0	0	50	23	23
C5	Brighton	7/10/2016	16/11/2016	40	194	55	27	0	0	100	11	14
			24/11/2016	8	269	100	50	0	10	90	9	13
C6	Presteria	16/12/2016	8/02/2017	54	192	50	0	0	0	100	9	11
			16/02/2017	8	260	65	31	0	0	100	11	12
C7	Lucrecia	27/03/2017	26/04/2017	30	255	70	21	0	0	100	15	16
			3/05/2017	7	384	80	19	0	19	100	14	16
D1	Cosmopolia	3/05/2016	2/06/2016	30	324	35	100	0	0	0	18	19
			9/06/2016	7	455	93	100	0	0	0	21	21
Rumbeke-Beitem												
E1	Presteria	10/09/2015	14/10/2015	34	255	-	0	0	100	0	16	17
E2	Brighton	14/10/2015	24/11/2015	41	127	7	0	0	100	0	13	14
E3	Presteria	6/01/2016	10/03/2016	64	195	5	0	0	100	0	10	11
			22/03/2016	12	530	35	0	0	100	0	12	12
E4	Lucrecia	5/04/2016	4/05/2016	29	151	25	0	0	100	0	14	16
			4/05/2016	0	348	75	0	1	100	0	21	21
E5	Cosmopolia	25/05/2016	21/06/2016	27	164	85	0	0	100	0	18	18
			28/06/2016	7	285	100	0	0	100	0	20	18
E6	Cosmopolia	6/07/2016	26/07/2016	20	157	20	0	0	100	0	22	21
			2/08/2016	7	341	85	0	5	60	30	20	20
E7	Motivo	11/08/2016	6/09/2016	26	196	30	0	0	100	17	21	21
			15/09/2016	9	347	100	0	0	30	80	22	22
F1	Gardia	22/09/2015	21/10/2015	29	-	-	0	0	100	0	14	16
	Halewyn/Presteria				227							
F2		10/11/2015	27/01/2016	78		-	0	0	100	0	10	11
			4/02/2016	8	302	100	0	0	100	0	10	11
F3	Lucrecia	24/02/2016	12/04/2016	48	305	5	0	0	100	0	12	13
			19/04/2016	7	483	98	0	0	90	10	15	16
F4	Lucrecia	27/04/2016	24/05/2016	27	257	45	0	0	56	44	17	19
			30/05/2016	6	442	95	0	0	89	21	17	18
F5	Cosmopolia	9/06/2016	28/06/2016	19	98	5	0	0	100	0	19	19
			5/07/2016	7	267	30	0	0	83	17	18	19
F6	Lucrecia	20/07/2016	9/08/2016	20	132	10	0	0	100	0	21	21
			16/08/2016	7	306	100	0	0	77	23	19	20
G1	Brighton	7/10/2015	9/11/2015	33	108	4	0	0	100	0	13	15
			18/11/2015	9	217	8	0	0	100	0	14	14
G2	Presteria	10/12/2015	10/02/2016	62	172	25	0	0	100	0	10	10

			16/02/2016	6	220	24	0	0	100	0	9	10
G3	Lucrecia	24/03/2016	19/04/2016	26	88	5	0	0	100	0	14	15
			27/04/2016	8	237	73	0	0	100	0	14	16
G4	Cosmopolia	12/05/2016	6/06/2016	25	204	5	0	0	100	0	17	18
			14/06/2016	8	391	100	0	0	60	55	20	20
G5	Cosmopolia	29/06/2016	26/07/2016	27	334	90	0	1	83	17	21	20
			2/08/2016	7	508	100	0	0	55	95	20	20
G6	Cosmopolia	3/08/2016	23/08/2016	20	111	15	0	0	100	0	20	20
			30/08/2016	7	274	55	0	0	82	27	24	23
H1	Brighton	21/10/2015	21/12/2015	61	183	-	0	0	100	0	12	13
H2	Presteria	29/01/2016	22/03/2016	53	255	45	0	0	100	0	11	11
			30/03/2016	8	429	73	0	0	100	0	13	13

* The average air and soil temperature is calculated for the growing period; planting until the first symptoms appear and the appearance of the first symptoms until the second observation

Differences in morphology of the fungal colonies between *R. solani* isolates coming from different trials were noticed *in vitro*. Therefore, the anastomosis group of 26 different *R. solani* isolates, collected from butterhead lettuce with *Rhizoctonia*-like symptoms in the glasshouses in Kruishoutem and Sint-Katelijne-Waver, was analyzed using the sequence of the rDNA-*ITS* fragment (Table 4.3). A maximum likelihood phylogenetic tree with bootstrap 1000, was constructed with 42 representative isolates from Sharon *et al.* (2008) and our 26 isolates (Figure 4.4). Four different AGs could be distinguished, namely AG1-IB, AG-BI, AG4-HGI and AG2-1. Eight isolates corresponded to AG1-IB, nine isolates to AG-BI, five isolates to AG4-HGI and four isolates to AG2-1 with a sequence similarity of 92-99%, 95-96%, 97-100% and 93-98%, respectively, compared with the representative isolates. In the glasshouse in Kruishoutem AGs AG1-IB, AG-BI and AG4-HGI (three isolates) were found; while in Sint-Katelijne-Waver AG2-1 and AG4-HGI (two isolates) were found.

Table 3 Identification of *R. solani* and *Pythium* spp. isolated from butterhead lettuce with basal rot symptoms from the glasshouse trials. The first number of the isolate name refers to the glasshouse (2 = Kruishoutem, 3 = Rumbeke-Beitem, 4 = Sint-Katelijne-Waver).

Isolate	Pathogen	Anastomosis group	Glasshouse	Planting	Accession number
Rh2.01	*R. solani*	AG1-IB	Kruishoutem	A1	MK583626
Rh2.02	*R. solani*	AG1-IB	Kruishoutem	A1	MK583627
Rh2.03	*R. solani*	AG1-IB	Kruishoutem	A1	MK583628
Rh2.04	*R. solani*	AG1-IB	Kruishoutem	B1	MK583629
Rh2.05	*R. solani*	AG-BI	Kruishoutem	AB2	MK583630
Rh2.06	*R. solani*	AG-BI	Kruishoutem	AB2	MK583631
Rh2.07	*R. solani*	AG-BI	Kruishoutem	AB2	MK583632
Rh2.08	*R. solani*	AG-BI	Kruishoutem	AB2	MK583633
Rh2.09	*R. solani*	AG-BI	Kruishoutem	AB2	MK583634
Rh2.10	*R. solani*	AG4-HGI	Kruishoutem	A3	MK583635
Rh2.11	*R. solani*	AG4-HGI	Kruishoutem	A5	MK583636
Rh2.12	*R. solani*	AG4-HGI	Kruishoutem	B4	MK583637
Rh2.13	*R. solani*	AG-BI	Kruishoutem	A6	MK583638
Rh2.14	*R. solani*	AG1-IB	Kruishoutem	B6	MK583639
Rh2.15	*R. solani*	AG-BI	Kruishoutem	B6	MK583640
Rh2.16	*R. solani*	AG-BI	Kruishoutem	B6	MK583641
Rh2.17	*R. solani*	AG-BI	Kruishoutem	A8	MK583642
Rh2.18	*R. solani*	AG1-IB	Kruishoutem	A8	MK583643
Rh2.19	*R. solani*	AG1-IB	Kruishoutem	B7	MK583644
Rh2.20	*R. solani*	AG1-IB	Kruishoutem	B7	MK583645
Rh4.01	*R. solani*	AG4-HGI	Sint-Katelijne-Waver	C3	MK583646
Rh4.02	*R. solani*	AG4-HGI	Sint-Katelijne-Waver	C3	MK583647
Rh4.03	*R. solani*	AG2-1	Sint-Katelijne-Waver	C3	MK583648
Rh4.04	*R. solani*	AG2-1	Sint-Katelijne-Waver	C3	MK583649
Rh4.05	*R. solani*	AG2-1	Sint-Katelijne-Waver	C5	MK583650
Rh4.06	*R. solani*	AG2-1	Sint-Katelijne-Waver	C6	MK583651
Pyt3.01	*P. sylvaticum*		Rumbeke-Beitem	F3	MK583652
Pyt3.02	*P. ultimum*		Rumbeke-Beitem	F4	MK583653
Pyt3.03	*P. ultimum*		Rumbeke-Beitem	F5	MK583654
Pyt3.04	*P. ultimum*		Rumbeke-Beitem	E7	MK583655
Pyt3.05	*P. ultimum*		Rumbeke-Beitem	E7	MK583656
Pyt3.06	*P. ultimum*		Rumbeke-Beitem	E7	MK583657
Pyt4.03	*P. ultimum*		Sint-Katelijne-Waver	C3	MK583658

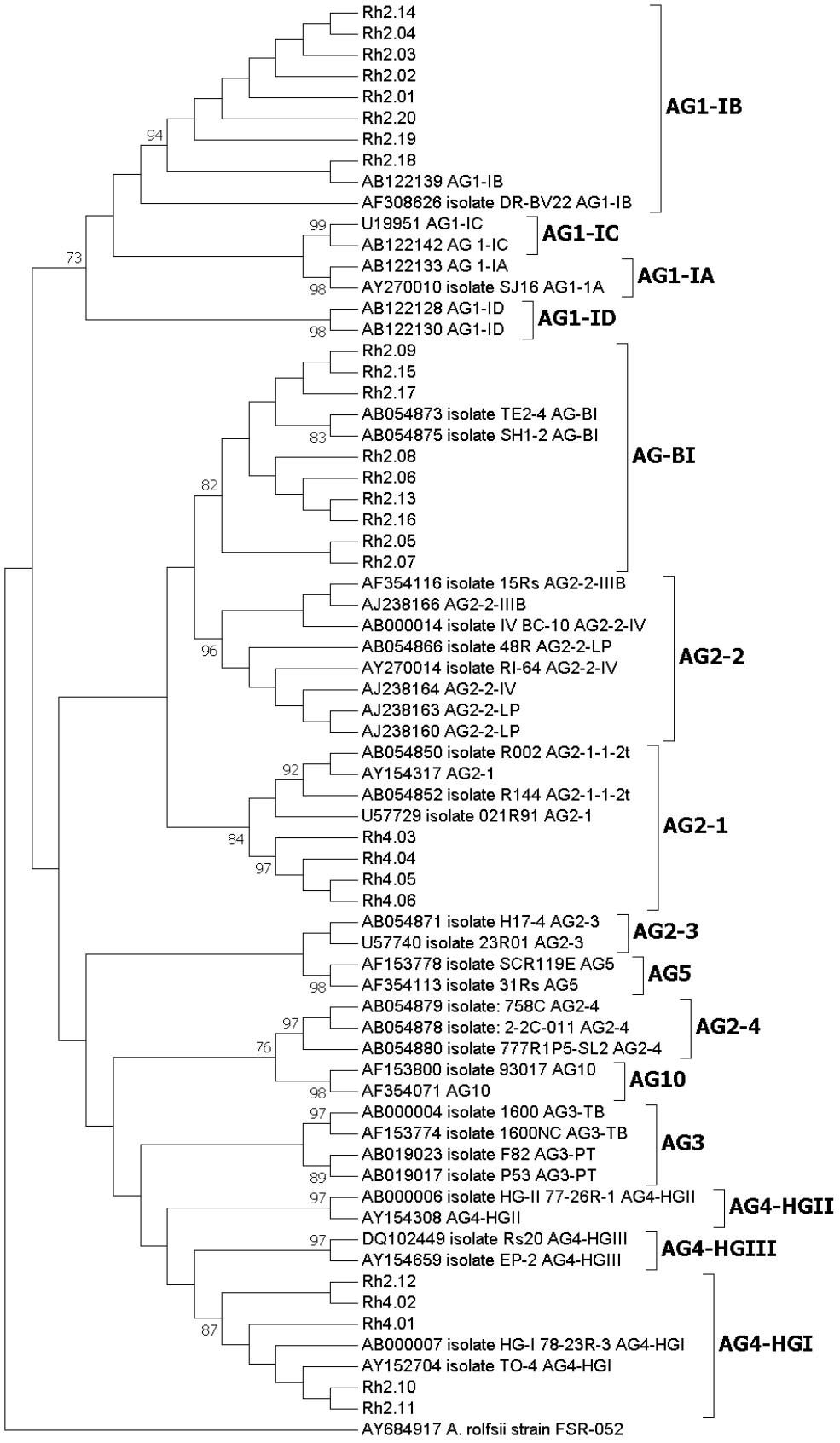

Figure 4.4 Maximum likelihood phylogenetic tree based on the rDNA-*ITS* region. The tree was derived from alignment of 26 *R. solani* isolates from Belgian greenhouses (indicated with prefix Rh) and 41 reference isolates and the outgroup *Athelia rolfsii* (AY684917). Bootstraps indicated on the branched nodes are only given for those branches with a value higher than 70.

Furthermore, seven *Pythium* isolates from the glasshouse in Rumbeke-Beitem and Sint-Katelijne-Waver were identified up to the species level based on their rDNA-*ITS* region. One isolate from the glasshouse in Rumbeke-Beitem was identified as *P. sylvaticum* with sequence similarity of 99.6% compared with KU211488.1. The other isolates belonged to *P. ultimum* with a sequence similarity of 98-100% compared with KU211465.1 (Rojas *et al.*, 2017) (Table 4.3).

We observed the first symptoms of basal rot caused by *R. solani* anastomosis group AG-BI in the glasshouse in Kruishoutem in February 2016 (Table 4.2, AB2) when the average soil temperature was low (11°C) (Table 4.2). The soil temperature rose to 14°C in March and April, during plantings A1 and B1, and AG1-IB was detected with moderate and high disease incidences of basal rot (85 and 48%, respectively), when the first symptoms were observed. During summer and early autumn, anastomosis group AG4-HGI appeared with low to high disease incidence of basal rot (13, 40 and 78%) (Table 4.2, A3, A5, B4). Later in the year, AG-BI was observed again (Table 4.2, A6). In February and March 2017, AG-BI and AG1-IB only occurred when the average soil temperatures ranged between 9 and 12°C, in plantings B6 and A8. The anastomosis group AG1-IB was observed again when the average soil temperature increased to 14°C in April 2017 in planting B7.

In the glasshouse in Sint-Katelijne-Waver, AG4-HGI and AG2-1 appeared together in May when the average soil temperature was 17°C (Table 4.2, C3). Only AG2-1 was observed in November and February when the average soil temperature was 14 and 11°C, respectively (Table 4.2, C5 and C6).

In the glasshouse of Rumbeke-Beitem, *P. sylvaticum* was found in April, one week after the first symptoms were noticed (Table 4.2, F3) when the average soil temperature was 16°C. *Pythium ultimum*, which was also observed in the glasshouse in Sint-Katelijne-Waver, was found in May, June and September (Table 4.2, C3, F4, F5 and E7) when the average soil temperature was 17-22°C.

In summary, a clear seasonal pattern of *R. solani* AGs could be observed in two glasshouses with anastomosis group AG4-HGI only appearing in warmer periods (17-20°C) and AG-BI, AG2-1 and AG1-IB appearing in colder periods (9-14°C, 11-20°C and 9-16°C, respectively). In addition, *P. ultimum* was observed in warmer periods (17-22°C) in two glasshouses, while *P. sylvaticum* was observed only once, in a colder period (16°C).

Different symptoms of *R. solani* on butterhead lettuce could be observed (Figure 4.5). Dark chocolate-brown necrotic lesions on the veins were observed on lettuce infected with AG2-1 and AG4-HGI, while AG1-IB caused light rust-colored necrotic lesions. When lettuce was infected with AG-BI necrotic lesions on the nerves were never observed, but typically the border of outer leaves was affected. In general, plants infected with AG-BI showed fewer symptoms.

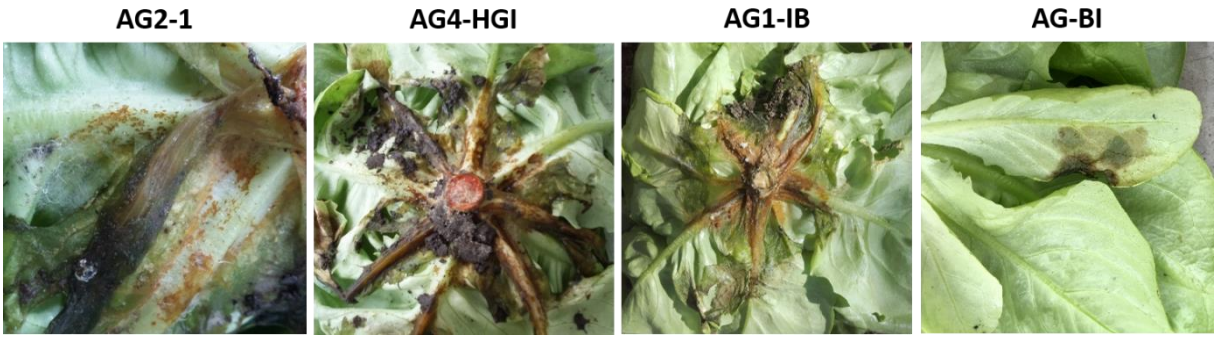

Figure 4.5 Symptoms caused by different *R. solani* anastomosis groups on butterhead lettuce. From left to right: AG2-1, AG4-HGI, AG1-IB and AG-BI are shown.

4.4.2 Mycelial growth rate and temperature range of *Rhizoctonia solani*

Since differences in appearances of anastomosis groups were observed during the year in the glasshouse trials, an *in vitro* experiment was set up to measure the mycelial growth rate at different temperatures. The mycelial growth rate varied between isolates and temperature from 0.0 to 25.8 mm day^{-1} (Supplementary data Table S4.1). A factorial analysis revealed significant effects due to the temperature and anastomosis group and interaction between temperature and anastomosis group on the mycelial growth rate ($P < 0.001$). A summary of the average mycelial growth rate of the tested AGs at different temperatures is shown in Figure 4.6.

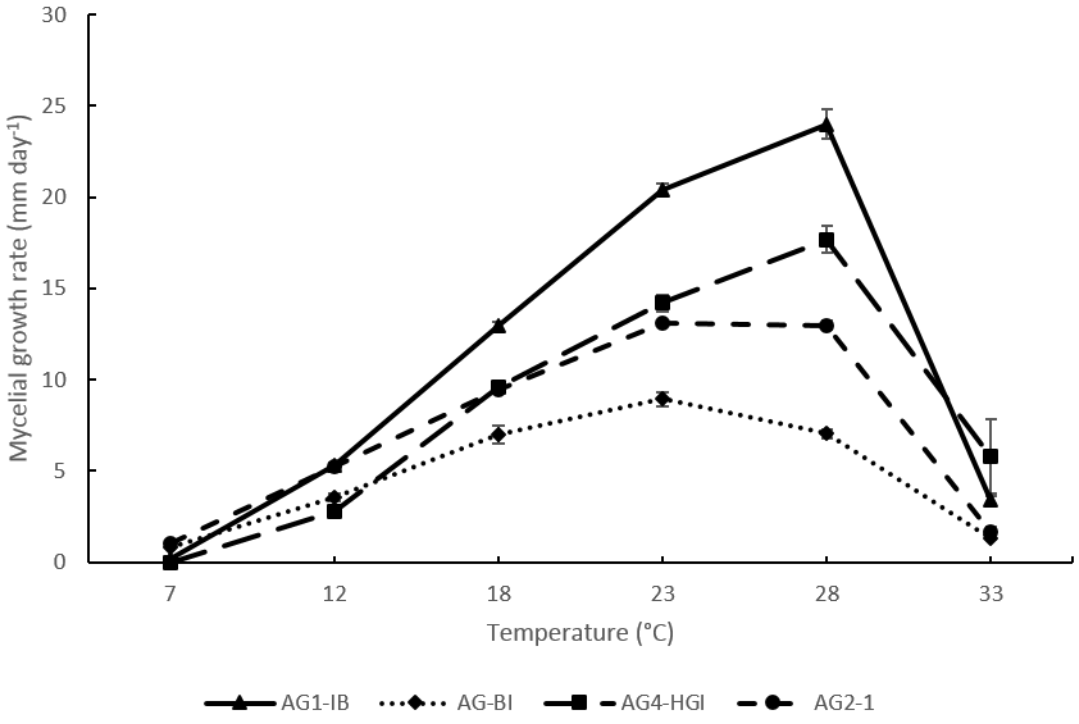

Figure 4.6 Mycelial growth rate (mm day^{-1}) at different temperatures (°C) of the anastomosis group of *R. solani* (AG1-IB, AG-BI, AG4-HGI and AG2-1) found in three glasshouses. Data points are averages of four isolates per group and error bars represent the standard error (n = 4).

The optimal growth temperature for isolates belonging to AG1-IB was 28°C (22.3-25.8 mm day^{-1}). Lower temperatures resulted in slower growth. At 7°C, mycelial growth was strongly inhibited (0.1-0.2 mm day^{-1}). Isolates Rh2.01 and Rh2.14 stopped growing after 18 days, while isolates Rh2.18 and Rh2.19 stopped growing after 30 days (Supplementary data Table S4.2). The maximum colony diameter was on average 13.2 mm. The growth was also strongly inhibited at 33°C (2.9-3.9 mm day^{-1}). All isolates stopped growing after 4 days with a maximum colony diameter of 34.0 mm.

Most isolates of AG-BI had the highest growth rate at 23°C (7.9-9.6 mm day^{-1}), but no statistical difference in growth rate between 23 and 28°C was observed for isolate Rh2.16. At 33°C, isolates Rh2.05 and Rh2.16 stopped growing after 2 days and Rh2.13 and Rh2.17 after 4 days, with a maximum diameter of 17.9 mm. All isolates of AG-BI were able to fully cover the plate (85 mm) at 7°C (0.7-0.9 mm day^{-1}).

Anastomosis group AG4-HGI had an optimal growth temperature at 28°C (16.2-19.2 mm day^{-1}). This AG was the only AG that was not able to grow at 7°C and continued to grow at 33°C. At 33°C the growth rates ranged from 2.8 to 11.6 mm day^{-1}. Especially isolates Rh2.11 and Rh2.10 originating from the glasshouse in Kruishoutem grew very fast. These two isolates are genetically closely related (Figure 4.4).

Isolates belonging to AG2-1 had the highest growth rate at 23°C (12.9-13.4 mm day^{-1}) and 28°C (12.6-13.5 mm day^{-1}), depending on the isolate. At 33°C, growth was strongly inhibited (0.9-2.0 mm day^{-1}). The isolates stopped growing after 7 days with a maximum diameter of 31.4 mm. At 7°C, all isolates of AG2-1 reached the border of the Petri dish with growth rates of 0.9-1.2 mm day^{-1}.

Growth at different temperatures resulted in differences in morphology for isolates belonging to the same or different AGs (Figure 4.7). While the mycelial growth of AG1-IB isolates was inhibited at 7°C and 33°C, sclerotia were formed on the plug. At other temperatures the mycelial growth of AG1-IB did not stop and most sclerotia were formed at the border of the Petri dish. At 33°C, the mycelium of *R. solani* AG1-IB was denser than when it was incubated at lower temperatures.

Isolates belonging to AG-BI did not form sclerotia. The longer the AG-BI plates were incubated, the more rust colored and dense mycelium was formed compared to AG1-IB. Small differences in color were observed between isolates.

The mycelium of isolates belonging to AG4-HGI was paler compared to that of the other studied AGs. The isolates stopped growing at 7°C. At 33°C, isolates Rh2.10, Rh4.01 and Rh4.02 grew irregularly and formed sclerotia, while this was not the case for isolate Rh2.11. At temperatures lower than 33°C no sclerotia were observed.

Sclerotia were observed at 18, 23 and 28°C for AG2-1. These sclerotia were formed in concentric circles. At 28°C the mycelium started to grow in a flocked appearance, while this was not observed at lower temperatures.

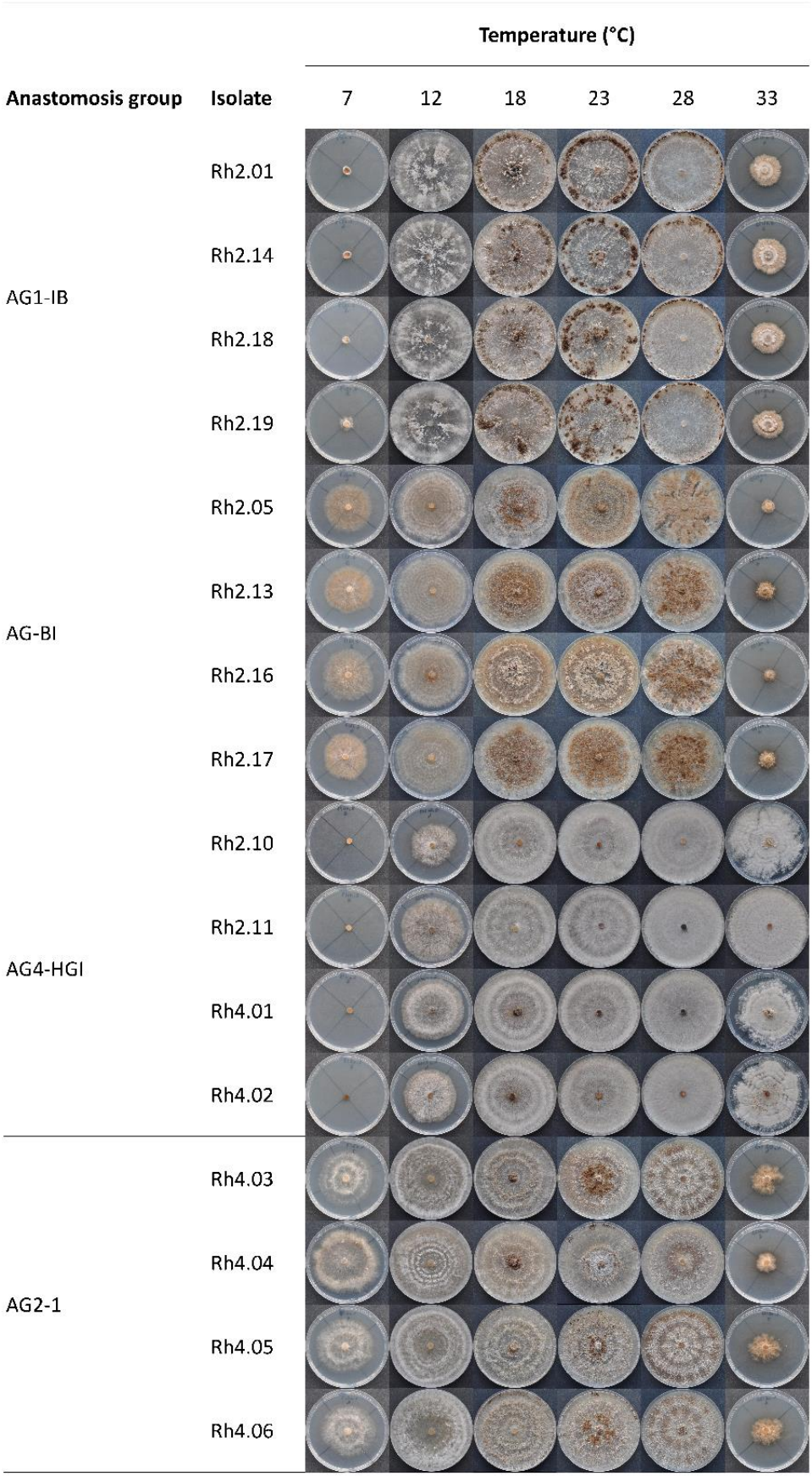

Figure 4.7 Differences in colony morphology between isolates from different anastomosis groups: AG1-IB, AG-BI, AG4-HGI and AG2-1 at different temperatures: 7, 12, 18, 23, 28 and 33°C. Mycelium was 10 days old for temperatures 12, 18, 23, 28 and 33°C; and 30 days old for temperature 7°C.

4.4.3 *R. solani* pathogenicity assays

A small subset of five isolates was tested in two detached leaf assays to verify if they were pathogenic towards lettuce. The lesions produced by *R. solani* were irregularly shaped and all isolates were able to infect lettuce leaves. Differences in aggressiveness between isolates were noted (data not shown). The detached leaf assay was carried out twice with the sixteen isolates used in the mycelial growth rate experiment to investigate the variability in pathogenicity between isolates within the same anastomosis group. No differences were found between the two repetitions (Kruskal-Wallis, $P > 0.05$), therefore data were pooled (Figure 4.8). In general, AG1-IB and AG4-HGI were most pathogenic (DI = 50-85% and DI = 28-63%, respectively), followed by AG2-1 (DI = 25-33%) and AG-BI (DI = 3-18%). Within anastomosis group AG2-1 no differences were found between isolates. For isolates belonging to AG-BI, mycelial growth was often observed on the surface of the leaf without penetration. For this AG-BI group, isolate Rh2.17 was slightly more aggressive than isolates Rh2.05 and Rh2.13. Also within the anastomosis groups AG1-IB and AG4-HGI, isolates differed in aggressiveness. Isolates Rh2.01 and Rh2.19 belonging to AG1-IB were the most aggressive (DI = 78 and 85%) followed by Rh4.01 (AG4-HGI) and Rh2.18 (AG1-IB) with a disease index of 63 and 58%, respectively.

For all sixteen isolates, a strong correlation ($r_s = 0.92$, $P < 0.01$) was found between the DI and mycelial growth rate at 23°C.

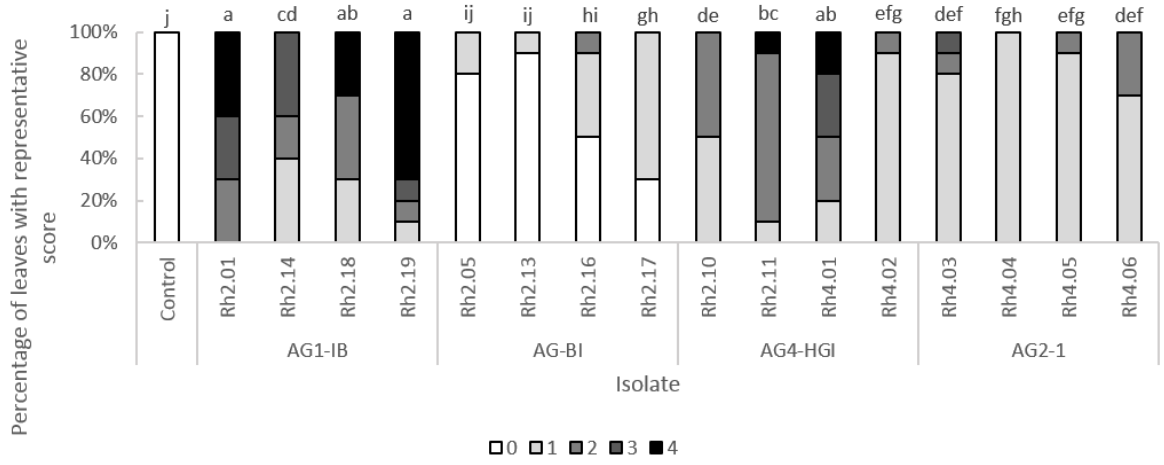

Figure 4.8 Pathogenicity of sixteen *R. solani* isolates belonging to different AGs on lettuce in a detached leaf assay five days after inoculation at 23°C. The figure represents pooled data from two experiments (n = 10). Different letters above the bars indicate differences in pathogenicity between isolates (Wilcoxon rank sum test, $P < 0.05$). Scores 0-5 stand for 0 = 0%, 1 = 1-25%, 2 = 26-50%, 3 = 51-75% and 4 = 76-100% of the leaf covered with symptoms caused by *R. solani*.

4.5 Discussion

This research was initiated to test whether fungicide spray schedules to control basal rot in intensive lettuce production can be optimized taking into account the seasonal occurrence of specific basal rot pathogens. To determine the active basal rot pathogens in different seasons, butterhead lettuce was continuously planted without any fungal disease control in three different glasshouses. The occurrence

of the different basal rot pathogens appeared to be glasshouse specific. In Kruishoutem, *R. solani* was the predominant pathogen, while *R. solani* and *Pythium* spp. were predominant in Sint-Katelijne-Waver and *B. cinerea* in Rumbeke-Beitem. Soil texture is known to have an influence on the development of fungi. *Rhizoctonia solani* spreads better in a sandy soil than in a heavy soil (Gill *et al.*, 2000; Hua & Höfte, 2015), while sandy soils seem to be more suppressive than clayey soils towards *Pythium* diseases (Knudsen *et al.*, 2002). This could explain the predominance of *R. solani* and the low occurrence of *Pythium* spp. in the sandy soil of Kruishoutem. Moreover, each glasshouse had its own disease management history prior to the set-up of the trials. Soil disinfestation products were used one to two years before the trials, which have different potencies (Mao *et al.*, 2012). In Sint-Katelijne-Waver and Kruishoutem, the product Contans, containing *Coniothyrium minitans*, was used prior to the trials. This fungus can kill sclerotia of *Sclerotinia* spp. (Van Beneden *et al.*, 2010), which can explain the low prevalence of this basal rot pathogen. These standard practices and competition between pathogens may have had an influence on basal rot pathogens resulting in glasshouse specificity.

Van Beneden *et al.* (2009) and Kooistra (1983) observed a correlation between the occurrence of specific basal rot pathogens and the time of the year. *Botrytis cinerea* was mainly found in winter, *R. solani* in summer and *Sclerotinia* spp. and *Pythium* spp. in spring, summer and autumn. These results could not be confirmed in this study. While in the glasshouse in Sint-Katelijne-Waver, *R. solani* was indeed observed during warmer periods, *Pythium* spp. appeared during colder periods. *Rhizoctonia solani* was the predominant pathogen in the glasshouse in Kruishoutem and *B. cinerea* in Rumbeke-Beitem, irrespective of the time of the year. Wareing *et al.* (1986) also found that *B. cinerea* was year-round the most common pathogen in protected and outdoor lettuce production areas in the UK. In our study, *R. solani* already caused symptoms at lower temperatures than *B. cinerea* and *Pythium* spp., while at higher temperatures, this pathogen induced symptoms faster when compared with *B. cinerea* and *Pythium* spp. High variability was observed for *R. solani*, probably due to different AGs.

The precise characterization and identification of isolates that cause bottom rot in lettuce is a prerequisite to understand the dynamics of *R. solani*. In the current study, AG4-HGI, AG1-IB, AG2-1 and AG-BI were found. AG4-HGI was found in two of the three glasshouses in warmer periods when soil temperatures ranged between 17 and 20°C, which is in accordance with Van Beneden *et al.* (2009) who also observed this anastomosis group in summer in Belgium. AG4 was also found in the Netherlands, the UK and the US, but with low prevalence and the subgroup was not identified (Herr, 1992; Kooistra, 1983; Wareing *et al.*, 1986). AG1-IB was found in the glasshouse trials when temperatures ranged between 9 and 16°C from February to April, in contrast to the former Belgian study where this anastomosis group was observed from May until August (Van Beneden *et al.*, 2009). AG1-IB was the most commonly observed AG in lettuce in Brazil and in outdoor-grown lettuce in summer in Germany and in Ohio in the US (Grosch *et al.*, 2004; Herr, 1992; Kuramae *et al.*, 2003). AG2-1 was observed in our glasshouse trials when temperatures ranged between 11 and 20°C, while it was only observed once in September in the prior Belgian study (Van Beneden *et al.*, 2009). This AG was found with low prevalence in outdoor-grown lettuce produced in summer in Germany (Grosch *et al.*, 2004) and in the US when the summer was cooler and wetter than normal (Herr, 1992). In the UK, however AG2-1 is commonly observed in protected lettuce (Wareing *et al.*, 1986). In our study AG-BI was for the first

time directly isolated from naturally infected plants when temperatures ranged between 9 and 14°C. Generally, this AG is considered to be non-pathogenic (Sneh *et al.*, 1996), but Schneider *et al.* (1997) described AG-BI as a pathogen based on a pathogenicity test with tulip, iris and hyacinth. Carling *et al.* (2002b) observed that AG-BI was a weak pathogen on seedlings of cauliflower, romaine lettuce, sugar beet and radish in an *in vitro* test. They also proposed that AG-BI should be part of the AG2 group. We also found that AG2-1 and AG-BI are genetically closely related and behave in a similar way.

The seasonal shift between the anastomosis groups with predominance of AG4-HGI in warmer periods and AG2-1, AG1-IB and AG-BI present in colder periods, can be explained by the preferred growth temperature of each AG (Anguiz & Martin, 1989; Balali *et al.*, 1995; Doornik, 1981; Harikrishnan & Yang, 2004; Kumar *et al.*, 1999; Schneider *et al.*, 1997). Previous studies showed that the abundance of each anastomosis group can depend on the climate (Balali *et al.*, 1995; Carling & Leiner, 1990; Harikrishnan & Yang, 2004) and that *in vitro* mycelial growth rate corresponds well with the mycelial growth rate in soil (Ritchie *et al.*, 2009). The *in vitro* growth optima for the different AGs correspond well with the optimum mycelial growth ranges observed in previous studies (Grosch & Kofoet, 2003; Grosch, *et al.* 2004; Kuninaga *et al.*, 1979; Ritchie *et al.*, 2009; Schneider *et al.*, 1997). We noticed that AG4-HGI is the only AG that grows very well at high temperatures (33°C) and does not grow at low temperatures (7°C) *in vitro*, which can explain its predominance in warmer periods.

The pathogenic potential of the tested isolates varied a lot, in the detached leaf assay as well as in the field. While AG4-HGI, AG2-1 and AG1-IB were associated with clear, but variable bottom rot symptoms in the glasshouse, AG-BI typically affected the leaf borders. Kooistra (1983) also observed differences in bottom rot symptoms on lettuce between *R. solani* isolates, but did not correlate this to the anastomosis groups. AG1-IB and AG4-HGI were more pathogenic towards lettuce than AG2-1 and AG-BI on detached leaves. This difference in aggressiveness between AG4-HGI, AG1-IB and AG2-1 was also reported by Van Beneden *et al.* (2009). In addition, we observed within-group differences in pathogenicity between isolates belonging to AG1-IB, AG4-HGI and AG2-1. Grosch *et al.* (2004) noticed differences in the pathogenic potential of different AG1-IB isolates from lettuce in Germany on detached leaves. A strong correlation (r_s = 0.92) between the damage on detached leaves and hyphal mycelial growth rate at 23°C was observed for all AGs we tested. However, no correlation between disease severity and hyphal growth rate was found for AG1-IB isolates from lettuce in Germany (Grosch *et al.*, 2004).

Our study confirms the weak pathogenic potential of AG-BI reported by Carling *et al.* (2002b). Although all AG-BI isolates were obtained from symptomatic plants, some isolates only caused few symptoms on detached leaves. In the field, AG-BI occurred at temperatures between 9 and 14°C, while the detached leaf assay was done at 23°C. It remains to be investigated whether AG-BI is more virulent at lower temperatures. We were unable to test this with the leaf assay because symptom development is slow at low temperatures and it is impossible to keep detached leaves fresh for long periods. Schneider *et al.* (1997) conducted their pathogenicity tests with AG-BI at 18°C which is probably a more favorable temperature for this AG than the 23°C used in our assays. A considerable variation in virulence between AGs at different temperatures was already observed by Doornik (1981).

We isolated two species of *Pythium* spp. in the glasshouse trials: *P. sylvaticum* in April, when temperatures averaged 16°C, and *P. ultimum* in May, July and September when temperatures ranged between 17 and 22°C, similar to the findings of Van Beneden *et al.* (2009). Wei *et al.* (2011) showed that *P. ultimum* was highly pathogenic on soybean at 4, 12, 20 and 28°C, while the pathogenic potential of *P. sylvaticum* decreased at higher temperatures. This relation with temperature is probably the same for our isolates and can explain why we found *P. sylvaticum* only in April and *P. ultimum* in warmer periods.

Our study demonstrates that fungicide spray schedules to control basal rot cannot be optimized according to the seasonal occurrence of specific pathogens. In certain glasshouses, *R. solani* and *B. cinerea* can be predominant year-round. This research shows that basal rot pathogens appear to be glasshouse specific and indicates that disease management schemes need to be mainly adapted for each glasshouse. However, the choice of fungicides in the spray schedules could be optimized to target the specific pathogens present in the soil of each glasshouse, which can lead to a reduction in fungicide use. The different basal rot pathogens can be identified with the help of agricultural advisory experts or the FUNSLA-app. Tracking down the most important pathogens during the year should help the grower to use appropriate fungicide sprayings for the coming crops. This is of high importance when applying specific fungicides that are only permitted to be used a few times a year. Moreover, we found that seasonal shifts in *R. solani* anastomosis groups and *Pythium* spp. occur, with predominance of *R. solani* AG4-HGI and *P. ultimum* in warmer periods and *R. solani* AG1-IB, AG2-1 and AG-BI and *P. sylvaticum* in colder periods. In addition, *R. solani* AGs differ in their pathogenic potential and sensitivity towards fungicides (Ajayi-oyetunde *et al.*, 2017; Sneh *et al.*, 1996). Further studies are needed to test the sensitivity to fungicides of different AGs affecting lettuce and to find out whether chemical control of weakly pathogenic AGs is essential to prevent economic losses. This would allow the use of appropriate fungicides only when necessary.

4.6 Acknowledgments

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4.7 Supplementary data

Table S4.1 Mycelial growth rate (mm day^{-1}) of different isolates of *R. solani* found in Belgian lettuce, and belonging to anastomosis groups AG1-IB, AG-BI, AG4-HGI and AG2-1, at temperatures ranging from 7 to 33°C. Within columns, different letters indicate differences in mycelial growth rates between isolates Within rows, different letters between brackets indicate differences in growth rate between temperatures (Tukey's test, $P < 0.05$).

	Temperature (°C)											
Isolate	**7**		**12**		**18**		**23**		**28**		**33**	
AG1-IB												
Rh2.01	0.1	fg (f)	5.7	a (d)	13.4	a (c)	20.2	ab (b)	22.3	b (a)	3.1	de (e)
Rh2.14	0.1	g (e)	5.3	ab (d)	13.0	a (c)	19.5	b (b)	22.9	b (a)	3.9	c (d)
Rh2.18	0.1	fg (f)	5.2	ab (d)	12.3	b (c)	20.7	ab (b)	25.0	a (a)	3.7	cd (d)
Rh.2.19	0.2	f (f)	5.0	ab (d)	13.0	a (c)	21.1	a (b)	25.8	a (a)	2.9	e (e)
AG-BI												
Rh2.05	0.9	cd (d)	3.6	cde (c)	7.5	f (b)	9.6	f (a)	7.4	f (b)	1.2	gh (d)
Rh2.13	0.8	de (f)	3.8	cd (d)	7.5	f (b)	8.7	fg (a)	6.7	f (c)	1.4	fgh (e)
Rh2.16	0.9	cd (d)	3.0	ef (c)	5.5	g (b)	7.9	g (a)	7.5	f (a)	1.1	h (d)
Rh2.17	0.7	e (f)	3.9	c (d)	7.4	f (b)	9.5	f (a)	6.5	f (c)	21.4	fgh (e)
AG4-HGI												
Rh2.10	0.0	h (f)	2.1	g (e)	8.9	e (c)	13.2	e (b)	16.2	d (a)	5.5	b (d)
Rh2.11	0.0	h (f)	3.1	def (e)	10.1	c (d)	15.3	c (b)	18.7	c (a)	11.6	a (c)
Rh4.01	0.0	h (e)	3.2	cdef (d)	9.9	cd (c)	14.6	cd (b)	19.2	c (a)	3.3	cde (d)
Rh4.02	0.0	h (e)	2.7	fg (d)	9.5	cde (c)	13.7	de (b)	16.6	d (a)	2.8	e (d)
AG2-1												
Rh4.03	1.0	b (f)	5.1	ab (d)	9.2	de (c)	13.4	de (a)	12.6	e (b)	1.9	fg (e)
Rh4.04	1.2	a (e)	5.5	a (d)	10.0	c (c)	12.9	e (b)	13.5	e (a)	0.9	h (e)
Rh4.05	0.9	bc (e)	4.8	b (c)	9.1	e (b)	13.1	e (a)	13.3	e (a)	2.0	f (d)
Rh4.06	0.9	bc (e)	5.5	ab (c)	9.6	cde (b)	13.0	e (a)	12.6	e (a)	1.9	fg (d)

Table S4.2 Maximum mycelial diameter at 7 and 33°C and time when growth stopped. NG = no growth observed. - = a continuous growth was observed.

	Temperature (°C)					
	7			**33**		
Isolate	Mycelial diameter (mm)	Stdev	Time (days)	Mycelial diameter (mm)	Stdev	Time (days)
AG1-IB						
Rh2.01	10.9	2.2	18	32.0	3.0	4
Rh2.14	9.8	0.4	18	37.3	3.0	4
Rh2.18	14.2	1.5	30	34.8	2.2	4
Rh2.19	18.0	1.7	30	31.8	1.2	4
Average	*13.2*	*3.7*	*24*	*34.0*	*2.6*	*4*
AG-BI						
Rh2.05	-	-		14.3	0.7	2
Rh2.13	-	-		21.4	1.4	4
Rh2.16	-	-		14.6	0.7	2
Rh2.17	-	-		21.5	0.3	4
Average	-	-		*17.9*	*4.0*	*3*
AG4-HGI						
Rh2.10	NG	NG		-	-	
Rh2.11	NG	NG		-	-	
Rh4.01	NG	NG		-	-	
Rh4.02	NG	NG		-	-	
Average	*NG*	*NG*		-	-	
AG2-1						
Rh4.03	-	-		32.6	4.3	7
Rh4.04	-	-		23.3	0.8	7
Rh4.05	-	-		35.8	1.0	7
Rh4.06	-	-		34.0	2.5	7
Average	-	-		*31.4*	*5.6*	*7*

CHAPTER

5

Belgian *Fusarium* isolates causing wilt in lettuce show genetic and pathogenic diversity

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5.1 Abstract

Fusarium oxysporum f. sp. *lactucae* race 4, causing browning of the vascular tissue and wilting of lettuce heads, was first observed in Belgium in 2015. Within four years, the pathogen spread to nearly the entire production area of butterhead lettuce and caused serious losses. However, differences in the disease development were observed between commercial glasshouses. To explain these differences, we collected 78 *Fusarium* isolates from diseased lettuce heads and characterized them physiologically and genetically. Based on molecular race identification assays, 91% of the isolates belonged to *F. oxysporum* f. sp. *lactucae* race 4, clearly making this the dominant race, but 6% of the isolates belonged to race 1, which had not yet been reported from Belgium. Pathogenicity assays using differential cultivars confirmed the molecular race identification of selected isolates. The cultivar 'Patriot' was a good new differential cultivar as it was susceptible to race 1 and not to race 4. Race 4 isolates were highly aggressive compared to race 1 isolates when a susceptible lettuce cultivar was planted in a substrate with chlamydospores, while this difference was not observed when using a root dip assay containing microconidia, emphasizing the importance of the inoculation method. The differential pathogenicity of the two races explained most of the difference in disease development in the glasshouses. Based on genotyping-by-sequencing, Belgian race 1 and race 4 isolates were highly similar to reference isolates from Japan and the Netherlands, respectively. There was very little intra-race genotypic diversity except that within race 4, two groups could be differentiated, suggesting at least two separate introductions. The main race 4 group also contained an old European isolate of *F. curvatum.* Conspecificity of *F. oxysporum* f. sp. *lactucae* race 4 (and race 1) with *F. curvatum* was confirmed using multilocus sequence analysis and implies a need to rename *F. oxysporum* f. sp. *lactucae* race 4 and race 1 to *F. curvatum*. This finding also disproves the hypothesis of a recent exotic origin of race 4 in Europe. The limited genetic diversity of this *Fusarium* population is consistent with the fast human-mediated spread of the pathogen and emphasizes the importance of hygienic measures. Race identification of isolates is crucial for the deployment of lettuce cultivars with appropriate resistance profiles.

5.2 Introduction

Butterhead lettuce (*Lactuca sativa* L.) is the sixth most important crop on the Belgian vegetable market. It is mainly produced in soil in glasshouses in an intensive production system with up to five croppings per year. Recently, the production of butterhead lettuce is threatened by Fusarium wilt, which over a period of four years spread over the entire production area. Typical symptoms are yellowing of the leaves, vascular browning and wilting. The causal organism is *Fusarium oxysporum* f. sp. *lactucae* (Matuo *et al.*, 1967). Until recently three races were reported. Race 1 is widely spread and has been reported in Japan (Fujinaga *et al.*, 2003), USA (Hubbard & Gerik, 1993), Taiwan (Lin *et al.*, 2014), Iran (Millani *et al.*, 1999), Brazil (Ventura & Costa, 2008), Argentina (Malbrán *et al.*, 2014), Portugal (Pasquali *et al.*, 2007), Italy (Garibaldi *et al.*, 2002) and France (Gilardi *et al.*, 2017). Race 2 has only been found in Japan and race 3 has been reported in Taiwan and Japan (Fujinaga *et al.*, 2003; Lin *et al.*, 2014). In 2015, a new race of *F. oxysporum* f. sp. *lactucae* was reported in the Netherlands and defined as race 4 (Gilardi *et al.*, 2016). This race is spreading very rapidly in Europe. It was subsequently observed in Belgium in 2015 (Claerbout *et al.*, 2018), Ireland in 2016, England in 2017 (Taylor *et al.*, 2019) and in Italy in 2018 (Gilardi *et al.*, 2019).

The four races of *F. oxysporum* f. sp. *lactucae* can be distinguished based on their differential pathogenicity on lettuce cultivars (Gilardi *et al.*, 2016). Race 1 is pathogenic on 'Patriot' and 'Banchu Red Fire', while race 2 is pathogenic on 'Patriot' and 'Costa Rica'; and race 3 is pathogenic on all three cultivars (Fujinaga *et al.*, 2003). Gilardi *et al.* (2016) showed that 'Banchu Red Fire' is partially resistant and 'Costa Rica' is susceptible to race 4. They included 'Romana romabella' as a good differential cultivar as it is susceptible to race 3 and 4 and resistant to race 1 and 2. The pathogenicity of race 4 on 'Patriot' has never been reported. Moreover, vegetative compatibility groups (VCGs) based on the heterokaryon compatibility are used to classify *F. oxysporum* formae speciales and non-pathogenic strains (Correl, 1991; Leslie, 1993). Four different VCGs are known for *F. oxysporum* f. sp. *lactucae*; VCG 0300, VCG 0301, VCG 0302 and VCG 0303 which correspond to race 1, 2, 3 and 4, respectively (Fujinaga *et al.*, 2005; Pintore *et al.*, 2017). These VCG studies indicate that the four races have been selected locally and have been spread by seeds, plant material, soil… (Garibaldi *et al.*, 2004; Gordon & Koike, 2015; Pintore *et al.*, 2017).

Race identification based on differential cultivars is often difficult as symptoms development depends on environmental factors. Identifying VCGs requires a lot of expertise. Therefore, molecular assays, such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphisms (RFLP) and inter-retrotransposon amplified polymorphism (IRAP), have been developed to determine the races of *F. oxysporum* f. sp. *lactucae* isolates faster and more efficiently (Gilardi *et al.*, 2016; Pasquali *et al.*, 2007; Shimazu *et al.*, 2005). Specific primers for race 1 and race 4 using IRAP on transposable elements have been developed by Pasquali *et al.* (2007) and Gilardi *et al.* (2016). Race-specific primers were also developed for race 1 and for race 2 based on RAPD analysis and derived sequence tagged site markers (Shimazu *et al.*, 2005).

Race 1 and 4 cannot be distinguished based on sequence divergence in the translation elongation factor 1-alpha (*tef1*) and intergenic spacer region (*IGS*), while race 2 and 3 can clearly be discriminated (Gilardi

et al., 2016). Race 1 and 4 are closely related and are the only races present in Europe. Hence, high resolution genotyping approaches should be used to study the genetic diversity within *F. oxysporum* f. sp. *lactucae* races 1 and 4. Genotyping-by-sequencing (GBS) is fit for this purpose and can be applied to a relatively large number of samples: it uses restriction enzymes to reduce genome complexity and combines this with high throughput sequencing to get representative DNA sequence information. GBS has already been performed on several plants, such as watermelon (Lambel *et al.*, 2014) and wheat (Xiao *et al.*, 2016); fungi, such as *F. graminearum* (Talas & McDonald, 2015), *Rhizophagus irregularis* (Savary *et al.*, 2018) and *Magnaporthe oryzae* (Korinsak *et al.*, 2019); and oomycetes such as *Phytophthora infestans* (Hansen *et al.*, 2016). While it is traditionally used to determine within-species genetic diversity, the resulting data can also be analysed in a reference-free manner, to simultaneously determine intra- and interspecific genetic diversity in the absence of reference genome data (Van Poucke *et al.*, submitted).

Differences of Fusarium wilt development were observed in several Belgian commercial lettuce glasshouses. Our first objective was to obtain a collection of isolates and use controlled inoculation experiments with a susceptible butterhead lettuce cultivar to see if this variability in disease development was due to differences in virulence between those isolates. Our second objective was to genetically characterize the isolate collection using a variety of methods. We used the race-specific assays from Pasquali *et al.* (2007) and Gilardi *et al.* (2016), together with inoculation assays on differential lettuce cultivars to determine the race of each isolate. We then studied the genetic diversity of our collection of Belgian *F. oxysporum* f. sp. *lactucae* isolates using GBS. An additional phylogenetic analysis based on the sequences of the translation elongation factor 1-alpha (*tef1*), calmodulin (*cmdA*) and RNA polymerase II second largest subunit (*rpb2*) was carried out to verify the GBS analysis for selected isolates. Finally, we related the differences in pathogenicity to the observed genotypic differences.

5.3 Materials and methods

5.3.1 Fungal isolation and DNA-extraction

Butterhead lettuce plants with Fusarium wilt symptoms grown in soil in commercial glasshouses were collected from 2015 until 2018 in Belgium. Six symptomatic pieces of roots or leaves (0.5-1 cm^2) from one plant were surface sterilized for 30 s with 1% NaOCl, washed three times with distilled sterile water. The pieces were briefly dried and plated on potato dextrose agar (PDA) amended with streptomycin sulphate (100 mg l^{-1}). A plug of mycelium was transferred to fresh PDA in order to obtain pure cultures. A single spore culture was prepared from each isolate that originated from a unique lettuce head and stored at -80°C.

For extraction of genomic DNA, a plug of actively growing mycelium of each isolate was transferred to potato dextrose broth and incubated for one week at 28°C. The mycelial mats were filtered and crushed into fine powder using liquid nitrogen. The genomic DNA was extracted using the Invisorb Spin Plant Mini Kit (Stratec Molecular).

5.3.2 Race identification

5.3.2.1 PCR assasys with specific primers

Race identification of each isolate was done using the race-specific PCR assays developed for race 1 with primers Hani3' (5' CCCTCCAACATTCAACAACTG 3') and Hanilatt3rev (5' ATTCACTGTACACCAACCTTTT 3') (Pasquali *et al.*, 2007), and for race 4 with primers FPUF (5' GGAACCAACCGTCACAATAAC 3') and FPUR (5' GTCGTAACACTAACTCGCTT 3') (Gilardi *et al.*, 2016). Amplification was performed using a Flexcycler PCR Thermal Cycler (Analytik Jena) using the cycling programs described in Pasquali *et al.* (2007) and Gilardi *et al.* (2016). The PCR products were analyzed by electrophoresis (100 V, 25 min) on 2% agarose gels in TAE buffer.

5.3.2.2 Plant assay with differential cultivar set

Four isolates belonging to race 1 (Fus1.39, Fus1.59, Fus1.60 and Fus6.01) and five isolates belonging to race 4 (Fus1.01, Fus1.33, Fus1.34, Fus1.56 and Fus1.58) were selected to perform a pathogenicity assay with a differential set of plants to verify the molecular race identification. Four lettuce cultivars, 'Costa Rica No. 4', 'Banchu Red Fire', 'Romana Romabella 30CN' and 'Patriot' were used. Seeds were surface sterilized for 30 s in 1% NaOCl and washed three times with sterile water. The seeds were sown in sterilized potting soil (Structural, Universal type 1, M. Snebbout n.v./s.a.) and grown for 2 weeks at 18°C (16 h light, 8 h dark). The roots of the 3 to 4 leaf stage lettuce plants were carefully washed before inoculation. A spore suspension mainly containing microconidia was prepared from a two-week old culture grown on PDA at room temperature (19-22°C) in the dark. The roots were dipped in a spore suspension of 5×10^5 spores ml^{-1} for 10 min at 150 rpm. Water was used as a control and each treatment had five replicates. The plants were planted in 100 g of sterilized potting soil and grown for 3 weeks at 24°C (16 h light, 8 h dark). Wilting symptoms were scored on a scale from 0 to 4; with 0 = no symptoms, 1 = limited growth reduction and oldest leaves show minor wilting symptoms, 2 = severe growth reduction and oldest leaves show clear wilting symptoms, 3 = severe wilting symptoms, only the center of the plant is alive, and 4 = dead plant (Figure 5.1). The disease index (DI) was calculated for each isolate for every cultivar as follows: [(0 × number of plants within class 0)+(1 × number of plants within class 1)+(2 × number of plants within class 2)+(3 × number of plants within class 3)+(4 × number of plants within class 4)]/(total number of plants within treatment × 4) × 100. The plant experiment was conducted twice.

Figure 5.1 Disease scale (0-4) to score Fusarium wilt on lettuce with 0 = no symptoms, 1 = limited growth reduction and oldest leaves show minor wilting symptoms, 2 = severe growth reduction and oldest leaves show clear wilting symptoms, 3 = severe wilting symptoms, only the center of the plant is alive, and 4 = dead plant.

5.3.3 Pathogenicity of race 1 and 4

The pathogenicity of the two races was compared using four isolates of race 1 (Fus1.39, Fus1.59, Fus1.60 and Fus6.01) and six isolates of race 4 (Fus1.01, Fus1.02, Fus1.33, Fus1.34, Fus1.56 and Fus1.58). A susceptible butterhead lettuce cv. 'Cosmopolia' was used in these trials and grown as described before. We used two inoculation methods: a root dip with microconidia and mixing of chlamydospores in the potting soil.

5.3.3.1 Root dip assay with microconidia

In a first experiment, the roots were dipped in a spore suspension of 5×10^5 spores ml^{-1} as described above. Since no clear differences in symptoms were observed, lower concentrations (5×10^5, 5×10^4, 5×10^3 and 5×10^2 spores ml^{-1}) were tested in a second experiment with isolates Fus1.01 (race 4) and Fus1.39 (race 1) only. Each treatment had eight replicates. The plants were grown for 3 weeks at 24°C and disease was scored as described above.

5.3.3.2 Chlamydospore-based inoculation

For the second inoculation method, the lettuce plants were grown in sterilized potting soil inoculated with chlamydospores. The chlamydospore inoculum was prepared according to Smith & Snyder (1971). Air-dried sandy loam soil (200 g) was autoclaved in glass jars on two consecutive days and subsequently inoculated with 10 ml of a microconidia-suspension of 10^7 spores ml^{-1}. The lid was loosely closed to allow evaporation. The soil was incubated for 3 weeks at 23°C until it was completely dried out. The final inoculum density was checked by dilution plating on PDA supplemented with 100 mg l^{-1} streptomycine sulphate. The chlamydospore inoculum was serially diluted with sterile potting soil to achieve final concentrations of 500, 100 and 10 cfu g^{-1} of potting soil. Lettuce plants were planted in 100 g of inoculated potting soil. Sterile potting soil was used as a control treatment. Every treatment had eight replicates. The plants were grown for 3 weeks at 24°C and scored as described above. This experiment was conducted twice.

5.3.4 Genetic diversity of race 1 and 4 and phylogenomic analysis

GBS was conducted on the *Fusarium* isolates listed in Table 5.1, wherefore the genomic DNA was extracted as described above. The used method was described by Van Poucke *et al.* (submitted), except that the libraries were sequenced by different sequencing providers (SEQme Dobříš, Czech Republic and Admera, New Jersey, USA). The loci identification was also conducted as described in Van Poucke *et al.* (submitted) except that for the indloc module of GIbPSs the default settings were used and that loci with a length between 32 and 300 bp were selected using data selector. For the phylogenomic analysis based on the GBS data, the loci shared by the selected isolates (see Table 5.1) were concatenated and aligned with a Perl script (Van Poucke *et al.*, submitted). The resulting fasta file was converted into a phylip file using jModeltest2 v2.1.10 (Darriba *et al.*, 2012). The invariant single-nucleotide polymporphisms (SNPs) were subsequently removed with the "ascbias.py" script from https://github.com/btmartin721/raxml_ascbias and resulted in a total of 646 variable SNPs. A maximum likelihood (ML) phylogenetic tree was constructed using the GTRCAT model without rate heterogeneity with a correction for ascertainment bias in the program RAxML v8.2.10 (Stamatakis, 2014). The Lewis correction was used since there were no invariant SNPs present in the dataset. Hundred bootstrap

analyses were applied to assess the statistical support of the branches. The resulting tree was visualized using iTOL (Letunic & Bork, 2019).

5.3.5 Phylogenetic analysis based on *tef1*, *cmdA* and *rpb2* sequence data

Partial sequences from translation elongation factor 1-alpha (*tef1*), calmodulin (*cmdA*) and RNA polymerase II second largest subunit (*rpb2*) were amplified from *F. oxysporum* f. sp. *lactucae* isolates Fus1.01, Fus1.39, Fus1.56, Fus6.01 and PD01504750896; *F. oxysporum* f. sp. *rhois* CBS 220.49 and *F. oxysporum* f. sp. *tulipae* CBS 242.59 (Table 5.1). A 50-µl reaction volume with 25 ng genomic DNA and 10 mM dNTPs, 0.5 µM of each primer, 1× PCR buffer and 1 U Taq DNA polymerase (Qiagen) was used for every PCR assay. *Tef1* was amplified with the primers EF1 (5' ATGGGTAAGGARGACAAGAC 3') and EF2 (5' GGARGTACCAGTSATCATGTT 3') (O'Donnell *et al.*, 1998) and with the following program: 94°C for 30 s, 35 cycles of 94°C for 30 s, 53°C for 45 s and 72°C for 1 min; and a final extension of 5 min at 72°C.

CmdA was amplified with the primers CAL228F (5' GAGTTCAAGGAGGCCTTCTCCC 3', Carbone & Kohn, 1999) and CAL2Rd (5' TGRTCNGCCTCDCGGATCATCTC 3', Groenewald *et al.*, 2003) with the following program: 94°C for 10 min, 35 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min; and a final extension of 10 min at 72°C.

Rpb2 was amplified with the primers RPB2-5F2 (5' GGGGWGAYCAGAAGAAGGC 3', Sung *et al.*, 2007) and fRPB2-7cR (5' CCCATRGCTTGTYYRCCCAT 3', Liu *et al.*, 1999) with the following program: 94°C for 10 min, 35 cycles of 94°C for 1 min, 53 or 56°C for 1 min and 72°C for 1 min; and a final extension of 10 min at 72°C.

The PCR products were analyzed by electrophoresis (100 V, 25 min) on 2% agarose gels in TAE buffer. Purification of PCR products with Exosap was carried out to remove excess of primers and dNTPs. The amplified fragments were sent for sequencing in both directions to LGC genomics (Berlin, Germany). Consensus sequences were obtained by assembling forward and reverse sequences using BioEdit version 7 (Hall, 1999). For each locus, multiple alignments of the DNA sequences of the 7 strains together with 32 reference strains from Lombard *et al.* (2019) were made using MUSCLE in MEGA 7 (Kumar *et al.*, 2016) and corrected manually. All sequences used are summarized in Table 5.2. The Kimura's two parameter model was selected for all loci based on the BIC criterion. Phylogenetic analysis with the ML method including 1000 bootstraps replicates were conducted on the three loci, either separately or as a multilocus sequence dataset.

Table 5.1 *Fusarium* isolates collected in this study from butterhead lettuce grown in Belgian glasshouses (Fus1.01 till Fus1.80) and reference *Fusarium* isolates with race identification using specific PCR-assays for race 4 (Gilardi *et al.*, 2016) and race 1 (Pasquali *et al.*, 2007).

Species	Isolate	Grower	Origin	Collection date	Host	Race[a]	Phylogeny[b]	Disease development[c]
Isolated from Belgian glasshouse-grown butterhead lettuce								
F. oxysporum f. sp. *lactucae*	Fus1.01	1	Emblem	2015	*Lactuca sativa* cv. 'Halewyn'	4	1,2	+
F. oxysporum f. sp. *lactucae*	Fus1.02	2	Sint-Katelijne-Waver	17/11/2015	*Lactuca sativa*	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.03	2	Sint-Katelijne-Waver	17/11/2015	*Lactuca sativa*	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.04	2	Sint-Katelijne-Waver	17/11/2015	*Lactuca sativa*	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.05	1	Emblem	2015	*Lactuca sativa* cv. 'Halewyn'	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.06	1	Emblem	2015	*Lactuca sativa* cv. 'Halewyn'	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.09	4	Kapelle-op-den-Bos	12/07/2016	*Lactuca sativa*	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.10	4	Kapelle-op-den-Bos	12/07/2016	*Lactuca sativa*	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.11	4	Kapelle-op-den-Bos	12/07/2016	*Lactuca sativa*	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.12	5	Hamme	16/08/2016	*Lactuca sativa*	4	1	
F. oxysporum f. sp. *lactucae*	Fus1.13	5	Hamme	16/08/2016	*Lactuca sativa*	4	1	
F. oxysporum f. sp. *lactucae*	Fus1.14	5	Hamme	16/08/2016	*Lactuca sativa*	4	1	
F. oxysporum f. sp. *lactucae*	Fus1.15	5	Hamme	16/08/2016	*Lactuca sativa*	4	1	
F. oxysporum f. sp. *lactucae*	Fus1.16	4	Kapelle-op-den-Bos	2/08/2017	*Lactuca sativa* cv. 'Lucrecia'	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.17	4	Kapelle-op-den-Bos	2/08/2017	*Lactuca sativa* cv. 'Lucrecia'	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.18	6	Reet	24/07/2017	*Lactuca sativa*	4	1	
F. oxysporum f. sp. *lactucae*	Fus1.19	6	Reet	24/07/2017	*Lactuca sativa*	4	1	
F. oxysporum f. sp. *lactucae*	Fus1.20	6	Reet	24/07/2017	*Lactuca sativa*	4	1	
Fusarium sp.	Fus1.21	7	Onze-Lieve-Vrouw-Waver	14/07/2017	*Lactuca sativa*	-		+
F. oxysporum f. sp. *lactucae*	Fus1.22	7	Onze-Lieve-Vrouw-Waver	14/07/2017	*Lactuca sativa*	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.23	8	Melsele	27/10/2017	*Lactuca sativa*	4	1	-
F. oxysporum f. sp. *lactucae*	Fus1.24	8	Melsele	27/10/2017	*Lactuca sativa*	4	1	-
F. oxysporum f. sp. *lactucae*	Fus1.25	8	Melsele	27/10/2017	*Lactuca sativa*	4	1	-
F. oxysporum f. sp. *lactucae*	Fus1.26	9	Rummen	26/10/2017	*Lactuca sativa*	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.27	9	Rummen	26/10/2017	*Lactuca sativa*	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.28	9	Rummen	26/10/2017	*Lactuca sativa*	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.29	10	Duffel	21/10/2017	*Lactuca sativa*	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.30	11_A	Hooglede	28/09/2017	*Lactuca sativa*	4	1	-
F. oxysporum f. sp. *lactucae*	Fus1.31	11_A	Hooglede	28/09/2017	*Lactuca sativa*	4	1	-
F. oxysporum f. sp. *lactucae*	Fus1.32	11_A	Hooglede	28/09/2017	*Lactuca sativa*	4	1	-
F. oxysporum f. sp. *lactucae*	Fus1.33	11_B	Hooglede	23/10/2017	*Lactuca sativa*	4	1	-/+

F. oxysporum f. sp. *lactucae*	Fus1.34	11_B	Hooglede	23/10/2017	*Lactuca sativa*	4	1	-/+
F. oxysporum f. sp. *lactucae*	Fus1.35	12	Torhout	2/11/2017	*Lactuca sativa*	4	1	-
F. oxysporum f. sp. *lactucae*	Fus1.36	12	Torhout	2/11/2017	*Lactuca sativa*	4	1	-
F. oxysporum f. sp. *lactucae*	Fus1.37	12	Torhout	2/11/2017	*Lactuca sativa*	4	1	-
Fusarium sp.	Fus1.38	13	Roeselare	24/11/2017	*Lactuca sativa*	-		-
F. oxysporum f. sp. *lactucae*	Fus1.39	13	Roeselare	24/11/2017	*Lactuca sativa*	1	1,2	-
F. oxysporum f. sp. *lactucae*	Fus1.40	14	Putte	28/11/2017	*Lactuca sativa*	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.41	14	Putte	28/11/2017	*Lactuca sativa*	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.42	15	Onze-Lieve-Vrouw-Waver	16/04/2018	*Lactuca sativa*	4	1	++
F. oxysporum f. sp. *lactucae*	Fus1.43	15	Onze-Lieve-Vrouw-Waver	16/04/2018	*Lactuca sativa*	4	1	++
F. oxysporum f. sp. *lactucae*	Fus1.44	15	Onze-Lieve-Vrouw-Waver	16/04/2018	*Lactuca sativa*	4	1	++
F. oxysporum f. sp. *lactucae*	Fus1.45	15	Onze-Lieve-Vrouw-Waver	16/04/2018	*Lactuca sativa*	4	1	++
F. oxysporum f. sp. *lactucae*	Fus1.46	16_A	Vrasene	16/05/2016	*Lactuca sativa*	4	1	++
F. oxysporum f. sp. *lactucae*	Fus1.47	16_A	Vrasene	16/05/2016	*Lactuca sativa*	4	1	++
F. oxysporum f. sp. *lactucae*	Fus1.48	16_B	Vrasene	16/05/2016	*Lactuca sativa*	4	1	++
F. oxysporum f. sp. *lactucae*	Fus1.49	16_B	Vrasene	16/05/2016	*Lactuca sativa*	4	1	++
F. oxysporum f. sp. *lactucae*	Fus1.50	17	Deinze	12/07/2018	*Lactuca sativa*	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.51	17	Deinze	12/07/2018	*Lactuca sativa*	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.52	17	Deinze	12/07/2018	*Lactuca sativa*	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.53	18	Lichtervelde	2018	*Lactuca sativa*	4	1	-
F. oxysporum f. sp. *lactucae*	Fus1.54	18	Lichtervelde	2018	*Lactuca sativa*	4	1	-
F. oxysporum f. sp. *lactucae*	Fus1.55	18	Lichtervelde	2018	*Lactuca sativa*	4	1	-
F. oxysporum f. sp. *lactucae*	Fus1.56	19	Gits	21/08/2018	*Lactuca sativa*	4	1,2	-
F. oxysporum f. sp. *lactucae*	Fus1.57	19	Gits	21/08/2018	*Lactuca sativa*	4	1	-
F. oxysporum f. sp. *lactucae*	Fus1.58	19	Gits	21/08/2018	*Lactuca sativa*	4	1	-
F. oxysporum f. sp. *lactucae*	Fus1.59	20	Gits	2018	*Lactuca sativa*	1	1	-
F. oxysporum f. sp. *lactucae*	Fus1.60	20	Gits	2018	*Lactuca sativa*	1	1	-
F. oxysporum f. sp. *lactucae*	Fus1.61	21	Ardooie	23/10/2018	*Lactuca sativa*	4	1	-
F. oxysporum f. sp. *lactucae*	Fus1.62	21	Ardooie	23/10/2018	*Lactuca sativa*	4	1	-
F. oxysporum f. sp. *lactucae*	Fus1.63	22	Putte	9-11/2018	*Lactuca sativa*	4	1	-
F. oxysporum f. sp. *lactucae*	Fus1.64	22	Putte	9-11/2018	*Lactuca sativa*	4	1	-
F. oxysporum f. sp. *lactucae*	Fus1.65	22	Putte	9-11/2018	*Lactuca sativa*	4	1	-
F. oxysporum f. sp. *lactucae*	Fus1.66	22	Putte	9-11/2018	*Lactuca sativa*	4	1	-
F. oxysporum f. sp. *lactucae*	Fus1.67	22	Putte	9-11/2018	*Lactuca sativa*	4	1	-
F. oxysporum f. sp. *lactucae*	Fus1.68	23	Sijsele	9-11/2018	*Lactuca sativa*	4	1	+

F. oxysporum f. sp. *lactucae*	Fus1.69	23	Sijsele	9-11/2018	*Lactuca sativa*	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.70	23	Sijsele	9-11/2018	*Lactuca sativa*	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.71	23	Sijsele	9-11/2018	*Lactuca sativa*	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.71	23	Sijsele	9-11/2018	*Lactuca sativa*	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.72	24	Gits	9-11/2018	*Lactuca sativa*	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.73	24	Gits	9-11/2018	*Lactuca sativa*	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.74	24	Gits	9-11/2018	*Lactuca sativa*	4	1	+
F. oxysporum f. sp. *lactucae*	Fus1.75	24	Gits	9-11/2018	*Lactuca sativa*	1	1	+
F. oxysporum f. sp. *lactucae*	Fus1.76	24	Gits	9-11/2018	*Lactuca sativa*	1	1	+
F. oxysporum f. sp. *lactucae*	Fus1.77	25	Broechem	28/12/2018	*Lactuca sativa*	4	1	-
F. oxysporum f. sp. *lactucae*	Fus1.78	25	Broechem	28/12/2018	*Lactuca sativa*	4	1	-
F. oxysporum f. sp. *lactucae*	Fus1.79	25	Broechem	28/12/2018	*Lactuca sativa*	4	1	-
F. oxysporum f. sp. *lactucae*	Fus1.80	25	Broechem	28/12/2018	*Lactuca sativa*	4	1	-
Reference isolates								
F. curvatum	CBS 247.61		Germany	1957	*Matthiola incana*	4	1,2	
F. nirenbergiae	CBS 130303		USA	Unknown	*Solanum lycopersicum*	Nt	1,2	
F. oxysporum f. sp. *asparagi*	CBS 143081		The Netherlands	2017	*Asparagus*	Nt		
F. oxysporum f. sp. *cepae*	CBS 148.25		Unknown	Unknown	*Allium cepa*	Nt		
F. oxysporum f. sp. *conglutinans*	CBS 186.53		USA	Unknown	*Brassica oleracea*	Nt		
F. oxysporum f. sp. *lactucae*	PD01504750896		The Netherlands	2015	*Lactuca sativa*	4	1,2	
F. oxysporum f. sp. *lactucae*	PD01504750888		The Netherlands	2015	*Lactuca sativa*	4	1	
F. oxysporum f. sp. *lactucae*	Fus6.01		Japan	Unknown	*Lactuca sativa*	1	1,2	
F. oxysporum f. sp. *lilii*	CBS 130322		USA	Unknown	*Lilium*	Nt		
F. oxysporum f. sp. *melonis*	CBS 420.90		Israel	1986	*Cucumis melo*	Nt		
F. oxysporum f. sp. *opuntiarum*	CBS 743.79		Germany	1959	*Zygocactus truncatus*	Nt		
F. oxysporum f. sp. *phaseoli*	CBS 935.73		USA	1969	*Phaseolus*	Nt		
F. oxysporum f. sp. *rhois*	CBS 220.49		Unknown	Unknown	*Rhus typhina*	Nt	1,2	
F. oxysporum f. sp. *tulipae*	CBS 242.59		Germany	1957	*Tulipa*	Nt	1,2	
F. oxysporum f. sp. *vasinfectum*	CBS 116615		Ivory Coast	1995	*Gossipium hirsutum*	Nt		

[a] Nt = not tested; - = not detected

[b] 1 = used in phylogenomic tree based on GBS data; 2 = used in phylogenetic analysis based on tef1, cmdA and rpb2 sequence data

[c] ++ = very fast; + = fast; +/- = moderate; - = slow

Table 5.2 References of the *cmdA*, *rpb2* and *tef1* sequences of the selected *Fusarium* isolates that were included in the phylogenetic analysis. Genbank accession numbers in bold were obtained in this study.

Species	Isolate	Host/substrate	Origin	GenBank accession number		
				cmdA	***rpb2***	***tef1***
F. callistephi	CBS 115423	*Agathosma betulina*	South Africa	MH484723	MH484905	MH484996
F. cugenangense	CBS 620.72	*Crocus* sp.	Germany	MH484697	MH484879	MH484970
F. curvatum	CBS 247.61	*Matthiola incana*	Germany	MH484694	MH484876	MH484967
	CBS 238.94	*Beaucarnia* sp.	The Netherlands	MH484711	MH484893	MH484984
	CBS 141.95	*Hedera helix*	The Netherlands	MH484712	MH484894	MH484985
F. duoseptatum	CBS 102026	*Musa sapientum* cv. *Pisang ambon*	Malaysia	MH484714	MH484896	MH484987
F. elaeidis	CBS 217.49	*Elaeis* sp.	Congo DR	MH484688	MH484870	MH484961
	CBS 218.49	*Elaeis* sp.	Congo DR	MH484689	MH484871	MH484962
	CBS 255.52	*Elaeis guineensis*	Unknown	MH484692	MH484874	MH484965
F. foetens	CBS 120665	*Nicotiana tabacum*	Iran	MH484736	MH484918	MH485009
F. glycines	CBS 176.33	*Linum usitatissimum*	Unknown	MH484686	MH484868	MH484959
	CBS 214.49	Unknown	Argentina	MH484687	MH484869	MH484960
	CBS 200.89	*Ocimum basilicum*	Italy	MH484706	MH484888	MH484979
F. gossypinum	CBS 116611	*Gossypium hirsutum*	Ivory Coast	MH484725	MH484907	MH484998
	CBS 116612	*Gossypium hirsutum*	Ivory Coast	MH484726	MH484908	MH484999
	CBS 116613	*Gossypium hirsutum*	Ivory Coast	MH484727	MH484909	MH485000
F. hoodiae	CBS 132474	*Hoodia gordonii*	South Africa	MH484747	MH484929	MH485020
	CBS 132476	*Hoodia gordonii*	South Africa	MH484748	MH484930	MH485021
	CBS 132477	*Hoodia gordonii*	South Africa	MH484749	MH484931	MH485022
F. languescens	CBS 645.78	*Solanum lycopersicum*	Morocco	MH484698	MH484880	MH484971
	CBS 302.91	*Solanum lycopersicum*	The Netherlands	MH484710	MH484892	MH484983
	CBS 872.95	*Solanum lycopersicum*	Unknown	MH484713	MH484895	MH484986
F. nirenbergiae	CBS 130303	*Solanum lycopersicum*	USA	MH484741	MH484923	MH485014
	CBS 840.88	*Dianthus caryophyllus*	The Netherlands	MH484705	MH484887	MH484978
	CBS 149.25	*Musa* sp.	Unknown	MH484683	MH484865	MH484956
F. oxysporum	CBS 221.49	*Camellia sinensis*	South East Asia	MH484690	MH484872	MH484963
	CPC 25822	*Protea* sp.	South Africa	MH484761	MH484943	MH485034

	CBS 144134	*Solanum tuberosum*	Germany	MH484771	MH484953	MH485044
	CBS 144135	*Solanum tuberosum*	Germany	MH484772	MH484954	MH485045
F. oxysporum f. sp. *lactucae*	PD0150475896	*Lactuca sativa*	The Netherlands	**MN837479**	**MN837486**	**MN837493**
	Fus1.01	*Lactuca sativa*	Belgium	**MN837480**	**MN837487**	**MN837494**
	Fus1.39	*Lactuca sativa*	Belgium	**MN837482**	**MN837489**	**MN837496**
	Fus1.56	*Lactuca sativa*	Belgium	**MN837481**	**MN837488**	**MN837495**
	Fus6.01	*Lactuca sativa*	Japan	**MN837483**	**MN837490**	**MN837497**
F. oxysporum f. sp. *rhois*	CBS 220.49	*Rhus typhina*	Unknown	**MN837477**	**MN837484**	**MN837491**
F. oxysporum f. sp. *tulipae*	CBS 242.59	*Tulipa*	Germany	**MN837478**	**MN837485**	**MN837492**
F. trachichlamydosporum	CBS 102028	*Musa sapientum* cv. *Pisang awak legor*	Malaysia	MH484715	MH484897	MH484988
F. triseptatum	CBS 258.50	*Ipomoea batatas*	USA	MH484691	MH484873	MH484964
F. udum	CBS 177.31	*Digitaria eriantha*	South Africa	MH484684	MH484866	MH484957

5.3.6 Statistical analysis

The data were analyzed using scripts written in R studio and run in R-version 3.5.0. The statistical tests were performed at a confidence level of $P = 0.05$. The distribution of the data of the weights was first analyzed for normality with QQ plots and the Shapiro-Wilk test for every isolate. Homogeneity of variances was tested with Levene's test. A one-way analysis of variance was conducted using Tukey's multiple comparison test, when the assumptions were fulfilled, otherwise the non-parametric Wilcoxon rank sum test was used. The ordinal data of the pathogenicity assays were analyzed with non-parametric statistics. Differences between isolates were detected based on the Wilcoxon rank sum test for every inoculum concentration. The effect of the inoculum concentration, race and their interaction on the disease index from the pathogenicity assay with chlamydospores was analyzed with linear regression.

5.4 Results

5.4.1 Race identification

Between 2015 to 2018, 78 *Fusarium*-isolates were collected from lettuce heads showing wilting symptoms. They originated from 25 different lettuce growers in Belgium (Table 5.1 and Figure 5.2).

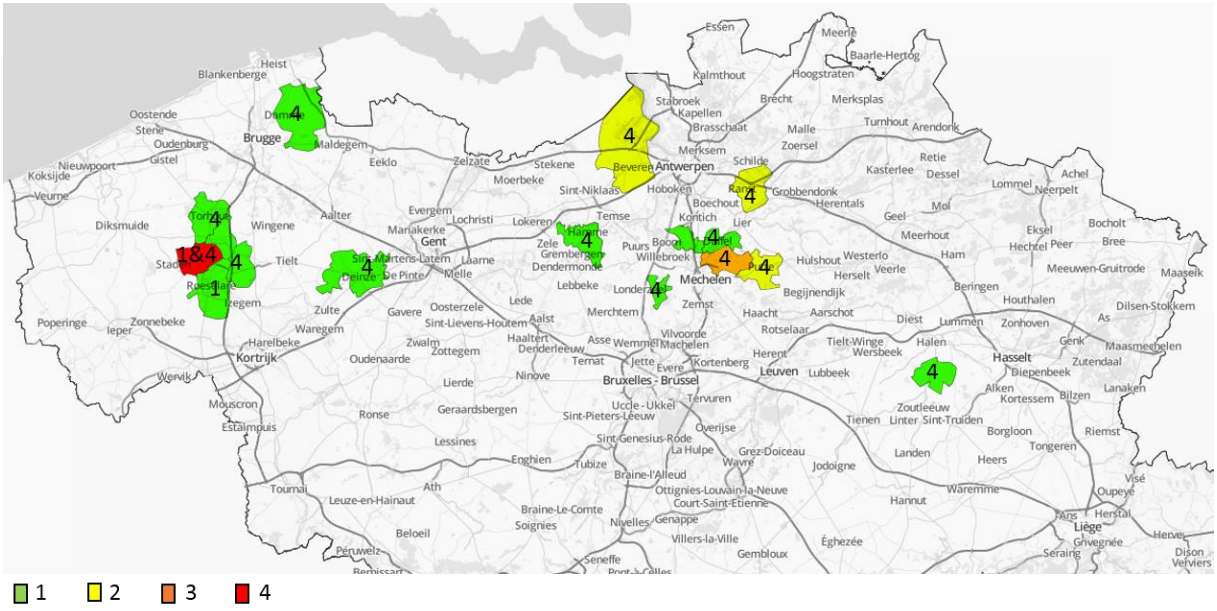

Figure 5.2 Geographical overview of locations where samples with Fusarium symptoms were collected. The number of growers in each town is indicated in green (1 grower), yellow (2 growers), orange (3 growers) or red (4 growers). The identified Fusarium races are marked on the map. (Map made with www.gemeentekaart.be).

5.4.1.1 PCR assays with specific primers

Using the race specific PCR-assays five isolates were assigned to race 1 and 71 isolates to race 4 (Table 5.1). Two isolates could not be attributed to race 1 or race 4. Race 1 was found at two lettuce growers and race 4 at 22 lettuce growers, while simultaneous occurrence of both races was only recorded at one grower.

5.4.1.2 Plant assay with differential cultivar set

Plant inoculation experiments with four race 1 and five race 4 isolates were conducted to verify the results of the race-specific PCR-assays (Figure 5.3). Race 4 isolates were highly pathogenic on 'Costa Rica No. 4' and 'Romana Romabella 30CN', while this was not the case for race 1 isolates. No clear differences in disease symptoms were observed on 'Banchu Red Fire', although race 1 isolates were on average more pathogenic than the control and race 4 isolates. Race 1 isolates were highly pathogenic on cultivar 'Patriot', while race 4 isolates were not.

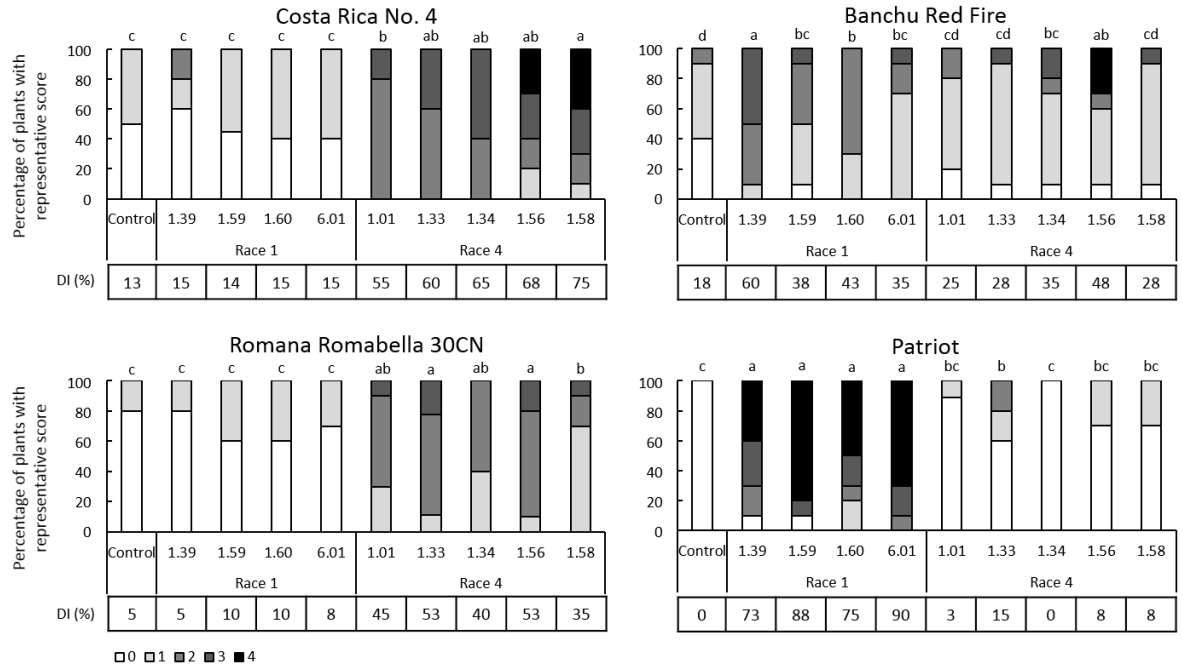

Figure 5.3 Pathogenicity of four race 1 and five race 4 isolates on the differential cultivars 'Costa Rica No. 4', 'Banchu Red Fire', 'Romana Romabella 30CN' and 'Patriot'. Plants were inoculated using the root dip method (in 5 × 10^5 cfu ml^{-1}) and grown at 24°C during 3 weeks. Results show pooled data from two experiments (n = 10). For each cultivar, different letters indicate statistical differences (Wilcoxon rank sum test, $P < 0.05$). DI = Disease index.

Consistent with the disease index, race 4 isolates clearly reduced the weight of the 'Costa Rica No. 4' cultivar in both experiments (Figure 5.4). In experiment 1, plants inoculated with race 4 isolates only weighed 0.4-1.0 g, while the control weighed 6.3 g and plants inoculated with race 1 isolates weighed 3.2-4.9 g. Race 1 isolates Fus1.39 and Fus1.60 also reduced the weight, compared to the control, although to a lesser extent than race 4 isolates. In cultivar 'Romana Romabella 30CN', all race 4 isolates reduced the weight in both experiments, except isolates Fus1.58 in experiment 1 and Fus1.34 in experiment 2 for which the reduction was not statistically significant. No differences in weight were observed between the control and the race 1 isolates in both experiments. Similar to the disease symptoms, no differences in weight were observed between races and the control on the cultivar 'Banchu Red Fire'. Cultivar 'Patriot' showed clear differences between race 1 and 4; the weight of plants inoculated with race 1 was strongly reduced, while the weight of plants inoculated with race 4 was not.

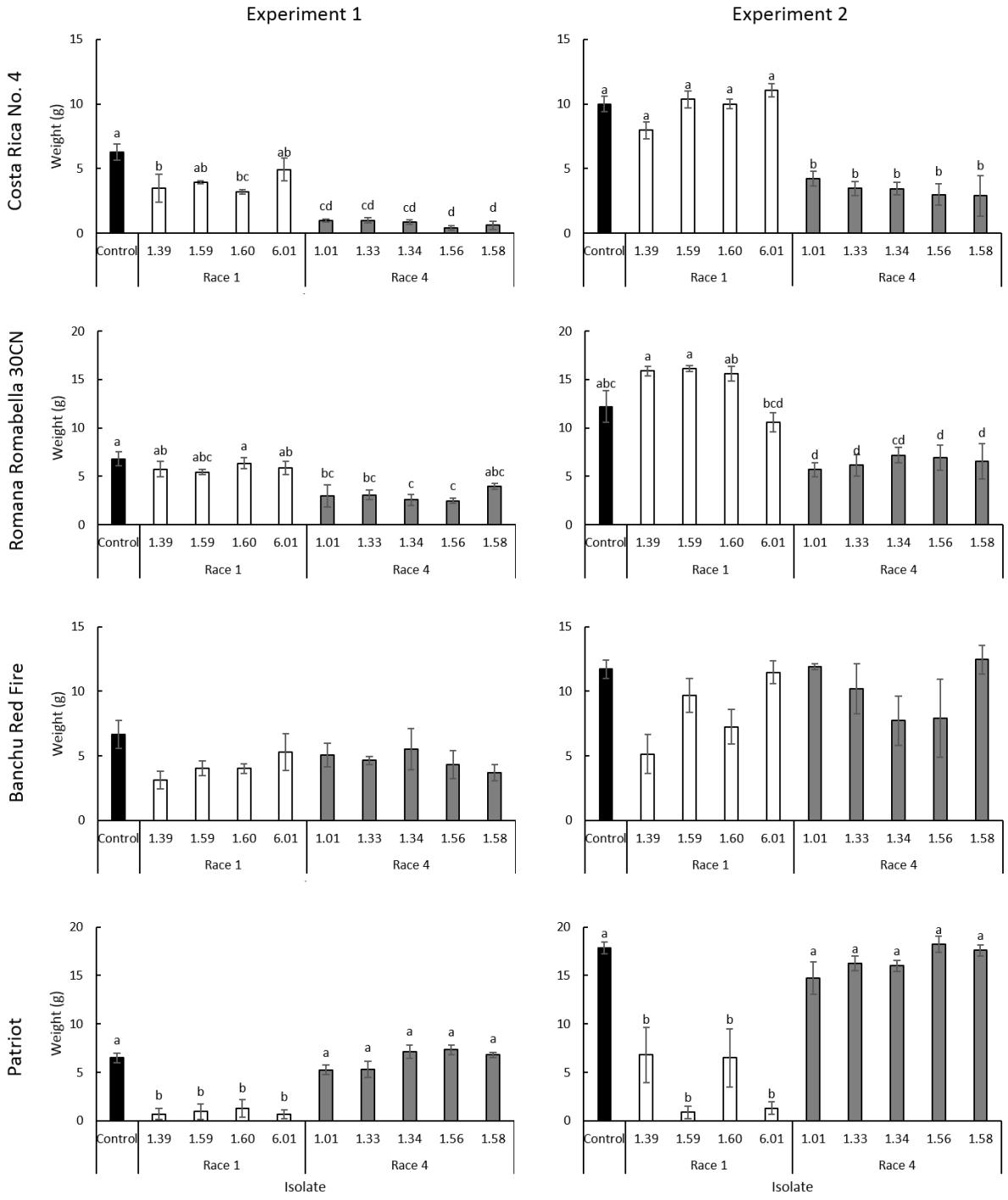

Figure 5.4 The weight of the differential cultivars 'Costa Rica No. 4', 'Banchu Red Fire', 'Romana Romabella 30CN' and 'Patriot' inoculated with race 1 or race 4 isolates using the root dip method (in 5×10^5 cfu ml^{-1}) and grown at 24°C during 3 weeks. Different letters indicate statistical differences (Tukey test, $P < 0.05$, n = 5). Error bars represent the standard error.

5.4.2 Pathogenicity of race 1 and 4

Differences in disease development were observed in commercial glasshouses, even on cultivars that are susceptible to both races (Table 5.1). Moreover, in colder periods, symptoms can be still observed where race 4 occurs, while this is not the case for race 1. Controlled inoculation experiments using two types of inoculum were used to verify if this difference was due to a variation in virulence of the two races.

5.4.2.1 Root dip assay with microconidia

A root dip assay with 5×10^5 conidia ml^{-1} with four race 1 and six race 4 isolates showed a high disease index of 72-97% for all isolates compared to the control (Figure 5.5 A). No differences were observed between isolates, only race 4 isolate Fus1.01 showed more symptoms than race 4 isolate Fus1.02 and race 1 isolates Fus1.59 and Fus1.60. All isolates reduced the weight considerably (Figure 5.5 B). Similar to the disease symptoms, plants inoculated with race 4 isolate Fus1.01 had a lower weight than plants inoculated with race 4 isolate Fus1.02, and race 1 isolates Fus1.59 and Fus1.60.

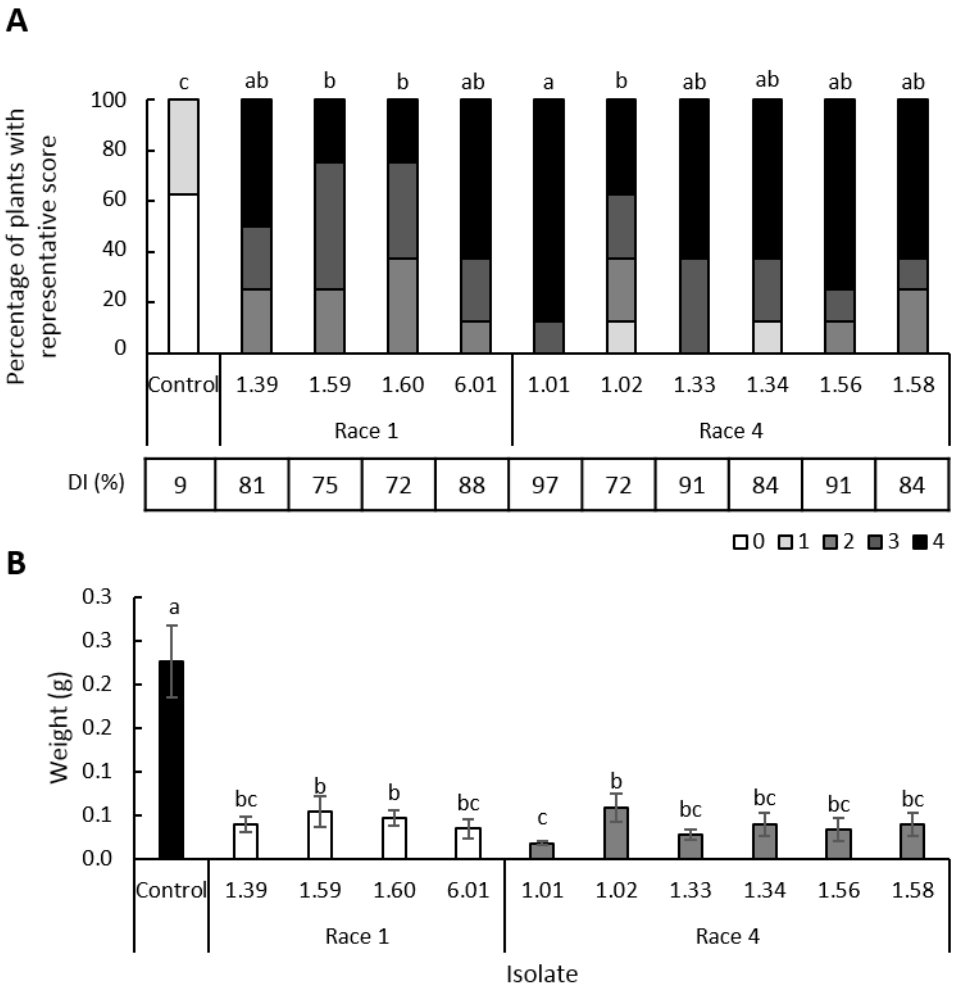

Figure 5.5 Four race 1 and six race 4 isolates inoculated using the root dip method at 5×10^5 cfu ml^{-1} on butterhead lettuce cv. 'Cosmopolia'. **A**: Pathogenicity of the isolates. DI = Disease index. **B**: Weight of the lettuce plants. The plants were grown at 24°C during 3 weeks. Different letters show statistical differences (Wilcoxon rank sum test, $P < 0.05$, n = 8). Error bars represent the standard error.

Subsequently, a root dip assay was carried out with one race 1 isolate (Fus1.39) and one race 4 isolate (Fus1.01) at lower spore suspension concentrations (Figure 5.6). Again the high spore concentration of 5×10^5 cfu ml^{-1} did not show any differences between the two isolates in symptoms or weight. This was also observed at 5×10^4 cfu ml^{-1}. Inoculation with the lower spore concentrations of 5×10^3 cfu ml^{-1} and 5×10^2 cfu ml^{-1} did not show a significant difference with the control in symptoms nor in weight.

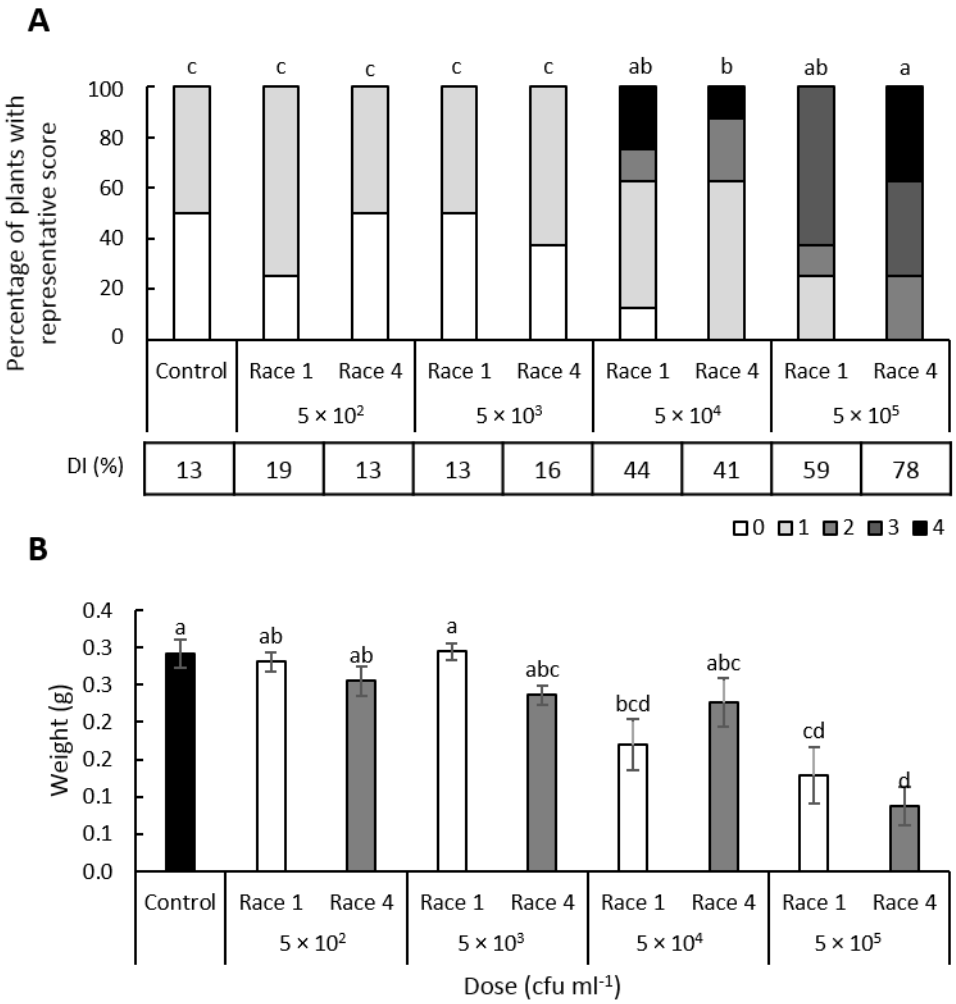

Figure 5.6 One race 1 isolate (Fus1.39) and one race 4 isolate (Fus1.01) inoculated with a root dip of at different concentrations (5×10^2, 5.10^3, 5×10^4 and 5×10^5 cfu ml^{-1}) on butterhead lettuce cv. 'Cosmopolia'. **A**: Pathogenicity of the isolates (Wilcoxon rank sum test, $P < 0.05$, n = 8). DI = Disease index. **B**: Weight of the lettuce plants (Tukey test, $P < 0.05$, n = 8). The plants were grown at 24°C during 3 weeks. Different letters show statistical differences and error bars represent the standard error.

5.4.2.2 Chlamydospore-based inoculation

Two experiments at 24°C were carried out with different concentrations of chlamydospores in the substrate (Supplementary data Figure S5.1 and Figure S5.2). For clarity, the results of the two experiments were pooled (Figure 5.7). Compared with the control, all isolates produced disease symptoms at all concentrations tested, even as low as 10 cfu g^{-1}. Race 4 isolates were consistently more pathogenic than race 1 isolates at all concentrations. In addition, the weight was strongly reduced by the race 4 isolates and not, or less, by the race 1 isolates (Supplementary data Figure S5.2).

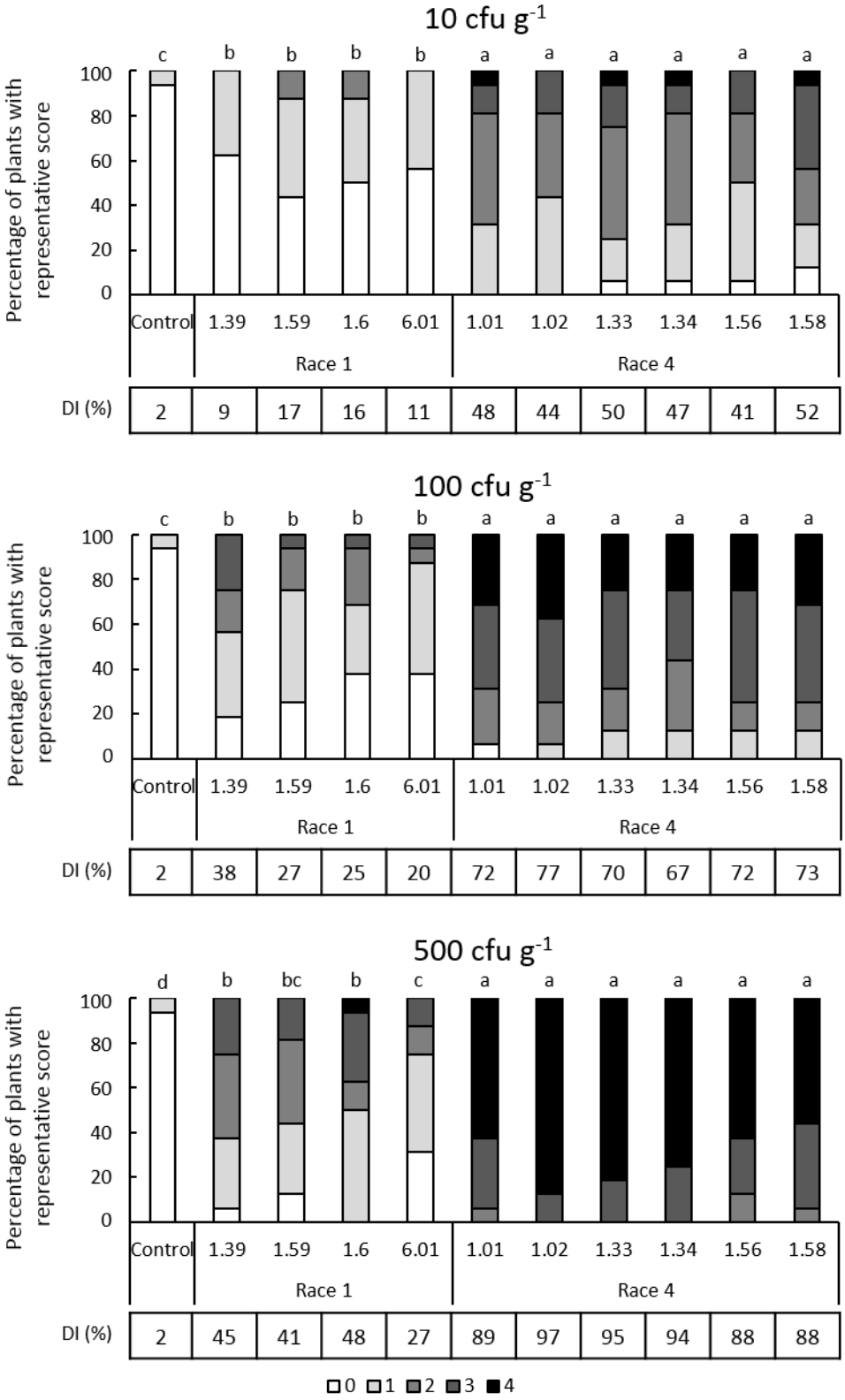

Figure 5.7 Pathogenicity on butterhead lettuce cv. 'Cosmopolia' inoculated with different concentrations of chlamydospores (10, 100 or 500 cfu g^{-1}) of four race 1 and six race 4 isolates. The plants were grown at 24°C during 3 weeks. Pooled results of two experiments. Different letters indicate statistical differences (Wilcoxon rank sum test, $P < 0.05$, n = 8). DI = Disease index.

In experiment 1, the race, dose and the interaction between race and dose had an effect on the disease index ($P < 0.01$) (Figure 5.8). The average disease index (DI) was 12, 13 and 23% for race 1, and 45, 69 and 92% for race 4 at 10, 100 and 500 cfu g^{-1}, respectively. In experiment 2, again the race and dose had an effect on the disease index ($P < 0.01$). The average DI was 15, 42, and 58% for race 1, and 49, 76 and 92% for race 4 at 10, 100 and 500 cfu g^{-1}, respectively. In experiment 2 more disease symptoms were observed at higher doses. This is probably due to a slightly higher temperature during the experiment.

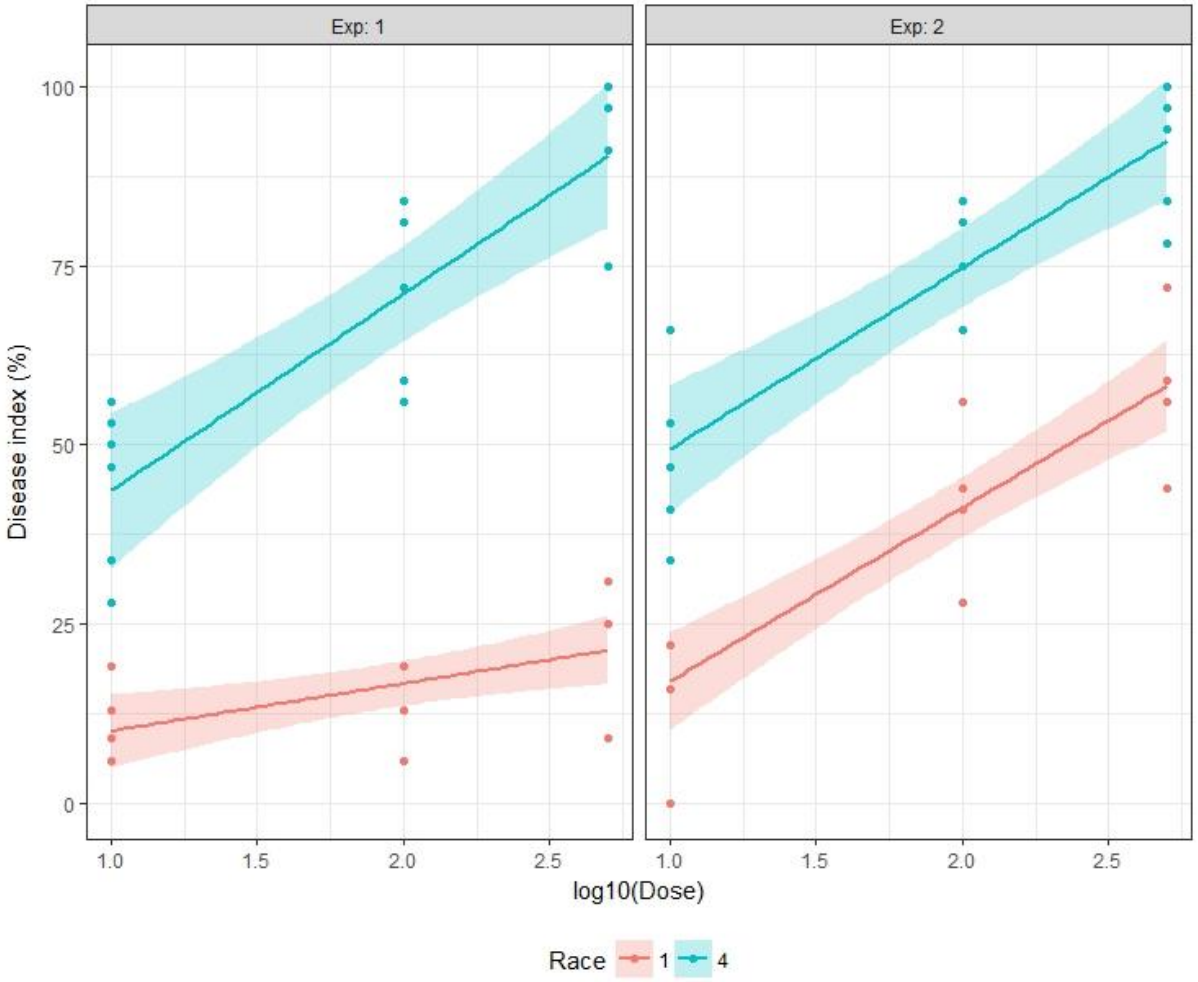

Figure 5.8 Dose response of different chlamydospores concentrations on the disease index of race 1 (n = 4) and 4 (n = 6) for experiment 1 and 2. Linear regressions with 95% confidence intervals are shown.

5.4.3 Genetic diversity of race 1 and 4 and phylogenomic analysis

The GBS analysis generated sequence data from 10,574 ± 257 loci for race 1 isolates and 10,832 ± 173 loci for the race 4 isolates. Given an average locus length of 149 bp, these loci represent a total of 1,575,526 bp sequence information for race 1 and 1,613,968 bp for race 4. The differential SNPs present in the common loci of all isolates were used to produce a ML phylogenetic tree (Figure 5.9). The race 4 isolates clearly grouped in a separate clade from the race 1 isolates. Within race 1 the diversity was very small (4 SNPs in 10,037 shared loci). In race 4 the diversity was slightly higher (40 SNPs in 9,596 shared loci) due to the presence of one subclade containing eight isolates. These eight isolates

originated from three lettuce growers (growers 18, 19 and 21 in Table 5.1), which are geographically less than 10 km apart. The forma specialis *F. oxysporum* f. sp. *rhois* and *F. oxysporum* f. sp. *tulipae* and the species *F. nirenbergiae*, which are closely related to non-distinguishable from *F. oxysporum* f. sp. *lactucae* race 1 and 4 based on *tef1*, *cmdA* and *rpb2* multilocus sequence information (see further), were well separated from *F. oxysporum* f. sp. *lactucae* race 1 and 4 using the GBS sequence data. *F. curvatum,* which is a new species that includes the redesignated *F. oxysporum* f. sp. *matthiolae* (Lombard *et al.*, 2019), clustered with the *F. oxysporum* f. sp. *lactucae* race 4 isolates. This is consistent with the result of the race-specific PCR assays, in which only *F. oxysporum* f. sp. *lactucae* race 4 isolates and *F. curvatum* CBS 247.61 produced a signal with the race 4 assay (Table 5.1).

5.4.4 Phylogenetic analysis based on *tef1*, *cmdA* and *rpb2* sequence data

The *tef1*, *cmdA*, and *rpb2* sequences consisted of 685-705, 569-618 and 771-880 nucleotides, respectively. The sequence datasets for all loci were congruent and were therefore combined to a dataset of 1939 characters, including gaps. The ML tree constructed using Kimura's two parameter model revealed that the *F. oxysporum* f. sp. *lactucae* isolates (race 1 and 4) grouped with *F. curvatum* CBS 247.61, CBS 238.94, CBS 141.95, *F. oxysporum* f. sp. *tulipae* CBS 242.59 and *F. oxysporum* f. sp. *rhois* CBS 220.49. This clade was supported with a bootstrap value of 88% (Figure 5.10). The sequences of the *Fusarium oxysporum* f. sp. *lactucae* isolates were identical to those of *F. curvatum* CBS 247.61 but differed 2 bp from the ones of *F. curvatum* CBS 238.94 and 4 bp from the ones of *F. curvatum* CBS 141.95.

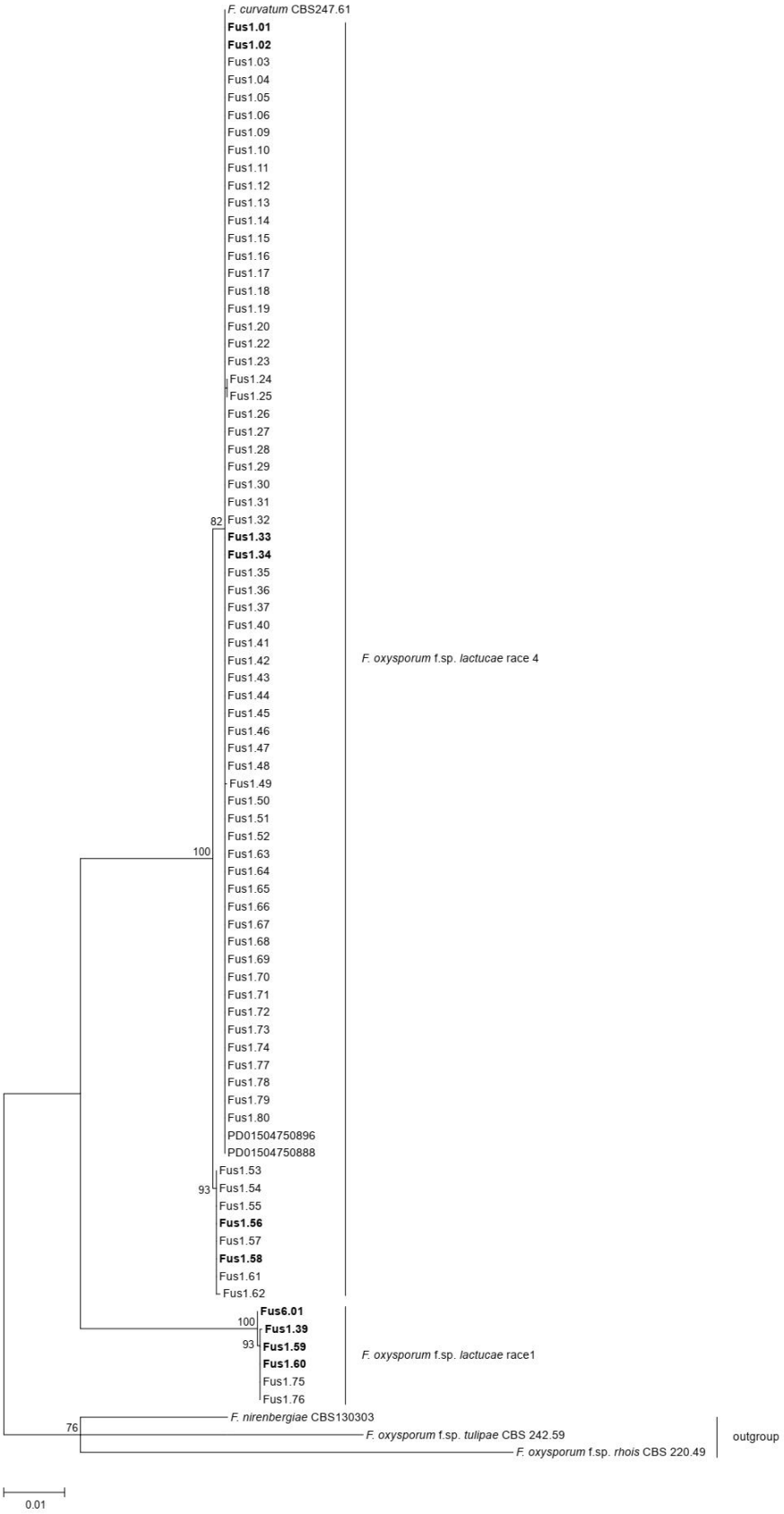

Figure 5.9 Maximum likelihood phylogenomic tree based on the variable SNPs in the GBS data of selected isolates. Numbers on or next to a branch indicate the bootstrap value of that branch. Only values higher than 70 are shown. Isolates that were used in pathogenicity tests are indicated in bold.

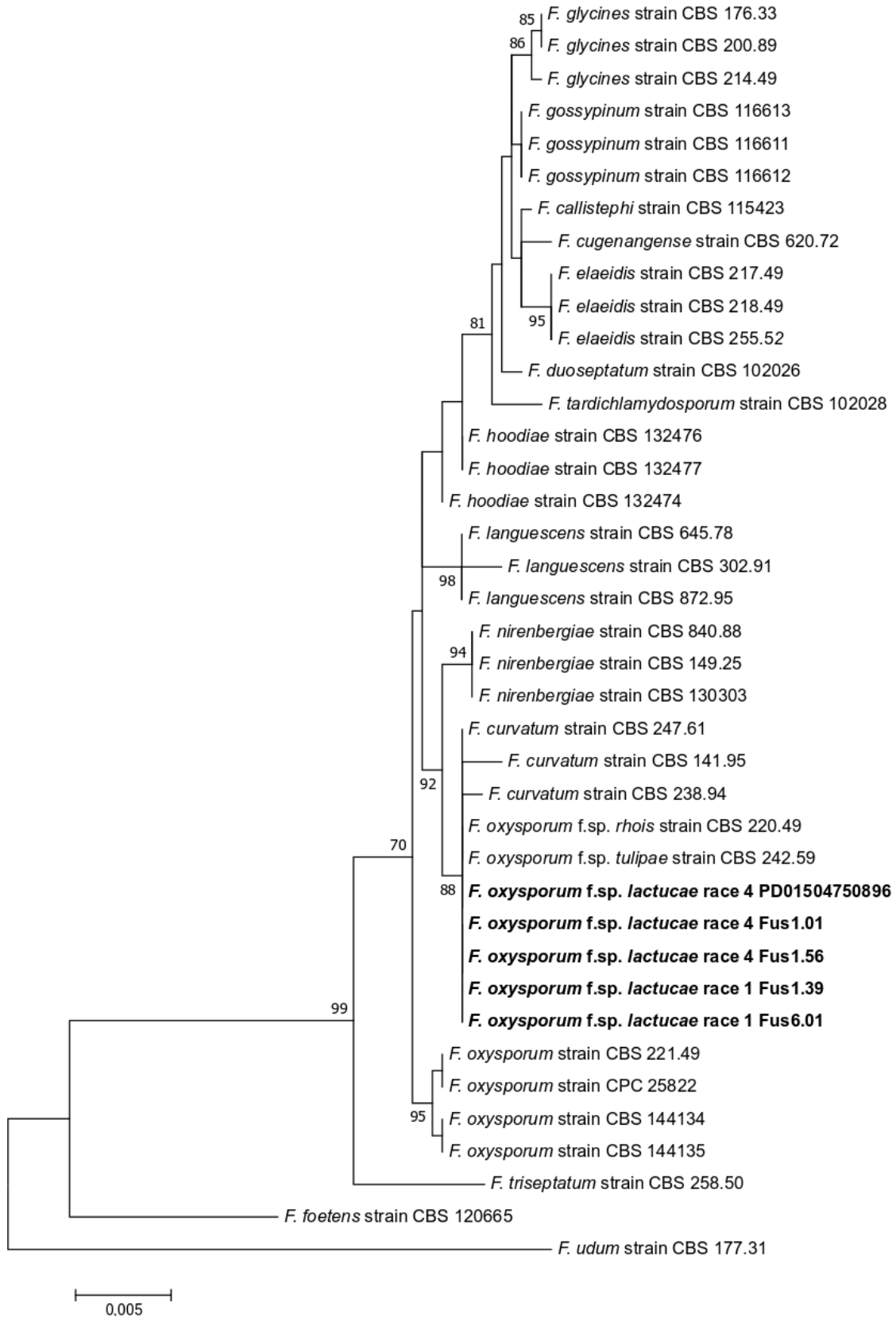

Figure 5.10 Maximum likelihood phylogenetic tree from the combined *cmdA*, *rpb2* and *tef1* sequence alignment. Numbers at the branches indicate the bootstrap value. Only values higher than 70 are shown. *Fusarium oxysporum* f. sp. *lactucae* isolates are indicated in bold.

5.5 Discussion

Seventy-eight *Fusarium*-isolates were collected from symptomatic butterhead lettuce plants in Belgian commercial glasshouses between 2015 and 2018. Specific PCR-assays and additional plant experiments with differential cultivars revealed that 91% of the isolates belonged to race 4 and 6% of the isolates to race 1. Race 1 has a worldwide distribution, but had not yet been confirmed in Belgium. In this work, we observed that race 4 is more virulent than race 1. It is possible that race 1 has been present for several years in Belgium, but that it did not draw much attention until the fast and widespread invasion of race 4.

Inoculation experiments with different lettuce cultivars revealed that 'Patriot' is a good cultivar to differentiate race 1 and 4, as it is susceptible to race 1 and resistant to race 4. Although this cultivar is commonly used as a susceptible control for races 1, 2 and 3, it had never been tested against race 4. The inoculation of the *Fusarium* isolates on cultivar 'Banchu Red Fire' did not show significant differences, while Gilardi *et al.* (2016) observed clear differences between race 1 and 4 on this cultivar. This could be explained by differing environmental conditions in both studies.

In general, disease development is influenced by several parameters such as the pathogenicity of the isolates (Srinivasan *et al.*, 2012), the inoculation method (Smith *et al.*, 2008) and environmental conditions (Burger *et al.*, 2003; Matheron *et al.*, 2005; Navas-cortés *et al.*, 2006; Scott *et al.*, 2010b). Consistent with previous studies (Paugh & Gordon, 2019; Scott *et al.*, 2010a,b) we showed a dose-response relationship during our inoculation trial on a susceptible cultivar. Most importantly, we showed the importance of the inoculation method to demonstrate the difference in virulence of the race 1 and 4 isolates. Using a root dip inoculation with mainly microconidia did not show any differences in virulence between isolates. However, mixing chlamydospore inoculum in the substrate, which is more closely related to a natural infection, clearly showed that race 4 isolates are more aggressive than race 1 isolates at 24°C. Symptoms did not differ between race 1 and 4, only they appeared earlier with race 4. Whether there is a difference in infection process between race 1 and 4 needs further investigation. The difference in virulence could explain why a slower disease development was observed in commercial glasshouses where only race 1 occurred, compared to glasshouses with only race 4, even on cultivars that are susceptible to both races.

Very low inoculum densities of chlamydospores (10 cfu g^{-1}) already lead to Fusarium wilt symptoms. Couteaudier & Alabouvette (1990) showed that *F. oxysporum* f. sp. *lini* chlamydospores are more pathogenic compared to microconidia and contain 100 times more energy based on fluorescein diacetate measurements. They also discovered that chlamydospores germinate at a higher rate than microconidia in the rhizosphere of flax seedlings. *Fusarium oxysporum* f. sp. *cubense* spore suspensions containing chlamydospores also produced higher and more consistent infection levels on banana compared to spore suspensions mainly containing microconidia (Smith *et al.*, 2008).

The genetic diversity analysis using GBS showed little genetic variation within *F. oxysporum* f. sp. *lactucae* race 4 isolates which confirms the epidemic spread of the pathogen from an original source with limited genetic diversity and/or the occurrence of a genetic bottleneck event, due to the recent introduction or spread of only a few genotypes of the pathogen. Despite this overall lack in genetic

diversity, still two subgroups could be distinguished. The minor group, containing isolates Fus1.53-1.58 and Fus1.61-1.62 originated from glasshouses belonging to three lettuce growers from the same geographical region. We have insufficient data to differentiate between the hypothesis of a single introduction in Belgium followed by local spread and the alternative hypothesis of separate introduction from a common source. However, given the relatively rare nature of this genotype, the first hypothesis is the more likely one. Different factors can be the reason for the local spread: a common plant nursery, traffic to a same auction house, a contractor which goes to different lettuce growers etc. No differences between the two groups could be detected in terms of virulence on susceptible butterhead lettuce cultivar or differential cultivars, even not on 'Costa Rica No. 4' ($P > 0.05$, Wilcoxon rank sum test). Based on the response of the differential cultivars tested, we also have no indications of physiological differences between the isolates of these two groups, hence there is no data to designate the smaller group to a new race. The identification of the two subgroups of race 4 does prove that at least two separate introductions of this race took place in Belgium. The relatively large number of SNPs between the two groups and the genetic homogeneity within the second group does not support the alternative hypothesis of local differentiation of the second group from the first group.

Surprisingly, isolates of the main group of *Fusarium oxysporum* f. sp. *lactucae* race 4 could not be differentiated from *F. curvatum* CBS 247.61 based on the GBS data. Consistent with this result, *Fusarium curvatum* CBS 247.61, which was formerly named *F. oxysporum* f. sp. *matthiolae* (Lombard *et al.*, 2019), gave a positive signal with the race 4 specific primers designed by Gilardi *et al.* (2016). In the phylogenetic analysis based on *cmdA*, *rpb2* and *tef1*, *Fusarium oxysporum* f. sp. *lactucae* race 4 isolates from Belgium and the Netherlands, *Fusarium oxysporum* f. sp. *lactucae* race 1 isolates from Belgium and Japan, *F. curvatum* CBS 247.61, *F. oxysporum* f. sp. *rhois* CBS 220.49 and *F. oxysporum* f. sp. *tulipae* CBS 242.59 could not be differentiated. The study of Gilardi *et al.* (2016) already showed that *Fusarium oxysporum* f. sp. *lactucae* race 4 isolates from the Netherlands and race 1 isolates from the USA, Italy and Japan belonged to the same clade in a phylogenetic analysis based on *tef1* and *IGS*. In addition, Mbofung *et al.* (2007) revealed the close relatedness between *F. oxysporum* f. sp. *lactucae* race 1 isolates from the USA and Japan, *F. oxysporum* f. sp. *matthiolae* NRRL 22545 (= *F. curvatum* CBS 247.61) and *F. oxysporum* f. sp. *rhois* NRRL 26227 (= CBS 220.49) in a phylogenetic analysis based on the mitochondrial small subunit gene, *tef1* and *IGS*. However, the diversity analysis in this study using the higher resolution GBS technique, did show genetic variation between race 4 and race 1, *F. oxysporum* f. sp. *rhois* and *F. oxysporum* f. sp. *tulipae*.

The identity between the main group of *F. oxysporum* f. sp. *lactucae* race 4 and *F. curvatum* CBS 247.61, which was isolated from glasshouse-grown column stock in Germany in 1957, suggests that this pathogen has been present in Europe for many years and does not represent a recent exotic introduction. Given the pathogenicity of this strain, we hypothesize that it was not associated with commercial greenhouse-grown lettuce until the recent epidemic in the Netherlands and spread very fast since. The pathogenicity of *F. curvatum* CBS 247.61 towards lettuce should be verified to confirm this hypothesis, as a recent gain of pathogenicity due to a relatively small genetic change cannot yet be excluded.

In accordance to the study of Lombard *et al.* (2019) who designated an epitype for *F. oxysporum* and resolved fifteen cryptic taxa as species based on multi-locus phylogenetic analysis and morphological properties, *F. oxysporum* f. sp. *lactucae* race 1 and 4 should be renamed to *F. curvatum* based on the phylogenetic analysis. However, based on morphological properties, Lombard *et al.* (2019) did not observe chlamydospores for *F. curvatum* ex-type isolate CBS 238.94 and observed strongly curved curved 3-septa macroconida, which is in contrast with *F. oxysporum* f. sp. *lactucae* race 4 isolates Fus1.01 and Fus1.02 (Claerbout *et al.*, 2018). Based on multi-locus phylogenetic analysis, *F. oxysporum* f. sp. *rhois* CBS 220.49 and *F. oxysporum* f. sp. *tulipae* CBS 242.59 also belong to *F. curvatum*, even though they are clearly different based on the GBS analysis. Detailed genetic and pathogenic analysis of additional *F. curvatum* isolates, including isolates CBS 141.95 and CBS 238.94, is necessary to determine appropriate species and pathogenic boundaries. This will then allow correct species and race designation to a number of isolates, including the ones that were formerly assigned to *Fusarium oxysporum* f. sp. *matthiolae*. The demonstrated genetic similarity of several isolates that were formerly designated as separate species or formae speciales explains the problems to differentiate them when developing and using molecular detection assays for *Fusarium oxysporum* f. sp. *lactucae* (Mbofung & Pryor, 2010). The SNPs identified using GBS now create possibilities as new targets for more specific detection and identification assays.

Fusarium oxysporum f. sp. *lactucae* race 2 and race 3 were not included in our study as they are not relevant for Europe. They are genetically quite distant from race 1 and race 4 (Gilardi *et al.*, 2016; Mbofung *et al.*, 2007) and therefore, unlike race 1 and race 4, they should not be renamed to *F. curvatum*.

Given the limited genetic diversity and the speed of the epidemic of *Fusarium oxysporum* f. sp. *lactucae* race 4 (*Fusarium curvatum*) in lettuce, the spread was presumably facilitated via human activities, such as transfer of planting material, soil and equipment. Our results show the importance of hygienic measures to prevent this type of spread of *Fusarium* in lettuce production areas. Furthermore, the occurrence of two races requires careful race identification measures so that appropriate (partially) resistant lettuce cultivars can be used. Differences in disease development in commercial glasshouses could be attributed to the different races, because race 4 is more virulent than race 1 at 24°C. However, the effect of differences in physical soil parameters, soil microbiome and other environmental factors on disease expression still deserves more study.

5.6 Acknowledgements

We are grateful to Rijk Zwaan (the Netherlands) for providing the Japanese race 1 isolate and the lettuce seeds for most plant experiments. We also thank Bejo zaden (the Netherlands) for providing the lettuce seeds of the cultivar ‘Romana romabella 30CN’. We thank Fran Focquet, Béatrice Moeneclaey, Ramize Xhaferi, Shirley Marcou and Nadia Lemeire for their technical assistance and Paul Quataert for statistical advice. This research was funded by grant no. 140984 from the ‘Flanders Innovation & Entrepeneurship (VLAIO)’.

5.7 Supplementary data

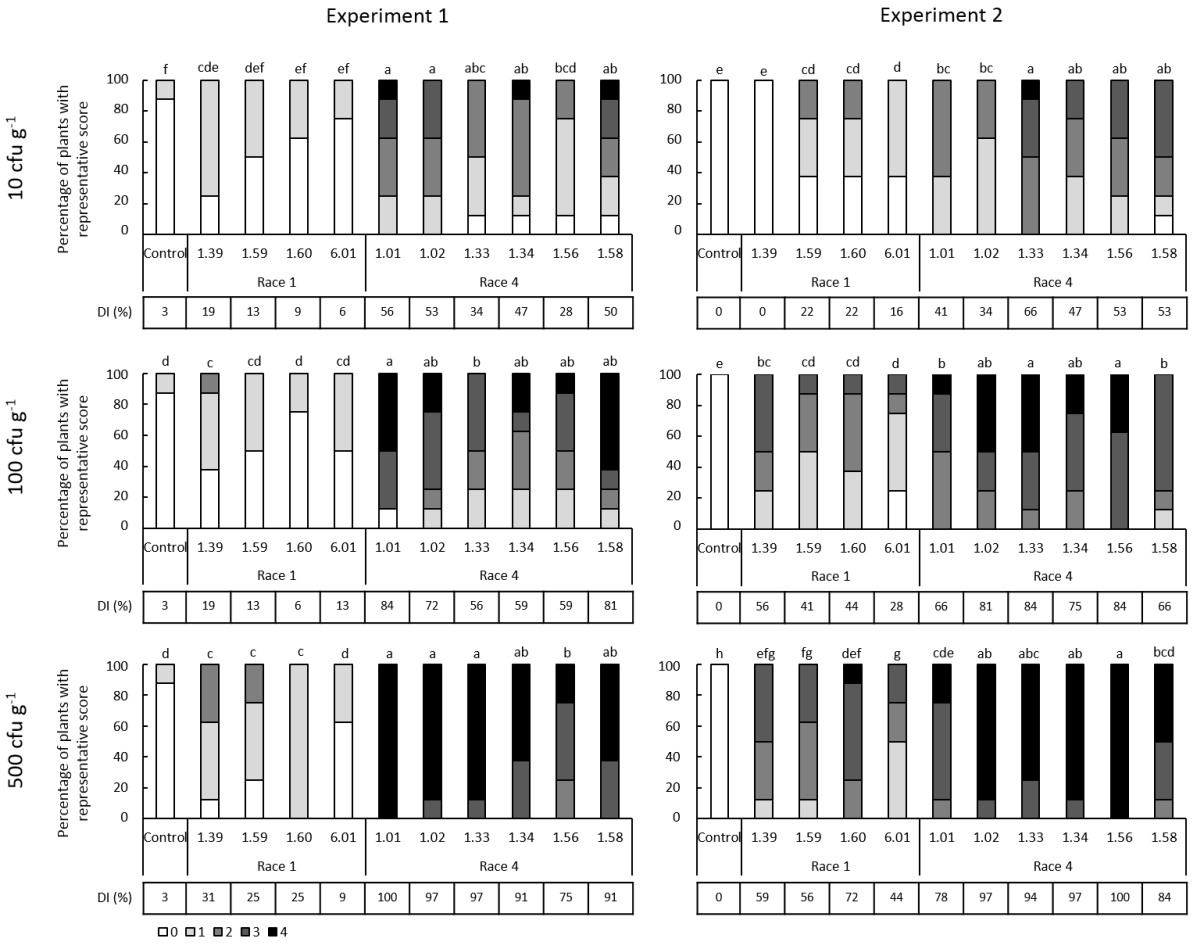

Figure S5.1 Pathogenicity on butterhead lettuce cv. 'Cosmopolia' inoculated with different concentrations of chlamydospores (10, 100 or 500 cfu g^{-1}) of four race 1 and six race 4 isolates. The plants were grown at 24°C during 3 weeks. Different letters show statistical differences (Wilcoxon rank sum test, $P < 0.05$, n = 8). DI = Disease index.

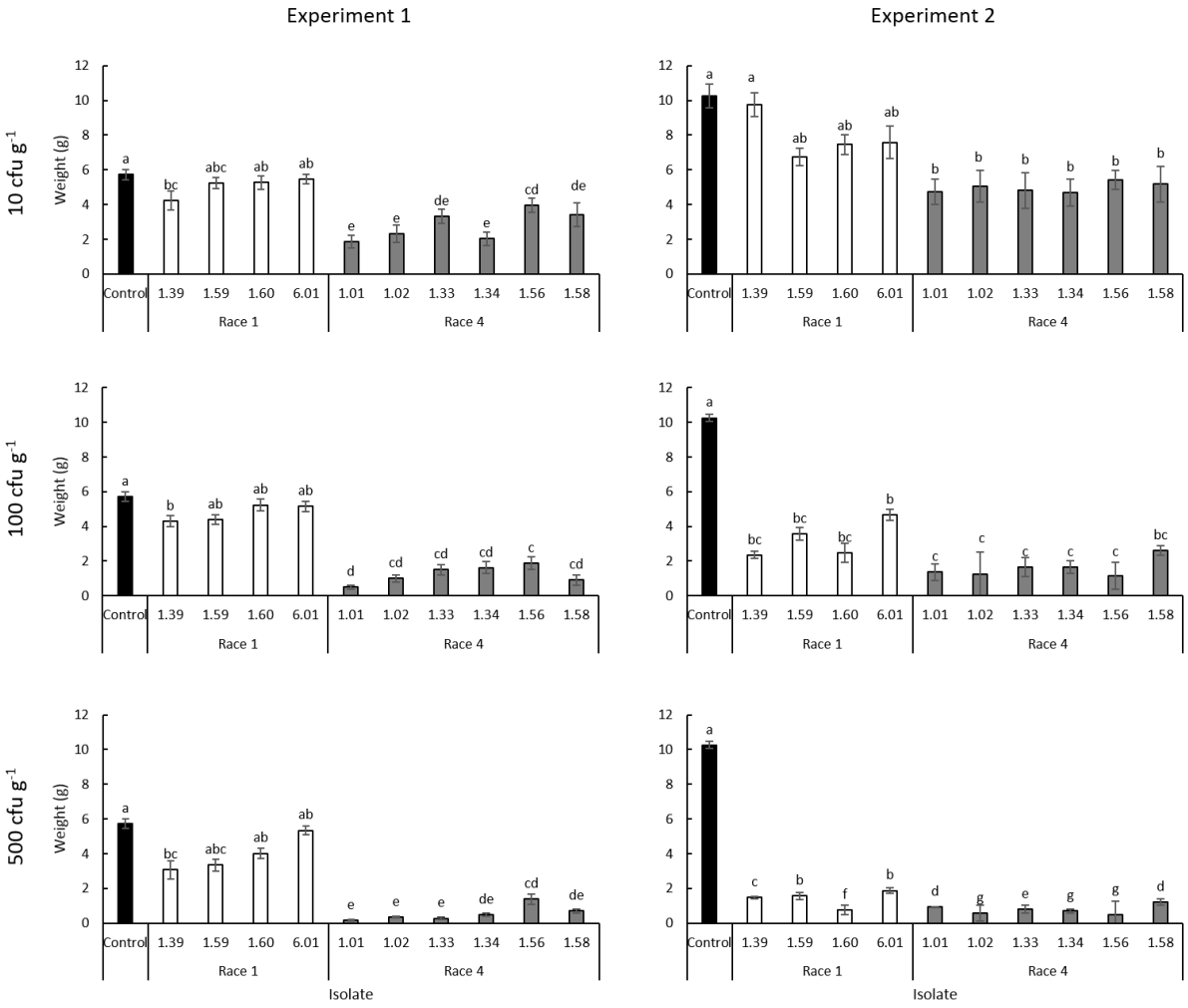

Figure S5.2 Weight of butterhead lettuce cv. 'Cosmopolia' inoculated with different concentrations of chlamydospores (500, 100 or 10 cfu g^{-1}) of four race 1 and six race 4 isolates. The plants were grown at 24°C during 3 weeks. Different letters show statistical differences (Tukey or Wilcoxon rank sum test, $P < 0.05$, n = 8). Error bars represent the standard error.

CHAPTER

6

Analysis of the lettuce production system in Belgium

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6.1 Abstract

The lettuce production in Belgium is known for its heavy and high qualitative lettuce heads. These are produced in an intensive production system, which increases the incidence of soil-borne pathogens. Harmful soil-borne pathogens are mainly managed by chemical soil disinfestation and pesticides, but the use of these chemicals has to be reduced due to stricter regulations and environmental concerns. Therefore, biopesticides, in particular microbial pesticides, are gaining popularity. The production system of lettuce was analyzed using systems thinking in order to understand why lettuce growers continue with this intensive production of lettuce. In addition, pesticides and microbial pesticides were explored using a SWOT-analysis, evaluating their strengths, weaknesses, opportunities and threats (SWOT). We found that an intensive production system is the most cost efficient and therefore widely practiced. Transferring to another production system is difficult due to several factors. The SWOT-analysis revealed that pesticide use should be reduced because of the residue problem on harvested lettuce and their toxicity to the environment and applicant, although they are highly effective and their use is independent of the environmental factors. Microbial pesticides are a recommended alternative, but more research is needed to increase their effectiveness and to understand how their application can be improved, so that food security and product quality can be ensured.

6.2 Introduction

Since the 1960s, agricultural production has increased due to improved technology, new high-yielding crop varieties, the use of synthetic fertilizers, irrigation etc. Together with this agricultural intensification there was an increase in soil-borne pathogens (Chellemi, 2002; Matson *et al.*, 1997). The management of soil-borne pathogens is of high importance to achieve an economic reduction of the disease. However, soil-borne pathogens are difficult to manage because of their very own nature: their existence in the soil (Katan, 2017).

This study focused on the production system of Belgian butterhead lettuce grown in soil in glasshouses: an intensive monoculture with up to five harvests per year. The most important soil-borne pathogens in Belgian lettuce are the organisms that cause basal rot: *Rhizoctonia solani*, *Pythium* spp., *Sclerotinia* spp. and *Botrytis cinerea* (Van Beneden *et al.*, 2009). Next to these, the nematodes *Pratylenchus penetrans* and *Paratylenchus* sp. can provoke yield loss. Since 2015, *Fusarium oxysporum* f. sp. *lactucae* has been reported in Belgium (Claerbout *et al.*, 2018), a dreaded soil-borne fungus that has been spreading very fast and is causing severe damage to almost the whole lettuce production area.

In general, a lettuce head must be appealing, without visible damage, and fulfill high quality requirements. Belgian lettuce is known for its high weight and quality compared with other countries. To reach these high standards, the management of soil-borne pathogens relies heavily on soil disinfestation and pesticide use. Nowadays, these strategies are under pressure because of stricter regulations due to environmental and health concerns. At the moment, only a limited number of chemicals for soil disinfestation are permitted and there are no fungicides available to control *F. oxysporum* f. sp. *lactucae* (Chapter 1). As a result, biopesticides are gaining more interest.

The intensive production of lettuce remains a common practice despite the fact that it favors the incidence of soil-borne pathogens. In order to understand why lettuce growers continue this intensive production, we analyzed the lettuce production system with a focus on disease pressure using the technique of systems thinking (Chapman, 2004). Systems thinking is holistic and is used to retain connection between variable keys (Chapman, 2004). As pesticides are frequently used to control soil-borne pathogens and biopesticides are gaining interest, a SWOT-analysis (Strengths, Weaknesses, Opportunities and Threats) was conducted for pesticides and microbial pesticides, and a confrontation matrix was made to come up with possible strategies aimed at improving the lettuce production system.

6.3 Research methods

6.3.1 The production system

The production system of glasshouse-grown lettuce in soil in Belgium was schematized in a causal loop diagram according to the guidelines of Kim (1992). The scheme was developed initially in workshops at ILVO (Flanders research institute for agriculture, fisheries and food) with researchers from different disciplines and was adjusted by the project partners of the FUNSLA-project and its steering committee. The FUNSLA-project was funded by the 'Flanders Innovation & Entrepeneurship (VLAIO)' and focusses on the integrated management of soil-borne fungi and nematodes of glasshouse-grown lettuce. The last adjustments were made in this thesis.

6.3.2 SWOT-analysis and confrontation matrix for pesticides and microbial pesticides

A SWOT-analysis was conducted to analyze the application of pesticides and microbial pesticides in Belgian lettuce production. A SWOT-analysis evaluates the internal factors Strengths and Weaknesses, and the external factors Opportunities and Threats. The internal factors measure the capabilities of the object being evaluated, while the external factors influence the sustainability of the object.

When mentioning pesticides below, we only refer to pesticides used to control pathogens, not to the general definition of pesticides covering chemically synthetized products used to protect crops from harmful organisms and weeds. No fumigants were included. With microbial pesticides we refer to those biopesticides which consist of bacteria, fungi, viruses, protozoa and nematodes, not to products originating from natural resources as metabolites from micro-organisms, plant extracts or pheromones (Fytoweb 2019).

A group of eight people from academia (3 people) and applied research institutes (5 people) from the FUNSLA-project enumerated all of these factors during two brainstorm sessions. The proposed factors were discussed, and adjusted where necessary, during a workshop with a group of eight people representing the government, breeding companies, crop protection industry, academia and applied research institutes. People from the steering commite of the FUNSLA-project which are closely related to the lettuce production were invited to participate in the SWOT-analysis. Eight people signed up to participate. Two of the eight people had already been involved in the brainstorm sessions. A confrontation matrix was filled out individually by these eight people and four other persons representing different sectors closely related to lettuce production. The following questions were answered for every combination: ‘Can we use our existing strength to take advantage of the opportunity?’, ‘Does the weakness prevent us from taking advantage of the opportunity?‘, ‘Can we use our existing strength to reduce likelihood and impact of the threat?’, ‘Does the weakness prevent us from overcoming a threat?’ These questions were answered with a score from 0 (not at all) to 3 (very strong). All the individual confrontation matrices were averaged in one general confrontation matrix.

6.4 Results

6.4.1 The production system

The production system of glasshouse-grown lettuce in soil is schematized in Figure 6.1. In general, particular soil cultivation practices lead to the specialization in one crop, here lettuce. This specialization on its turn lowers the long-term investment cost. Specialization leads to intensification of lettuce production which secures the grower’s revenue by a regular harvest spread over the year. The market price depends on several factors, such as supply, demand, weight and quality. The latter can be guaranteed by several quality labels, such as Flandria, Flandria +, Fine Fleur. Depending on the quality label, the market price can differ. Lettuce heads can only have maximum 70% of the European maximum residue level (MRL) and seven different active substances to fulfill the requirements of Flandria +. Flandria + lettuce heads can even be specified in different segments. Segment 1 lettuce heads have maximum one third of the European MRL and maximum five active substances. Segment 3 lettuce heads have also maximum one third of the European MRL but maximum seven active substances. As lettuce heads in segment 1 have a higher value, the presence of pesticide residues can reduce the

market price. Also the customer and retail want a low market price. The market price has an important influence on the revenue of the lettuce grower, together with the number of produced lettuce heads and the production costs. The revenue, revenue security and the low investment cost are drivers for the lettuce grower to continue lettuce monocropping.

The intensification of crops has led to an increased disease pressure of soil-borne pathogens (Chellemi, 2002), damaging roots and/or leaves and lowering the quality and weight of the lettuce crop. Furthermore, this intensive monoculture drives pathogen evolution which on its turn increases the disease pressure (Zhan *et al.*, 2015). Different disease management techniques, such as chemical, physical, biological and cultural approaches can be implemented to lower the disease pressure. Commonly used measures are fungicides and chemical soil disinfestation. However, nowadays chemical soil disinfestation is restricted and different applications of soil steaming are often used instead. Since the occurrence of *F. oxysporum* f. sp. *lactucae,* biopesticides are gaining more interest and partially resistant varieties are used.

The applied disease management practices depend first of all on the available tools which are strongly influenced by policy and research. Next to that, society wants food with less pesticides, produced in a sustainable way, and exerts pressure on the policy makers. Moreover, the attitude and vision of the lettuce grower and his financial capital influence the disease management.

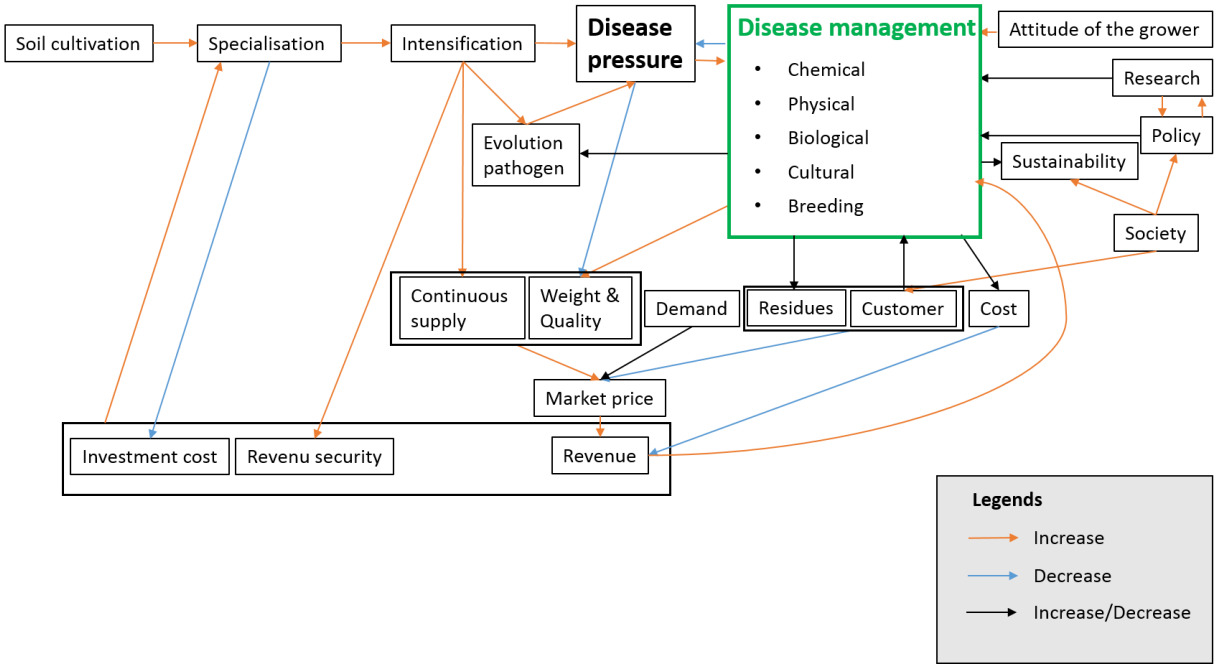

Figure 6.1 Causal loop diagram of the production system of lettuce in soil under glasshouse conditions

6.4.2 SWOT-analysis and confrontation matrix for pesticides and microbial pesticides

6.4.2.1 Pesticides

6.4.2.1.1 Strengths

Lettuce growers apply pesticides because they have a good and consistent effectiveness, which results in a good visual quality of the lettuce head (Barrière *et al.*, 2014). Pesticides cost less than microbial pesticides and lettuce growers are well acquainted with the application of pesticides and their ease of use. The efficacy of pesticides is less dependent on environmental factors, particularly temperature, than is the case for microbial pesticides. Pesticides are easier to combine with each other in spraying tanks or to apply in consecutive sprayings, while for microbial pesticides there are restrictions or information is missing. Prestop (a.s. *Gliocladium cantenulatum*) for instance has a waiting time of 4 days with the active substances cyprodinil and fludioxonil.

6.4.2.1.2 Weaknesses

Pesticides are sprayed on the lettuce heads and have contact or systemic actions. Hence they leave a residue on the lettuce crop and do not affect the survival structures of pathogens in the soil. Pesticides are toxic and can therefore affect the environment by disturbing natural enemies, beneficial micro-organisms or aquatic life. Because of their toxicity, the applicant should continuously be cautious during and after the application. The frequent use of pesticides can also enhance the adaptation of pathogens and lead to the development of pesticide resistance (Chandler *et al.*, 2011).

6.4.2.1.3 Opportunities

As there is a continuous demand for lettuce, the crop has a role in food security. The consumer wants an appealing lettuce heads of high quality. In general, pesticides are seen as an essential part in integrated pest management (IPM), because they are needed to intervene when alternatives fall short. Furthermore, as several pesticides are no longer registered for application in lettuce there is a demand for new active substances with a low ecotoxic profile and a narrow spectrum. The development of new application techniques for pesticides, such as precision agriculture, is also an opportunity.

6.4.2.1.4 Threats

The official regulation of pesticides is very strict and the approval of several active substances is not renewed because of their ecotoxicity. Moreover, society is not in favor of application of pesticides and wants residu-poor or even residu-free food (Chandler *et al.*, 2011). Non-statutory requirements, such as maximum residue levels, can also be considered as a threat. New diseases appear worldwide, also in leafy vegetables (Gullino *et al.*, 2019), exerting a time pressure on the development and marketing of new pesticides.

6.4.2.1.5 The confrontation matrix: How strengths and weaknesses can maximize opportunities and threats

Combining the different strengths and weaknesses with the opportunities and threats in a confrontation matrix revealed that overcoming the threats of pesticides is very much hampered by their weaknesses (Table 6.2 and supplementary data Table 6.4). The pesticide residue and the effect on the environment are the most important weaknesses, because they lead to stricter regulation, non-renewal of active substance approval and are not accepted by the society. The residues also lead to stricter non-statutory

requirements. Using new techniques such as precision agriculture could help to improve disease management and lower the residue on the lettuce heads. Moreover, pesticides are not safe to use for the applicant, which leads to stricter regulation and non-renewal of approvals. The frequent use of pesticides also leads to development of resistance in the pathogens, causing loss of registrations and more time pressure on the development of new active substances. None of the strengths of pesticides were found to be useful in handling the threats.

		External factors	
		Opportunities 1. New active substances 2. Demand for food security 3. Quality requirements 4. Development of new techniques 5. Essential in IPM	**Threats** 1. Strict regulation 2. Non-renewal of active sustance approval 3. Non-statutory requirements 4. Acceptance by the society 5. Time pressure
Internal factors	**Strengths** 1. Effectiveness and quality of crop 2. Low cost for lettuce grower 3. User friendly and familiarity 4. Combinability 5. Independent from environment	***What strengths can you use to capitalize on your opportunities?*** High effectiveness and independence from the environment ensure lettuce quality and food security.	***What strengths can you use to better handle your threats?*** None
	Weaknesses 1. Survival structures remain in soil 2. Safety of applicant 3. Residue 4. Development of resistance 5. Adverse effects on environment	***What weaknesses must be mitigated to capitalize on your opportunities?*** The safety of the applicant, the residue, the development of resistance and the effect on the environment prevent the development of new active substances.	***What weaknesses can be used by your external threats?*** The safety of the applicant, the residue and the effect on the environment provoke stricter regulations and lead to non-renewal of active substance approval. The residue also obstructs non-statutory requirements and the acceptance by the society. The development of resistance leads to non-renewal of approvals and puts time pressure on the development of new active substances.

Table 6.2 Confrontation matrix, analyzing the strengths, weaknesses, opportunities and threats of pesticides

Weaknesses of pesticides, such as the toxicity to the environment and applicant, as well as the residue, prevent the development of new active substances. These weaknesses must be mitigated to capitalize on the opportunities. Pesticides were seen as essential to intervene when alternatives fall short, but the residue and toxicity prohibit this.

Some strengths can be used to capitalize the opportunities. The high effectiveness of pesticides is important to secure the quality requirements and the food supply. Also, the independence of

environmental conditions to apply pesticides is important for food security and quality requirements. These strengths make pesticides essential in IPM.

6.4.2.2 Microbial pesticides

6.4.2.2.1 Strengths

Microbial pesticides are living organisms that are used to control pathogens. They can establish in the environment and destroy survival structures of pathogens and so have an effect over a long period. The product Contans with active substance *Coniothyrium minitans* for instance parasitizes sclerotia of *Sclerotinia* spp. (Whipps *et al.*, 2008). Furthermore, microbial pesticides can enhance soil suppressiveness and promote plant growth, while pesticide treatments often lead to a growth reduction after the application. Microbial pesticides reduce disease incidence by different modes of action, such as competition for space or nutrients, direct parasitism of the pathogen, plant growth promotion, and induced plant resistance (Harman, 2000). The multiple mode of actions reduce the risk of developing resistance. Furthermore, microbial pesticides have a good ecotoxic profile and produce little or no toxic residue (Marrone, 2019).

6.4.2.2.2 Weaknesses

Microbial pesticides are living organisms, hence, they are more complex than pesticides. Consequently, the application of microbial pesticides requires more knowledge to understand the underlying mechanisms (Marrone, 2019). Besides, microbial products need more time to establish in the soil and are less effective compared with chemical pesticides (Glare *et al.*, 2012). As a result, treated lettuce heads are of lower visible quality. The combinability of microbial pesticides with other treatments is more difficult than for pesticides. Chemical pesticides can have a negative effect against microbial pesticides. Lists with side effects do already exist for some microbial pesticides, but are sometimes also limited. In addition, the effectiveness of microbial pesticides depends on environmental factors such as temperature (Tomprefa *et al.*, 2011). In general, this control method is more expensive than pesticides (Glare *et al.*, 2012), although the price will decrease when the market expands.

6.4.2.2.3 Opportunities

The society demands sustainable products with less residues. Using microbial pesticides can make the lettuce growers less dependent on pesticides and can lead to a residue-free or residue-poor product (Marrone, 2019). A high demand will stimulate the development of microbial pesticides.

6.4.2.2.4 Threats

Microbial pesticides are expensive and lead to a higher cost of the lettuce heads products and lower profitability for the lettuce growers. Furthermore, they are less effective, limiting the quality compared to pesticides, and subsequently leading to an uncertain food security. The complex production process and the high production costs of microbial pesticides limit the supply of these products.

6.4.2.2.5 The confrontation matrix: How strengths and weaknesses can maximize the opportunities and threats?

Combining the strengths and weaknesses with the opportunities and threats revealed that the strengths take full advantage of the opportunities (Table 6.3 and supplementary data Table 6.5). The strengths

profit from the demand of the society for sustainable products. The increased soil suppressiveness, long term effect and slower disease development ensure that lettuce growers are less dependent on chemical pesticides, which results in less residues on the lettuce head. This leads to a residue poor/free product which can have a special position in the market.

Moreover, strengths can be used to handle the threats: the slow resistance development, long term effect and increased soil suppressiveness prevent problems with profitability and food security.

Some of the weaknesses should be mitigated to capitalize the opportunities. The required knowledge, the high price, the dependence on the environment, the slow action and low effectiveness hamper the development of new microbial pesticides, and the aim to be less dependent on pesticides. The low effectiveness of microbial pesticides can indirectly inhibit the obtainment of a residue poor/free product. Furthermore, some weaknesses can be used by the external threats, such as the dependency on the environment and the low effectivity can lead to profitability and food security problems. The cost of microbial pesticides will also lead to a higher price of the lettuce head.

		External factors	
		Opportunities 1. Less dependent on pesticides 2. Obtain residue poor/free product 3. Society demands sustainable products 4. Development	**Threats** 1. Supply 2. Profitability and food security 3. Quality requirements 4. Cost price
Internal factors	**Strengths** 1. Good ecotoxic profile 2. Slower resistance development 3. Long term effect 4. Less residues 5. Soil suppressiveness and less growth reduction	***What strengths can you use to capitalize on your opportunities?*** All strengths take advantage of the demand of the society for sustainable products. The increased soil suppressiveness, long term effect and slower disease development ensure a reduced dependence on pesticides. Less residues on the lettuce head can lead to a residue free/poor product.	***What strengths can you use to better handle your threats?*** The slow resistance development, long term effect and increased soil suppressiveness can prevent problems with profitability and food security.
	Weaknesses 1. More knowledge needed 2. Expensive 3. Depends on environment 4. Slower and less effective and less quality 5. Combinability	***What weaknesses must be mitigated to capitalize on your opportunities?*** The needed knowledge, the price, dependency on the environment, slower action and lower effectiveness hampers the development of new microbial pesticides. The lower effectiveness can inhibit the obtainment of a residue poor/free product.	***What weaknesses can be used by your external threats?*** The dependency on the environment and low effectiveness can lead to profitability and food security problems. The high cost of the product also leads to a higher price of the lettuce head.

Table 6.3 Confrontation matrix, analyzing the strengths, weaknesses, opportunities and threats of microbial pesticides

6.5 Discussion

Intensive cropping leads to an increased disease incidence of soil-borne pathogens (Chellemi, 2002) and is therefore not an appropriate cropping system. Analyzing the causal loop diagram of the production system of lettuce shows that the economic aspects are crucial. This highly specialized cropping system is cost efficient and therefore commonly used. Other crops in glasshouses, such as lamb's lettuce, pepper, cucumber, tomato or strawberry are also known for their specialized intensive production systems.

Accepting a lower weight and quality of lettuce heads on the market could ensure the sales for the lettuce grower. The lower weight of the lettuce heads would be interesting for the consumers as they prefer to eat fresh food and a small lettuce head could be enough for one meal. Next to that, a higher market price, which results in a higher revenue, could lead to more possibilities to choose an appropriate disease management. However, it could be difficult to convince the consumer to pay more. During the different workshops, two alternatives for the production system were proposed. One alternative is to grow lettuce in hydroponics instead of soil. The transition to hydroponics goes hand in hand with a high investment cost, which can prevent the change to this system. Moreover, hydroponic production is again an intensive system which will lead to a disease pressure of other pathogens. At the moment, *Phytophthora cryptogea* is an important emerging pathogen in hydroponics (Vandevelde, personal communication). The second alternative is to rotate with other lettuce types or other crops. Some oakleaf and Lollo cultivars are partially resistant against *F. oxysporum* f. sp. *lactucae*, but these lettuce types receive a lower market price (Vandevelde, personal communication). Moreover, growing other crops requires different knowledge and equipment, which most growers lack as they are specialized in producing lettuce only. Some lettuce growers, mainly in West-Flanders, also grow other crops outside and in the glasshouse. They can rotate more easily because they have the knowledge and equipment to grow other crops. However, the effect of these crops on the occurring pathogens should be taken into account. This can still lead to some difficulties for pathogens with a broad host range.

Pesticides to control pathogens have always been an important part of the disease management of soil-borne pathogens in lettuce, but they are under high pressure. The pesticide residues on the lettuce head and the adverse effects on the environment are the most important weaknesses, while the strict regulations and non-renewal of active substance approvals are the most important threats. The registration of pesticides becomes more difficult and costly. Around 140 000 chemicals are screened before one ultimately becomes a registered product (McDougall, 2016). Although pesticides are very effective, easy to apply and ensure the high quality requirements for lettuce heads, the current legislation drives lettuce growers to use fewer pesticides and grow lettuce in a more sustainable way. This can be achieved with an appropriate pesticide use (Chapter 4) or by precision agriculture. In addition, microbial pesticides can be used instead of pesticides. The biopesticide market is growing every year: estimated at 3.5% of the total pesticide market in 2009 and reaching 5-6% in 2018 (Glare *et al.*, 2012; Dunham & Trimmer, 2018). However, the effectiveness should be improved, therefore more research is needed to understand when and how biopesticides should be applied. In some cases, the available disease

management approaches fall short to handle the disease pressure. For instance, for *F. oxysporum* f. sp. *lactucae*, no effective pesticides nor biopesticides are available up to now.

In conclusion, the current intensive lettuce production system is economically seen the most appropriate way of growing lettuce as long as the disease pressure can be managed. The increasing disease pressure and new emerging diseases force to reconsider the production system, although it is difficult since several factors are important. Different studies encourage to use a systems-based approach with alternative strategies to manage soil-borne pathogens, since there is no silver-bullet solution. Therefore, the spread and the introduction of pathogens should be prevented by using pathogen-free planting material, and production area and preventative hygienic measures. This is of high importance, because several new emerging diseases are reported in leafy vegetables in Italy (Gilardi *et al.*, 2018) which can pose a future risk for the Belgian lettuce production. If an infection occurs, the inoculum should be reduced using soil disinfestation or integrating beneficial crop rotations to levels that ensure an economic valuable production. Disease suppressiveness should be promoted by stimulating shifts in the soil microbial community by crop rotation, organic amendments or other measures after soil disinfestation. The last intervention is a minimum use of disruptive actions like pesticides and bio-pesticides (Barriere *et al.*, 2015; Chellemi *et al.*, 2016). However, the use of these pesticides is restricted by the current legislation and the application of biopesticides is limited by the variability in effectiveness.

6.6 Acknowledgements

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6.7 Supplementary data

			External factors									
			Opportunities					Threats				
			O1. New active substances					T1. Strict regulation				
			O2. Demand for food security					T2. Non-renewal of active substance approval				
			O3. Quality requirements					T3. Non-statutory requirements				
			O4. Development of new techniques					T4. Acceptance by the society				
			O5. Essential in IPM					T5. Time pressure				
			O1	O2	O3	O4	O5	T1	T2	T3	T4	T5
Internal factors	Strengths	S1. Effectiveness and quality of crop	S1	1.7	2.3	2.8	1.1	2.2	1.6	1.0	0.9	1.1	1.3
		S2. Low cost for lettuce grower	S2	1.1	1.7	1.1	0.7	1.7	0.1	0.0	0.0	0.2	0.5
		S3. User friendly and familiarity	S3	1.4	1.3	0.5	0.9	1.6	0.1	0.3	0.3	0.8	0.4
		S4. Combinability	S4	1.9	1.7	1.7	1.3	1.9	0.2	0.3	0.8	0.5	1.3
		S5. Independent from environment	S5	1.4	2.5	2.1	1.4	2.0	0.3	0.4	0.7	0.6	0.9
	Weaknesses	W1. Survival structures remain in soil	W1	1.3	1.3	1.1	1.0	1.4	0.5	0.4	0.6	0.3	0.7
		W2. Safety of applicant	W2	2.1	0.9	0.5	0.8	1.6	2.4	2.2	0.8	1.4	0.5
		W3. Residue	W3	2.3	1.3	0.9	0.3	2.0	2.8	2.7	3.0	2.9	1.4
		W4. Development of resistance	W4	1.8	2.3	1.3	0.6	1.7	1.7	2.3	1.3	1.1	2.3
		W5. Effect on environment	W5	2.3	1.2	1.1	0.8	2.1	2.8	2.8	1.7	2.6	2.1

Table 6.4 Confrontation matrix, combining the internal factors (strengths and weaknesses) with the external factors (opportunities and threats) of pesticides. Numbers and colors show the average score given by the participants (n = 12) of 0 or red (not at all) to 3 or green (very strong) to the questions: 'Can we use our existing strength to take advantage of the opportunity?', 'Does the weakness prevent us from taking advantage of the opportunity?', 'Can we use our existing strength to reduce likelihood and impact of the threat?', 'Does the weakness prevent us of overcoming a threat?'.

			External factors							
			Opportunities				Threats			
			O1. Less dependent on pesticides				T1. Supply			
			O2. Obtain residue poor/free product				T2. Profitability and food security			
			O3. Society demands sustainable products				T3. Quality requirements			
			O4. Development				T4. Cost price			
			O1	O2	O3	O4	T1	T2	T3	T4
Internal factors	Strengths	S1. Good ecotoxic profile	S1	1.2	1.0	2.9	1.8	0.8	0.5	0.5	0.8
		S2. Slower resistance development	S2	2.2	1.3	1.8	1.9	1.5	1.7	1.2	1.3
		S3. Long term effect	S3	2.1	1.7	2.1	1.3	1.5	1.8	1.2	1.6
		S4. Less residues	S4	1.0	2.9	2.8	2.1	1.1	0.6	0.8	1.3
		S5. Soil suppressiveness and less growth reduction	S5	2.1	1.3	2.2	1.7	1.3	1.7	1.2	1.3
	Weaknesses	W1. More knowledge needed	W1	2.5	1.5	1.3	2.0	1.7	1.5	1.5	1.2
		W2. Expensive	W2	2.1	1.4	1.2	1.6	1.3	1.4	0.8	2.5
		W3. Depends on environment	W3	2.1	1.3	0.9	1.9	1.0	2.2	1.8	0.7
		W4. Slower and less effective and less quality	W4	2.3	1.8	1.2	2.0	1.3	2.3	2.5	1.4
		W5. Combinability	W5	1.6	1.0	0.8	1.2	0.7	1.0	1.0	0.9

Table 6.5 Confrontation matrix, combining the internal factors (strengths and weaknesses) with the external factors (opportunities and threats) of microbial pesticides. Numbers and colors show the average score given by the participants (n = 12) of 0 or red (not at all) to 3 or green (very strong) to the questions: 'Can we use our existing strength to take advantage of the opportunity?', 'Does the weakness prevent us from taking advantage of the opportunity?', 'Can we use our existing strength to reduce likelihood and impact of the threat?', 'Does the weakness prevent us of overcoming a threat?'.

CHAPTER

7

General discussion and recommendations for future research

7.1 General discussion

In Belgium lettuce is mainly produced in a continuous intensive production system with limited rotation cycles. This leads to an increased disease incidence of soil-borne pathogens (Chellemi, 2002). Until recently, the fungi *Rhizoctonia solani, Botrytis cinerea, Sclerotinia* spp., and oomycete *Pythium* spp. which cause basal rot and the nematodes *Paratylenchus* sp. and *Pratylenchus penetrans* used to be the most important soil-borne pathogens in Belgian butterhead lettuce. Since 2015, *Fusarium oxysporum* f. sp. *lactucae* is causing serious losses in the whole lettuce production area, affecting the supply of lettuce heads that has been declining since then (Figure 7.1). These pathogens occur worldwide, therefore the results obtained in this thesis can be extrapolated to other countries where lettuce is grown.

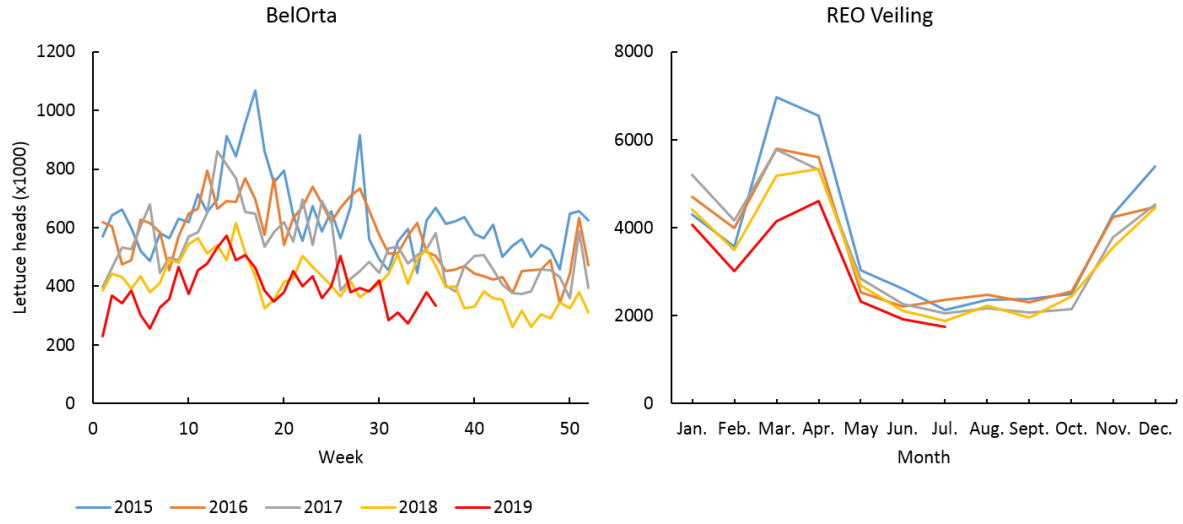

Figure 7.1 Supply of butterhead lettuce heads grown in soil and hydroponics in the auction houses BelOrta in Sint-Katelijne-Waver and REO Veiling in Roeselare (Numbers received from BelOrta and REO Veiling).

In Belgium, lettuce produce has to satisfy very high quality requirements. It has to be fresh and look appealing without visible damage or visible substances or fertilizers. Furthermore, the head needs to be compact and have a standardized weight and a good shelf life. This is in contrast with other countries such as France or the Netherlands, where lettuce heads with lower weights are accepted on the market. The high standards in Belgium make it more difficult to grow marketable lettuce heads than in other countries. In order to meet these high standards, lettuce growers used to rely heavily on chemical soil disinfestation and the application of fungicides to manage soil-borne pathogens. Nowadays, fewer chemical soil disinfestation products are authorized, which forces them to use other alternatives. Next to that, the use of fungicides has diminished because of the strict regulations, the non-renewal of active substance approvals and the pressure of the society. It is possible to reduce chemical soil disinfestation and fungicide application provided that knowledge is available about the conditions in which pathogens are active and are causing damage.

The research questions relating to the reduction of fungicide sprayings against basal rot pathogens and the avoidance of superfluous use of chemical soil disinfestation against nematodes are discussed in this chapter. Furthermore, the research questions with respect to the diversity and pathogenicity of *F.*

oxysporum f. sp. *lactucae* isolates and the Belgian lettuce production system are discussed. Besides, recommendations for future research are formulated.

RQ1: Which factors enable the populations of *P. penetrans* and *Paratylenchus* sp. to increase until the high numbers that cause damage? How can we avoid these high numbers and so reduce the frequency of chemical soil disinfestation?

In some cases the number of plant-parasitic nematodes can increase till very high numbers which results in a damaged crop. This increase can be due to biotic and abiotic factors (Bell & Watson, 2001b; 2001c). To gain insights into the factors influencing the nematode population dynamics, numbers of *P. penetrans* and *Paratylenchus* sp. were monitored during at least one year in commercial glasshouses. The population of *P. penetrans* increased slightly after chemical soil disinfestation and a maximum number of 124 (100 ml soil)$^{-1}$ was counted in a lettuce glasshouse rotated with leek and black fallow. The population of *Paratylenchus* sp. varied greatly in the glasshouses with continuous lettuce cropping, reaching a maximum number of 23,701 *Paratylenchus* sp. (100 ml soil)$^{-1}$. In contrast, glasshouses where lamb's lettuce was grown did not have high numbers nor fluctuations in numbers of *Paratylenchus* sp. The highest densities of *Paratylenchus* sp. were observed during winter and spring when the growing periods for lettuce are the longest. Moreover, the winter cultivar 'Brighton' showed the highest reproduction factor (2.26) for this pin nematode in a host status experiment. These findings emphasize the importance of the season and host status of the crop on nematode population dynamics. Seasonal appearance of plant-parasitic nematodes is commonly observed (Verschoor *et al.*, 2001). This was also the case for *Paratylenchus* sp. on butterhead lettuce, but not in the glasshouses with continuous cropping of lamb's lettuce. In addition, the food source is also important for the population build-up. Poor hosts will reduce the population build-up of plant-parasitic nematodes, while good hosts will stimulate it.

In general, chemical soil disinfestation is applied to obtain a drastic reduction in the numbers of nematodes (Haydock *et al.*, 2006), and this was confirmed in this study. However, a proportion of the population always survived in the 30-60 cm soil layer of the glasshouses, which involves a risk for future crops. Despite the high effectiveness of chemical soil disinfestation, this practice is no longer recommended due to environmental concerns. Steaming under negative pressure could be a good alternative as no *Paratylenchus* sp. was observed, even nine months later. Additional observations during the FUNSLA-project also revealed that sheet steaming reduced *Paratylenchus* sp. with almost 100% and 93% at 0-30 and 30-60 cm depth, respectively. Unfortunately, these methods are energy demanding. Black fallow is another option to reduce nematode populations (Viaene *et al.*, 2006; Pudasaini *et al.*, 2006). This was also observed in this study for *Paratylenchus* sp., where numbers declined after a period of 61-82 days without crops nor weeds. Drawbacks are that more time is needed than for steaming or chemical soil disinfestation, and the measure is less effective. Contradictory results were observed for *P. penetrans* where sometimes a slight population increase was noticed after fallow. Several studies already showed that monoculture enhances the build-up of plant-parasitic nematodes (Rahman *et al.*, 2007), as observed here with *Paratylenchus* sp. and the continuous cropping of butterhead lettuce. Crop rotation is another alternative to chemical soil disinfestation for the management of nematodes, and it provides revenues. Growing lamb's lettuce, wild rocket or parsley

resulted in a reduced *Paratylenchus* sp. population, while the *P. penetrans* population in the soil was reduced or remained the same after leek. Although rotation is a good alternative, the lettuce grower needs specific knowledge and equipment to cultivate crops other than lettuce. Besides, *P. penetrans* has a broad host range and could affect other crops in the rotation.

RQ2: At which densities do *P. penetrans* and *Paratylenchus* sp. cause damage to lettuce? Based on this information, growers can be advised when to take actions to manage these nematodes.

Several pot experiments were conducted to estimate the damage threshold for *P. penetrans* and *Paratylenchus* sp. using the Seinhorst equation. The first visible symptoms are root lesions, which affect root quality followed by a reduced root weight and later on a reduced crop weight. The damage threshold was 362 to 1,308 *Paratylenchus* sp. (100 ml soil)$^{-1}$ and 48 to 204 *P. penetrans* (100 ml soil)$^{-1}$ for root quality; 1,754 to 2,000 *Paratylenchus* sp. (100 ml soil)$^{-1}$ and 262 to 400 *P. penetrans* (100 ml soil)$^{-1}$ for root weight; and 669 to 3,834 *P. penetrans* (100 ml soil)$^{-1}$ for lettuce weight, while no reduction in lettuce head weight was observed with *Paratylenchus* sp. The lower damage thresholds for *P. penetrans* than for *Paratylenchus* sp. can be explained by differences in feeding habit. *Pratylenchus penetrans* is an endoparasitic nematode which enters the root to feed and causes typical lesions by its migratory behavior destroying multiple root cells (Castillo & Vovlas, 2007), while *Paratylenchus* sp. is an ectoparasitic nematode (Rhoades & Linford, 1961), puncturing root cells to feed but staying on the outside of the root system.

In general, large standard errors were observed for all damage thresholds and therefore the damage thresholds should be interpreted with caution. Including more replicates could lower these standard errors, but practical constraints make this difficult. Furthermore, the plants were grown in pots, in optimal conditions where stress is avoided for the plant, which can lead to actual higher damage thresholds than in glasshouse situations where plants grow in the ground. Moreover, the experiments with *P. penetrans* were conducted with sterile soil, which ignores the effect of secondary pathogens. Next to that, mixing the naturally infested soil with *Paratylenchus* sp. disturbed the population and resulted in high amounts of non-feeding fourth-stage juveniles. Therefore, validating the damage thresholds with field samples is important. However, the damage thresholds for root quality still give a good indication about the allowed nematode densities, because a good root quality ensures a healthy lettuce crop.

RQ3: Can we reduce the amount of fungicide sprayings by only targeting the active basal rot pathogens?

Kooistra (1983) and Van Beneden *et al.* (2009) observed a seasonal occurrence of the basal rot pathogens *B. cinerea, R. solani, Pythium* spp. and *Sclerotinia* spp. They noticed *B. cinerea* mainly in winter, *R. solani* in summer and *Sclerotinia* spp. and *Pythium* spp. in spring, summer and autumn. We verified these observations by continuously planting butterhead lettuce without any fungal disease control in three different glasshouses and determining the active basal rot pathogen. Our results showed that the occurrence of the different basal rot pathogens appeared to be mainly glasshouse specific and to a lesser extent seasonal. *Rhizoctonia solani* was predominant in Kruishoutem, while in Sint-Katelijne-

Waver *R. solani* was predominant only in warm periods and *Pythium* spp. in cold periods. In Rumbeke-Beitem it was *B. cinerea* that was predominant, through the whole year. Therefore, disease management schemes should be adapted for each glasshouse and cannot be based on a standard seasonal occurrence of pathogens.

However, *R. solani* induced symptoms at lower temperatures than *B. cinerea* and *Pythium* spp., and at higher temperatures, this pathogen induced symptoms faster when compared with *B. cinerea* and *Pythium* spp. High variability was observed for *R. solani*, probably due to different anastomosis groups. AG4-HGI was observed in warmer periods, while AG1-IB, AG2-1 and AG-BI were observed in colder periods. Also, differences between *Pythium* spp. were observed; *P. ultimum* was observed in warmer periods, while *P. sylvaticum* was observed in colder periods.

RQ4: What is the diversity of *Fusarium oxysporum* f. sp. *lactucae* isolates in Belgium? And are there differences in their pathogenicity?

From 2015 till 2018 several *Fusarium* isolates from butterhead lettuce with wilting symptoms were collected and identified. Most isolates belonged to *F. oxysporum* f. sp. *lactucae* race 4 and a minor proportion to race 1 based on specific primers designed by Pasquali *et al.* (2007) and Gilardi *et al.* (2016).

The genes *tef1* and *IGS* are mostly studied for *F. oxysporum* because of their better phylogenetic signal compared with other genes (O'Donnell *et al.*, 2009). However, *F. oxysporum* f. sp. *lactucae* race 1 and 4 isolates belonged to the same clade for these two genes (Gilardi *et al.*, 2016). Therefore, the genetic diversity of all the isolates was explored using genotyping-by-sequencing which has a high resolution. In general, race 1 and 4 were closely related and could be clearly distinguished from other *F. oxysporum* reference strains, except for one, *F. curvatum* CBS 247.61. Despite the low genetic variation, still two groups were noticed within race 4. The main race 4 group clustered with *F. oxysporum* f. sp. *lactucae* strains from the Netherlands and with the *F. curvatum* isolate CBS 247.61 from Germany. In addition, the Belgian race 1 isolates clustered with the race 1 isolate originating from Japan. These results suggest that these two races were probably introduced from a unique source and were further spread via plants, seeds, materials, machines with adhering soil. Moreover, *F. curvatum* CBS 247.61 was isolated in 1957 from the ornamental flower column stock (*Matthiola incana*) and clusters with the main *F. oxysporum* f. sp. *lactucae* race 4 group, which suggests the long occurrence of this pathogen in Europe and disproves the exotic origin of this race. However, the pathogenicity of *F. curvatum* CBS 247.61 should be investigated and detailed genetic studies are needed to confirm this hypothesis. In addition, the nomenclature based on formae speciales should be reconsidered as these can infect multiple hosts. The isolates from the small race 4 group were closely related to those of the main race 4 group. These isolates originated from lettuce growers from the same geographical region which illustrates their local spread.

Pathogenicity assays at 24°C using chlamydospores revealed that race 4 is more aggressive than race 1 isolates. This is in accordance with the severe disease symptoms typical for race 4 in commercial glasshouses, which is not the case for race 1. This study also showed that the inoculation protocol is of

high importance to study differences between *Fusarium* isolates. A classic root dip method in spore suspension was too aggressive to see differences between isolates, while a more natural method in which chlamydospores were mixed into the substrate could reveal clear differences between isolates.

RQ5: Why do growers opt for intensive lettuce production in Belgium? Can this system be maintained with the current disease pressure and what are the alternatives?

Lettuce growers are specialized and opt for intensive lettuce production because it is economically the most interesting system, as demonstrated in the causal loop diagram resulting from a systems thinking analysis. Revenue, revenue security and low investment costs make that growers continue their specialized production system. Unfortunately, this intensive production leads to an increased disease pressure of soil-borne pathogens (Chellemi, 2002). In this work, the population build-up of *Paratylenchus* sp. was clearly demonstrated in continuous butterhead lettuce cropping and Grosch *et al.* (2010) showed an increase in disease severity of *R. solani* when growing lettuce twice a year. Moreover, the occurrence of *F. oxysporum* f. sp. *lactucae* and the lack of effective control measurements for this fungus make it difficult to continue with the current production system. Most Dutch growers with a comparable production system as in Belgium already stopped growing butterhead lettuce due to the occurrence of this pathogen. Moreover, *F. oxysporum* f. sp. *lactucae* race 4 only occurs in glasshouses in Belgium, the Netherlands, England and Ireland, while it has not been observed in the outdoor production of lettuce. This could be due to the lower temperatures outside and, more important, the production system. Lettuce production outdoors is usually part of rotation system, while this is not the case in glasshouses. Crop rotation results in soils with a higher biodiversity, which could lead to a higher soil suppressiveness. This could be the reason why still no *F. oxysporum* f. sp. *lactucae* has been observed in Germany, where butterhead lettuce is grown in rotation with other lettuce types or other crops. Therefore, the intensive production system in glasshouses needs to be reconsidered. Several options for a different lettuce production system are illustrated in the decision tree in Figure 7.2. First of all, lettuce growers can stop growing lettuce. Due to the occurrence of Fusarium wilt in their glasshouse, some lettuce growers already gave up lettuce production all together. If a lettuce grower decides to continue growing lettuce, he can either continue to grow lettuce in soil or move away from soil. Many other glasshouse-grown horticulture crops moved away from soil, such as tomato, pepper, cucumber, strawberry. In the case of lettuce, hydroponic systems such as nutrient film technique and floating hydroponics can be installed, but this is accompanied with a high investment cost. The new system is again an intensive production system in which other pathogens, such as oomycetes, can cause high disease pressure. However, a hydroponic system is easier to maintain in terms of hygienic measures. Furthermore, less space is needed to grow the same numbers of lettuce heads compared to the production in soil. In soil, seedlings are immediately planted at a distance to allow the growth to a mature crop, while in hydroponics the space between the crops is adjusted to the growth of the lettuce head. From an ergonomic point of view, maintaining and harvesting lettuce from hydroponics is better compared with the production in soil.

In case the lettuce grower wants to continue producing lettuce in soil, he can choose between an intensive production or a systems-based approach. An intensive production, with regular soil disinfestation and continuous cropping, is not recommended in terms of disease management as shown in the causal loop diagram. The application of soil disinfestation disturbs the biological equilibrium of the soil and makes the soil vulnerable for the reinvasion of pathogens (Gamliel & Van Bruggen, 2016). Nowadays, the presence of *F. oxysporum* f. sp. *lactucae* makes it difficult to continue the intensive lettuce cropping system that has been in place for years. Even after soil disinfestation, wilting symptoms can appear in the next crops. Many growers grow lettuce cultivars with partial resistance to *F. oxysporum* f. sp. *lactucae*, but these do not always fulfill the high quality requirements. Highly resistant cultivars could be a solution to control Fusarium wilt in the future. This would not take away that the intensive system is still vulnerable to other pathogens and needs careful attention concerning disease management.

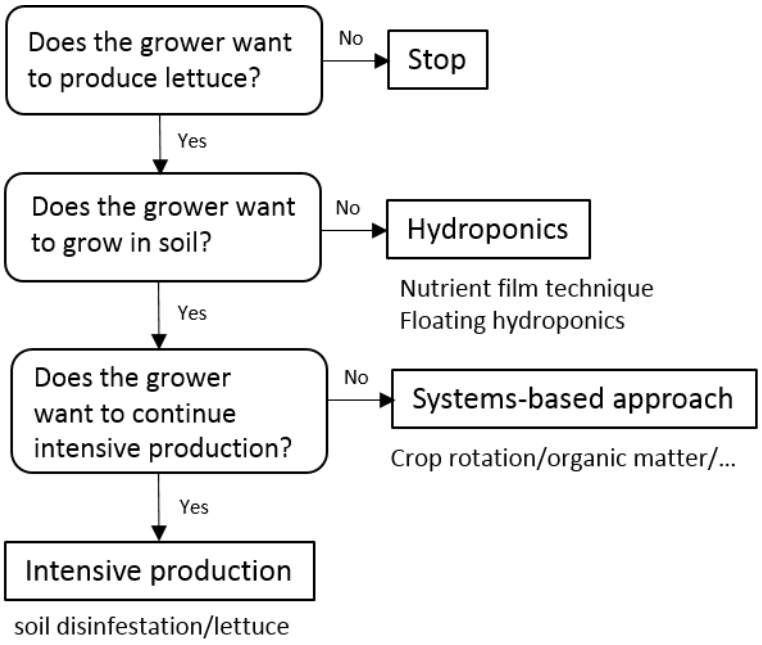

Figure 7.2 Decision tree production system

The second option for a grower who opts for butterhead lettuce production in soil is a sustainable production with a systems-based approach. Different studies encourage to use this approach with alternative strategies to manage soil-borne pathogens (Barriere *et al.*, 2015; Chellemi *et al.*, 2016). The spread and introduction of pathogens should be prevented by using clean planting material in a disease-free area and applying hygienic measures. If an infection occurs, the inoculum should be reduced, using soil disinfestation or integrating beneficial crop rotations, to levels that ensure an economic valuable production. The disease suppressiveness of the soil should be promoted by stimulating shifts in the soil microbial community by crop rotation, organic amendments or other measurements after soil disinfestation. Currently, some lettuce growers produce butterhead lettuce in rotation with alternative lettuce (different from butterhead lettuce), cucumber, zucchini, or implement a period of fallow in summer, the period with the most severe symptoms. However, implementing crop rotation can be

difficult because specialized knowledge and equipment is needed. The last strategy to manage soil-borne pathogens is a minimum use of pesticides and bio-pesticides, which were analyzed in a SWOT-analysis. Pesticides show a high effectiveness and their effects are independent from environmental factors. Pesticide application ensures a high quality of the lettuce heads and food security. However, pesticide residues, environmental effects, safety issues for the applicant and resistance development limit their use. Therefore, microbial pesticides are gaining interest. Microbial pesticides have a good ecotoxic profile, leave few residues, show slow or no resistance development, increase soil suppressiveness and may have a long-term effect. However, they are less effective and their effectiveness is more influenced by environmental factors compared with chemical pesticides. In addition, they are more expensive and difficult to combine. More knowledge about microbial pesticides is obviously needed to tackle these weaknesses.

Of course not only the grower is involved in the production of lettuce (Figure 7.3). The seed producer has an important role in providing resistant cultivars and pathogen-free seeds. Many pathogens are seed-borne and can be spread easily in this way. *Fusarium oxysporum* f. sp. *lactucae*, *B. cinerea*, *Verticillium dahliae* and *Microdochium panattonianum* have already been reported to contaminate lettuce seeds (Garibaldi *et al.*, 2004; Sowley *et al.*, 2010; Vallad *et al.*, 2005; Sutton & Holderness, 1986). Therefore seeds should be produced in areas with a low risk of pathogen occurrence. Pathogen detection in seeds and decontamination procedures can also limit the amount of diseased seeds.

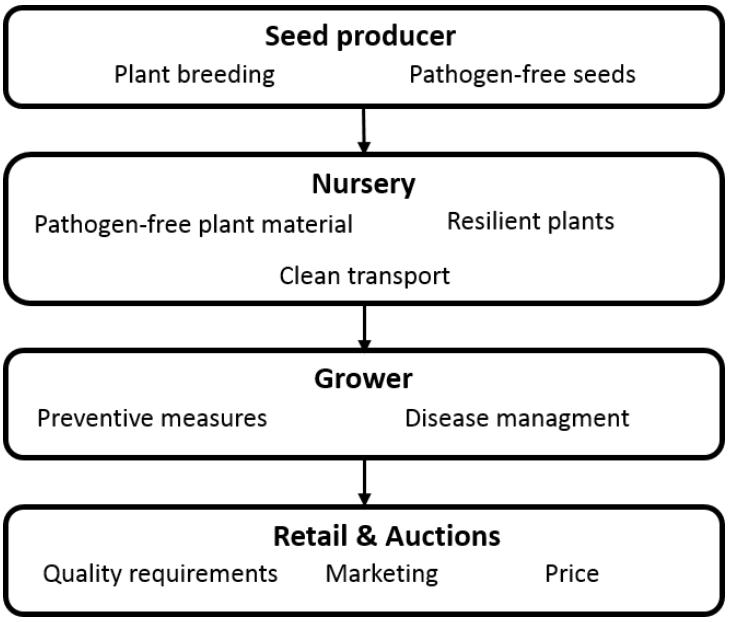

Figure 7.3 Lettuce from field to fork, the different actors and their role

Furthermore, the nursery should provide pathogen-free planting material and a clean transport. Therefore, preventive measures, such as limiting the number of visitors, regularly disinfecting machines and materials should be implemented. Next to that, we suggest to add organic matter, products or micro-organisms to the peat of the lettuce plant so resistance could be induced in the plants. Gilardi *et al.* (2016) already suggested the use of phosphite-based products, acibenzalor-S-methyl, pelleted *Brassica carinata* and biocontrol agents in nurseries to make plants more resilient to Fusarium wilt.

The grower should also implement preventive measures. If problems occur with soil-borne pathogens a correct diagnosis should be made in order to implement a proper management strategy. *Pratylenchus penetrans, Paratylenchus* sp., *R. solani, B. cinerea, Pythium* sp., *Sclerotinia* sp. and *F. oxysporum* f. sp. *lactucae* cause similar symptoms and are therefore difficult to distinguish (Table 7.1). Therefore, in the context of the FUNSLA-project, an app was designed based on pictures and fact sheets to assist lettuce growers in the identification of these pathogens (https://app.inagro.be/funsla/).

Table 7.1 Comparison of symptoms caused by common soil-borne pathogens of lettuce. Adapted and expanded from Gordon & Koike (2015) and Taylor & Clarkson (2018). Fus: *Fusarium oxysporum* f. sp. *lactucae*, Scle: *Sclerotinia* spp., Bot: *Botrytis cinerea*, Rhiz: *Rhizoctonia solani*, Pyth: *Pythium* spp., Nem: nematodes.

Symptoms	Fus	Scle	Bot	Rhiz	Pyth	Nem
Stunting	Yes	No	No	No	No	Yes
Plants collapse	Yes	Yes	Yes	No	Rare	No
Yellowing of older leaves	Yes	No	No	No	No	Yes
Vascular discoloration in taproot and crown	Yes	No	No	No	Rare	No
External crown and root tissue brown or rotted	Rare	Yes	Yes	Yes	Yes	No
Root lesions	No	No	No	No	No	Yes
Fungal mycelium and sclerotia on crown or soil	No	Yes	Yes	Yes	No	No

The last actor in the production of lettuce is the retail and auctions. Retail people and the auctions ask for a sustainably grown lettuce crop and request a high quality lettuce head. Accepting a lower quality could ensure the growers' sales. Moreover, they play an important role in marketing. Other lettuce types, such as lollo rossa, lollo bionda and oakleaf show resistance to Fusarium wilt and are good alternative for butterhead lettuce. Promoting these lettuce types could ensure the growers' sales and provide good prices. Moreover, VLAM ('Vlaams centrum voor agro- & visserijmarekting') can undertake a marketing campaign. Furthermore, the lettuce grower depends on the price the retail and auctions offer.

Intensive cropping with limited or no crop rotation is not the only reason for the occurrence of new pathogens in leafy vegetables. Several other parameters that can be responsible for the development of new diseases are listed by Gullino *et al.* (2019). Climate change, global travel and the international trade of seeds and products enhance the spread of pathogens. The entry of new pathogens in a glasshouse can disrupt the biological equilibrium in its soil and lead to devastating epidemics, such as *Fusarium* wilt. Climate change was also mentioned to influence the development of new diseases. In the past few years, *Allophoma tropica*, *Fusarium equiseti*, *F. oxysporum* f. sp. *lactucae* and *Pythium*

Cluster B2a have been reported to cause serious losses in Italy (Gilardi *et al.*, 2018; Gullino *et al.*, 2019). Apart from *F. oxysporum* f. sp. *lactucae,* these pathogens can also pose a threat for future Belgian lettuce cropping. Therefore, every actor should take his responsibility to guard against future threats.

7.2 Recommendations for future research

During this thesis we focused on several individual soil-borne pathogens, however their interactions should not be underestimated (Gracia *et al.*, 1991; Hassan, 1987; LaMondia, 2003; Kotcon *et al.*, 1984; Scholte & s'Jacob, 1989; Taheri *et al.*, 2016). The actual damage thresholds for the nematodes could be lower due to secondary infections. Furthermore, the disease development of *F. oxysporum* f. sp. *lactucae* or the basal rot pathogens in the lettuce plant may be enhanced by the presence of plant-parasitic nematodes. Therefore, the whole pathogen complex should be considered when lettuce is grown in soil.

A systems-based approach based on four pillars is recommended to control soil-borne pathogens (Barriere *et al.*, 2015; Chellemi *et al.*, 2016). First of all the introduction and spread of pathogens should be prevented by taking hygenic measures and using clean planting material and a disease-free producing area. The second pillar is the reduction of inoculum till an economic production can be ensured if infection occurs. This could be done by soil disinfestation or incorporating beneficial crop rotations. Thereafter, the disease suppressiveness should be promoted by stimulating shifts in the soil microbial community by crop rotation or incorporation of organic amendments. The last pillar is the intervention with pesticides and bio-pesticides to manage soil-borne pathogens. However, there are many ways to implement this and it is rather difficult for a highly specialized lettuce grower to transfer to this new production system. Therefore, more research is needed to investigate different ways of systems-based approaches and their influence on the soil-borne pathogens complex.

Identifying different routes of entry of pathogens such as seeds, plant material, machineries, work tools could help to understand what is needed to prevent pathogens from entering or spreading in the glasshouse. The effectiveness of several desinfectants should be examined to know which products kill pathogens most efficient.

Reducing the inoculum in the glasshouse can be realized in different ways: by chemical soil disinfestation, steaming, anaerobic soil disinfestation, biofumigation, organic amendments or crop rotation (Gamliel & van Bruggen, 2016). Different techniques could be compared in the glasshouse to reduce inoculum and the inoculum of the soil-borne pathogens should be measured after the application by plating, counting or qPCR. Also special attention should be given to the long term effect of the application. Disease symptoms of every pathogen could be scored in every cropping of lettuce.

The disease suppressiveness of the soil should be stimulated after reducing the inoculum. This could be conducted by incorporating organic amendments, micro-organisms or beneficial crop rotations. Pot experiments and field trials should be carried out to analyze the effect on the microbial community and nematode population density. It will also be necessary to guide growers in the implementation of this

approach. Therefore different techniques should be combined in longterm trials to demonstrate to growers.

This study resulted in several general conclusions for each pathogen, but also led to specific new research suggestions. Some future perspectives are discussed below.

- Black fallow reduced the population density of *Paratylenchus* sp., but was not effective against *P. penetrans* (Chapter 2 and 3). Surprisingly, often an increase of the population density of the root-lesion nematode was observed. The higher proportion of juveniles indicates that eggs had hatched. The grower only watered the soil at the end of black fallow. The increase of moisture content could explain the hatching of *P. penetrans,* as soil moisture is known to affect hatching of nematodes (Gaur & Haque, 1987). The influence of the timing of watering on hatching could be further investigated to avoid high population increase just before planting.

- Only a limited number of lettuce cultivars and alternative crops were tested for their host status for *Paratylenchus* sp. (Chapter 3). More knowledge on host status for this hitherto undescribed *Paratylenchus* sp. is necessary to incorporate good crop rotations in the management of this nematode.

- Estimated damage thresholds for nematodes should be interpreted with caution, because the experiments were conducted in an optimal environment for the plant. In addition, high standard errors were noted for the estimated parameters (Chapter 2 and 3). Other studies also showed that the standard error is mostly in the same order of magnitude as the estimated damage threshold (Teklu *et al.*, 2016; Heve *et al.*, 2015). This is due to the high variability in the growing process of lettuce, such as environmental factors, plant, nematodes, which all influence one number that is measured at the end: lettuce yield. This could be improved by including more replicates or avoiding variability during the set-up and during the experiment. Nevertheless, the estimated damage thresholds are still of high importance as a directive for the expected damage. As these experiments are conducted in optimal conditions for the plant, validation in the field is warranted. Several factors such as drought stress and secondary infections could lower the estimated damage threshold.

- Different *R. solani* AGs occur in Belgian glasshouses (Chapter 4; Van Beneden *et al.*, 2009). A seasonal appearance of the AGs was observed when growing butterhead lettuce in a continuous system without using fungicides. The AGs AG2-1, AG-BI and AG1-IB appeared mainly in colder periods, while AG4-HGI appeared in warmer periods (Chapter 4). Moreover, AG1-IB and AG4-HGI were highly aggressive on detached leaves, while AG2-1 and AG-BI were less aggressive. AG-BI was the least aggressive and only caused a disease index of 3 to 18%. It remains to be investigated whether AG-BI or AG2-1 cause economic damage when they occur

in a glasshouse in colder periods. In case they are not causing economic damage, fungicide spraying focusing on *R. solani* could be reduced or even omitted in these colder periods.

- It was already shown that *R. solani* AGs can differ in their sensitivity towards fungicides (Ajayi-oyetunde *et al.*, 2017; Sneh *et al.*, 1996). An adapted fungicide scheme with the most appropriate fungicides to control *R. solani* should be composed in function of the appearing AGs. Only a few active substances are allowed to control *R. solani.* These belong to different groups separated by FRAC (Fungicide Resistance Action Committee). It should be investigated if the AGs occurring in lettuce show different sensitivity towards the registered active substances so that an optimal use of the fungicides could be set up.

- Analyzing several *Fusarium* reference strains and *F. oxysporum* f. sp. *lactucae* isolates using GBS revealed that most of the *F. oxysporum* f. sp. *lactucae* isolates cluster with *F. curvatum* CBS 247.61 (Chapter 5). Pathogenicity trials on lettuce and column stock (*Matthiola incana*) could reveal if the genetic similarity based on the GBS data also results in a similar pathogenicity. Subsequently, sequencing the whole genome could provide more information on the similarity, although GBS has already a high resolution.

- A fast disease development was observed in commercial glasshouses were only race 4 occurred, while this was not the case for glasshouses with only race 1. This could be explained by the difference in their pathogenicity (Chapter 5). However, in some glasshouses with only race 4, disease development was slow, which may be due to soil suppressiveness. Soil suppressiveness to *Fusarium* wilt is often correlated with physical and chemical parameters, such as a high pH and high clay content; or microbial parameters, such as antagonistic bacteria (*Pseudomonas* spp. and actinomycetes) and fungi (non-pathogenic *F. oxysporum*) (Deltour *et al.*, 2017; Larkin *et al.*, 1996). Understanding which parameters are involved in the slow disease development could gain insights in the control of this dreading disease.

Summary

Butterhead lettuce (*Lactuca sativa* L.) is an important vegetable crop on the Belgian market with a turnover of 27.1 million euro in 2018. The Belgian production is characterized by the high quality and heavy weight of the lettuce head. The crop is grown mainly in soil in glasshouses in an intensive production system with up to five harvests per year. This intensive production leads to an increased disease incidence of several soil-borne pathogens, including fungi, oomycetes and nematodes. The most important nematodes are the root-lesion nematode *Pratylenchus penetrans* and the pin nematode *Paratylenchus* sp. These nematodes feed on the roots which leads to a damaged root system and a reduced growth of the lettuce head. Furthermore, rotting of the lower leaves, called basal rot, is an important disease in Belgian lettuce. The symptoms are caused by the fungi *Rhizoctonia solani, Botrytis cinerea,* and *Sclerotinia* spp. and the oomycete *Pythium* spp. These soil-borne pathogens are controlled mainly by chemical soil disinfestation and pesticides. Since 2015, *Fusarium oxysporum* f.sp. *lactucae* has been reported in several glasshouses in Belgium causing severe wilting of the lettuce head. Unfortunately, no effective control measures are available for this pathogen.

The use of pesticides and chemical soil disinfestation need to be restricted due to strict regulations, environmental concerns and consumer demand for pesticide-free food products. Moreover, according to the EC Directive on Sustainable Use of Pesticides (2009/128), growers should implement an integrated pest management (IPM) from 2014 onwards. In this doctoral thesis, nematodes and pathogens causing basal rot were investigated with the aim to reduce the application of pesticides and chemical soil disinfestation and to improve disease management strategies. In addition, special attention was given to a new lettuce pathogen, *F. oxysporum* f. sp. *lactucae,* which is threatening the Belgian lettuce production. Finally, the lettuce production system was analyzed to understand why lettuce growers opt for an intensive production system. A closer look was taken at chemical and biopesticides for an integrated control of lettuce pathogens, now and in the future, by conducting a SWOT-analysis.

Very high densities of nematodes, which are often associated with damage, are observed in commercial glasshouses. To gain insight into factors influencing these high numbers, nematode populations were monitored for at least one year by frequently sampling the same four 1-m² spots at 0-30 cm and 30-60 cm depth in Belgian commercial glasshouses to investigate the population dynamics of *P. penetrans* or *Paratylenchus* sp. Five glasshouses with continuous production of lettuce or lamb's lettuce were sampled for the study of the *Paratylenchus* sp. populations, while the *P. penetrans* population dynamics were monitored in one glasshouse where the grower grew lettuce in rotation with leek. The population densities of *P. penetrans* were rather low, while the numbers of *Paratylenchus* sp. fluctuated greatly with high densities occurring during winter and spring (> 20,000 nematodes (100 ml soil)$^{-1}$). Nematode populations were reduced drastically after chemical soil disinfestation, but a proportion survived in the lower layer, while with steaming under negative pressure no *Paratylenchus* sp. was observed, even after nine months. Three months of black fallow during winter resulted in a slight increase of the *P. penetrans* population, probably due to hatching, while two months of black fallow during spring resulted in 50 to 76% fewer pin nematodes. Growing leek reduced the *P. penetrans* population slightly, while a

small increase was observed for lettuce. The five monitored populations of *Paratylenchus* sp. were characterized based on morphological and molecular features to determine the species. Results indicated that it is a hitherto undescribed species. A host status experiment for this *Paratylenchus* sp. revealed that lettuce allowed nematode reproduction, with a factor of 1 to 2, while lamb's lettuce, parsley and wild rocket did not. Several damage threshold experiments were set up for *P. penetrans* and *Paratylenchus* sp. Two pot experiments with 10 densities of *P. penetrans* were conducted to estimate the damage threshold for a summer and autumn cultivar ('Cosmopolia' and 'Brighton', respectively). The thresholds for lettuce weight were 669 and 3834 *P. penetrans* (100 ml soil)$^{-1}$ in summer and autumn, respectively. The thresholds for root damage were much lower: 204 and 48 *P. penetrans* (100 ml soil)$^{-1}$. In three experiments with lettuce cv. 'Cosmopolia' in pots with a series of 9 or 10 densities of *Paratylenchus* sp. (up to 35,000 (100 ml soil)$^{-1}$), no damage to lettuce heads was observed. However, root weight and root quality were reduced, and the corresponding damage thresholds were 1,754 and 362 *Paratylenchus* sp. (100 ml soil)$^{-1}$, respectively. These results show that management strategies such as crop rotation, soil disinfestation or fallow are recommended to avoid population build-up.

A seasonal appearance of the basal rot pathogens: *R. solani*, *Sclerotinia* spp., *B. cinerea* and *Pythium* spp. has been reported, but lettuce growers use standard spraying schemes during the whole year. To investigate if the use of fungicides could be reduced by only controlling the pathogen active at the moment of spraying, lettuce was continuously grown in three glasshouses without any fungal disease control and the active pathogens causing basal rot were identified as soon as symptoms were visible. The occurrence of basal rot pathogens appeared to be mainly glasshouse specific and the different basal rot pathogens were active throughout the year. However, a seasonal appearance of *R. solani* anastomosis groups (AG) and *Pythium* spp. was observed with *R. solani* AG4-HGI and *Pythium ultimum* active at higher temperatures and *R. solani* AG2-1, AG-BI, AG1-IB and *Pythium sylvaticum* at lower temperatures. Each *R. solani* AG had its own optimal growth rate *in vitro* and the AGs differed in virulence on detached leaves. *Rhizoctonia solani* AG1-IB and AG4-HGI were the most pathogenic, followed by *R. solani* AG2-1 and AG-BI. These results show that the fungicide spraying scheme should be adapted to the pathogens occurring in the glasshouse, rather than restricting applications to a certain period generally applied in all glasshouses.

Since 2015, Fusarium wilt has spread rapidly in the lettuce production area. Seventy-eight *Fusarium* isolates were collected from lettuce heads with the typical wilting symptoms and identified to study their genetic variation and pathogenicity. Of these 78 isolates, 91% belonged to *F. oxysporum* f. sp. *lactucae* race 4 and 6% to race 1, which had not been reported before in Belgium. Pathogenicity assays revealed that the inoculation method is of importance to determine differences in virulence between races. These were only observed when chlamydospores were mixed into the substrate, not when applying a root dip in a spore suspension. At 24°C, race 4 isolates were more aggressive than race 1. By using the genotyping-by-sequencing technique it was shown that Belgian race 4 and race 1 isolates are highly similar to *F. oxysporum* f. sp. *lactucae* isolates from the Netherlands and Japan. This indicates the spread of these races from a unique source. Moreover, two genetic groups could be distinguished within race 4, of which the main isolates were highly similar to *F. curvatum* isolate CBS 247.61, a pathogen

isolated from column stock (*Matthiola incana*) in Germany in 1957. These results suggest that the pathogen has been present for a long time in Europe, but only recently became associated with lettuce.

The intensive lettuce production is a common practice, although it leads to a higher disease incidence of soil-borne pathogens. Analyzing the production system using systems thinking showed that this system is currently the most cost efficient, explaining why it is widely practiced. Strengths, weaknesses, opportunities and threats were evaluated for pesticide and microbial pesticide use. This SWOT analysis showed that pesticide use should be restricted due to the residues and toxicity to the environment and applicant, although they are highly effective and their use is largely independent from the environment. Microbial pesticides are of interest, because they have a good ecotoxic profile, leave less residues, show slower resistance development, increase soil suppressiveness and may have a long-term effect, but more research is needed to increase their effectiveness and to understand how their application could be improved, so that food security and lettuce quality could be ensured.

In conclusion, the intensive production of lettuce leads to an increased disease-incidence which is not appropriate in the terms of disease management. Moreover, this production system is nowadays hard to maintain due to the occurrence of *F. oxysporum* f.sp. *lactucae.* Therefore the production system should be reconsidered and adapted, taking into account the means and desires of the lettuce grower. Furthermore, an ensured future for the lettuce production is not only the responsibility of the lettuce grower. The seed producer, nursery, retail and auctions also play an important role.

Samenvatting

Kropsla (*Lactuca sativa* L.) is een belangrijk groente op de Belgische markt met een omzet van 27,1 miljoen euro in 2018. De Belgische productie wordt gekenmerkt door zware kroppen met een hoge kwaliteit. Sla wordt voornamelijk in grond in serres geteeld in een intensief productiesysteem met tot vijf oogsten per jaar. Deze intensieve productie leidt tot een verhoogde ziekte-incidentie van verschillende bodempathogenen, waaronder schimmels, oömyceten en nematoden. De belangrijkste nematoden zijn het wortellesieaaltje *Pratylenchus penetrans* en het speldaaltje *Paratylenchus* sp. Deze nematoden voeden zich op de wortel wat leidt tot een beschadigd wortelstelsel en een gereduceerde groei van de krop. Bovendien is verrotting van de oudste bladeren, ook wel smet genoemd, een belangrijke ziekte in Belgische sla. De symptomen worden veroorzaakt door de schimmels *Rhizoctonia solani*, *Botrytis cinerea* en *Sclerotinia* spp. en de oömyceet *Pythium* spp. Deze bodempathogenen worden hoofdzakelijk bestreden met chemische grondontsmetting en pesticiden. Sinds 2015 wordt *Fusarium oxysporum* f. sp. *lactucae* in verschillende serres in België geobserveerd en veroorzaakt ernstige verwelking. Er zijn echter geen effectieve beheersingsmaatregelen beschikbaar voor deze pathogeen.

Het gebruik van pesticiden en chemische grondontsmetting moet worden beperkt vanwege de strenge wetgeving, de bezorgdheid om het milieu en de vraag naar residuvrij voedsel van de consument. Bovendien moeten telers vanaf 2014 een geïntegreerd gewasbescherming (IPM) volgens de EG-richtlijn inzake duurzaam gebruik van pesticiden (2009/128/EG) implementeren. In dit doctoraatsproefschrift werden nematoden en smetpathogenen onderzocht met als doel de toepassing van pesticiden en chemische grondontsmetting te reduceren en strategieën voor ziektebeheer te verbeteren. Daarnaast werd *F. oxysporum* f. sp. *lactucae*, die de Belgische slaproductie bedreigt, extra onderzocht. Tot slot, werd het slaproductiesysteem geanalyseerd om te begrijpen waarom slatelers kiezen voor een intensief productiesysteem. Pesticiden en biopesticiden werden nader onderzocht via een SWOT-analyse om hun mogelijkheden voor een geïntegreerde beheersing van slapathogenen, nu en in de toekomst, na te gaan.

Hoge dichtheden aan nematoden, vaak geassocieerd met schade, worden waargenomen in commerciële serres. Om inzicht te krijgen waarom deze nematoden in hoge aantallen voorkomen, werden populaties van *P. penetrans* en *Paratylenchus* sp. gedurende minstens één jaar opgevolgd door regelmatig dezelfde vier plaatsen van 1 m² op 0-30 cm en 30-60 cm diepte te bemonsteren in Belgische commerciële serres. Vijf serres met een continue productie van sla of veldsla werden bemonsterd voor de studie van de *Paratylenchus* sp. populaties, terwijl de *P. penetrans* populatiedynamica werd gevolgd in één serre waar de teler sla roteert met prei. De *P. penetrans* populatiedichtheden waren vrij laag, terwijl het aantal *Paratylenchus* sp. sterk fluctueerde met hoge dichtheden in de winter en lente (> 20.000 nematoden (100 ml grond)$^{-1}$). De populaties werden sterk gereduceerd na chemische grondontsmetting, maar een deel overleefde in de onderste laag, terwijl bij stomen met onderdruk geen *Paratylenchus* sp. werd waargenomen, zelfs na negen maanden. Drie maanden zwarte braak in de winter resulteerde in een lichte toename van de *P. penetrans* populatie, waarschijnlijk als gevolg van het uitkomen van eitjes, terwijl twee maanden zwarte braak in de lente resulteerde in 50 tot 76% minder

speldaaltjes. Een preiteelt verminderde de *P. penetrans* populatie enigszins, terwijl een kleine toename werd waargenomen voor sla. De vijf gemonitorde populaties van *Paratylenchus* sp. werden gekarakteriseerd op basis van morfologische en moleculaire kenmerken om de soort te bepalen. Resultaten gaven aan dat het een tot nu toe onbeschreven soort is. Een waardplantproef voor deze *Paratylenchus* sp. toonde aan dat de populatie steeg na sla, met een factor 1 tot 2, terwijl bij veldsla, peterselie en rucola de populatie daalde. Verschillende schadedrempelproeven werden opgezet voor *P. penetrans* en *Paratylenchus* sp. Twee pot-experimenten met 10 dichtheden van *P. penetrans* werden uitgevoerd om de schadedrempel voor een zomer- en herfstras te bepalen ('Cosmopolia' en 'Brighton', respectievelijk). De schadedrempels voor het kropgewicht waren respectievelijk 669 en 3834 *P. penetrans* (100 ml grond)$^{-1}$ in de zomer en herfst. De schadedrempels voor wortelschade waren veel lager: 204 en 48 *P. penetrans* (100 ml grond)$^{-1}$. In drie experimenten met sla cv. 'Cosmopolia' in potten met een reeks van 9 of 10 dichtheden van *Paratylenchus* sp. (tot 35.000 (100 ml grond)$^{-1}$), werd geen schade aan de slakroppen waargenomen. Wortelgewicht en kwaliteit van de wortels waren echter verminderd en de overeenkomstige schadedrempels waren 1.754 en 362 *Paratylenchus* sp. (100 ml grond)$^{-1}$, respectievelijk. Deze resultaten tonen aan dat een populatietoename kan worden voorkomen met beheersingsmaatregelen zoals vruchtwisseling, grondontsmetting of braak.

Een seizoenaal voorkomen van de smetpathogenen: *R. solani*, *Sclerotinia* spp., *B. cinerea* en *Pythium* spp. werd waargenomen, maar slatelers gebruiken het hele jaar door standaard spuitschema's. Om na te gaan of het gebruik van fungiciden kon worden verminderd door alleen de actieve pathogeen te beheersen, werd sla continu gekweekt in drie serres zonder enige fungicidentoepassing en werden de pathogenen die smet veroorzaakten geïdentificeerd zodra de symptomen zichtbaar waren. Het voorkomen van smetpathogenen bleek voornamelijk serrespecifiek te zijn en de verschillende smetpathogenen waren het hele jaar door actief. Een seizoenaal voorkomen van *R. solani* anastomosegroepen (AG) en *Pythium* spp. werd waargenomen, met *R. solani* AG4-HGI en *Pythium ultimum* actief bij hogere temperaturen en *R. solani* AG2-1, AG-BI, AG1-IB en *Pythium sylvaticum* bij lagere temperaturen. Elke *R. solani* AG had zijn eigen optimale groeisnelheid *in vitro* en de AG's verschilden in virulentie in biotoetsen op afgetrokken bladeren. *Rhizoctonia solani* AG1-IB en AG4-HGI waren het meest pathogeen, gevolgd door *R. solani* AG2-1 en AG-BI. Deze resultaten tonen aan dat het spuitschema voor fungiciden moet worden aangepast aan de pathogenen die in de serre voorkomen, in plaats van toepassingen te beperken tot een bepaalde periode die algemeen in alle serres wordt toegepast.

Sinds 2015 heeft Fusarium verwelking zich snel verspreid in het slaproductiegebied. Achtenzeventig *Fusarium*-isolaten werden verzameld van kroppen met de typische verwelkingssymptomen en geïdentificeerd om hun genetische variatie en pathogeniteit te bestuderen. Van deze 78 isolaten behoorde 91% tot *F. oxysporum* f. sp. *lactucae* fysio 4 en 6% tot fysio 1, die nog niet eerder in België werd gerapporteerd. Pathogeniteitstesten toonden aan dat de inoculatiemethode van belang is om verschillen in virulentie tussen fysio's te bepalen. Deze werden alleen waargenomen wanneer chlamydosporen in het substraat werden gemengd en niet bij het uitvoeren van een worteldip in een sporenoplossing. Bij 24°C waren fysio 4 isolaten agressiever dan fysio 1 isolaten. Door gebruik te maken van de "genotyping-by-sequencing" technologie werd aangetoond dat Belgische fysio 4 en 1 isolaten

zeer vergelijkbaar zijn met *F. oxysporum* f. sp. *lactucae* isolaten uit Nederland en Japan. Dit wijst op een verspreiding van deze fysio's vanuit een unieke bron. Bovendien konden binnen fysio 4 twee genetische groepen worden onderscheiden, waarvan de grootste groep isolaten sterk vergelijkbaar waren met *F. curvatum* isolaat CBS 247.61, een pathogeen geïsoleerd uit zomerviolier (*Matthiola incana*) in Duitsland in 1957. Deze resultaten suggereren dat de pathogeen reeds langer aanwezig is in Europa, maar pas sinds kort geassocieerd is met sla.

De intensieve slaproductie is een gangbare praktijk, hoewel het leidt tot een hogere ziekte-incidentie van bodempathogenen. Een analyse met behulp van systeemdenken toonde aan dat het intensieve productiesysteem momenteel het meest kostenefficiënt is, wat verklaart waarom het op grote schaal wordt toegepast. Sterkes, zwaktes, kansen en bedreigingen werden geëvalueerd voor gebruik van pesticiden en microbiële pesticiden. Deze SWOT-analyse toonde aan dat het gebruik van pesticiden moet worden beperkt vanwege de residuen en toxiciteit voor het milieu en de toepasser, hoewel ze zeer effectief zijn en hun gebruik grotendeels onafhankelijk is van het milieu. Microbiële pesticiden zijn interessant, omdat ze een goed ecotoxisch profiel hebben, minder residuen achterlaten, een langzamere resistentieontwikkeling vertonen, de bodemweerbaarheid verhogen en mogelijks een effect op lange termijn hebben, maar meer onderzoek is nodig om hun effectiviteit te verhogen en te begrijpen hoe hun toepassing kan worden verbeterd, zodat voedselveiligheid en slakwaliteit kan worden gewaarborgd.

In conclusie, de intensieve slaproductie leidt tot een verhoogde ziekte-incidentie waardoor ziektebeheersing zeer moeilijk wordt. Vandaag de dag is dit productiesysteem moeilijk vol te houden vanwege het optreden van *F. oxysporum* f. sp. *lactucae*. Daarom moet het productiesysteem worden herbekeken en aangepast, rekening houdend met de middelen en wensen van de slateler. Bovendien is een gegarandeerde toekomst voor de slaproductie niet alleen de verantwoordelijkheid van de slateler. De zaadproducenten, de opkwekers van slaplanten, de veilingen en grootwarenhuizen spelen ook een belangrijke rol.

References

Agrios, G. N. (2005). Plant Pathology. Fifth edition. Burlington, Elsevier Academic Press.

Ajayi-oyetunde, O. O., Butts-wilmsmeyer, C. J., & Bradley, C. A. (2017). Sensitivity of *Rhizoctonia solani* to succinate dehydrogenase inhibitor and demethylation inhibitor fungicides. *Plant Disease*, **101**, 487–495.

Akyazi, F., Felek, A. F., Cermák, V., Cudejková, M., Foit, J., Yildiz, S., & Hánel, L. (2015). Description of *Paratylenchus (Gracilacus) straeleni* (De Coninck, 1931) Oostenbrink, 1960 (Nematoda: Criconematoidea, Tylenchulidae) from hazelnut in Turkey and its comparison with other world populations. *Helminthologia*, **52**, 270–279.

Andrássy, I. (2007). Free-living nematodes of Hungary (Nematoda Errantia), Volume II. *Hungarian Natural History Museum and Systematic Zoology Research Group of the Hungarian Academy of Sciences,* Budapest, 277-279.

Anguiz, R., & Martin, C. (1989). Anastomosis groups, pathogenicity and other characteristics of *Rhizoctonia solani* isolated from potatoes in Peru. *Plant Disease*, **73** (3), 199–201.

Ankrom, K. E., Lopez-Nicora, H., Niblack, T. L., & Lindsey, L. E. (2017). First report of a pin nematode (*Paratylenchus neoamblycephalus*) from soybean in Ohio. *Plant Disease*, **101**, 1330.

Armstrong, G. M., & Armstrong, J. K. (1978). Formae speciales and races of *Fusarium oxysporum* causing wilts of the *Cucurbitaceae*. *Phytopathology*, **68**, 19–28.

Balali, G. R., Neate, S. M., Scott, E. S., & Whisson, D. L. (1995). Anastomosis group and pathogenicity of isolates of *Rhizoctonia solani* from potato crops in South Australia. *Plant Pathology*, **44** (6), 1050-1057.

Barriere, V., Lecompte, F., & Lescourret, F. (2015). Efficacy of pest and pathogen control, yield and quality of winter lettuce crops managed with reduced pesticide applications. *European Journal of Agronomy*, **71**, 34–43.

Barrière, V., Lecompte, F., & Nicot, P. C. (2014). Lettuce cropping with less pesticides. A review. *Agronomy for Sustainable Development*, **34** (1), 175–198.

Bell, N. L., & Watson, R. N. (2001a). Dynamics of sympatric *Paratylenchus nanus* and *Paratrichodorus minor* populations in soil under pasture. *Nematology*, **3**, 267–275.

Bell, N. L., & Watson, R. N. (2001b). Population dynamics of *Paratylenchus nanus* in soil under pasture: 1. Aggregation and abiotic factors. *Nematology*, **3**, 187–197.

Bell, N. L., & Watson, R. N. (2001c). Population dynamics of *Paratylenchus nanus* in soil under pasture: 2. Biotic factors and population modelling. *Nematology,* **3** (3), 255-265.

Berkelmans, R., Ferris, H., Tenuta, M. & van Bruggen, A. H. C. (2003). Effects of long-term crop management on nematode trophic levels other than plant feeders disappear after 1 year of disruptive soil management. *Applied Soil Ecology,* **23**, 223–235.

Billard, A., Fillinger, S., Leroux, P., Bach, J., Lanen, C., Lachaise, H., Beffa, R., & Debieu, D. (2011). Fenhexamid Resistance in the *Botrytis* species complex , responsible for grey mould disease. *Fungicides - Beneficial and Harmful Aspects*, 61-78.

Blancard D, Lot H., & Maisonneuve B. (2006). A color atlas of diseases of lettuce and related salad crops. Observation, biology and control. Manson Publishing Ltd. ISBN 1-84076-050-8.

Blok, I., & Van der Plaats-Niterink, A. J. (1978). *Pythium uncinulatum* sp. nov. and *P. tracheiphilum* pathogenic to lettuce. *Netherlands Journal of Plant Pathology*, **84**, 135–136.

Boag, B., & Alphey, T. J. W. (1988). Influence of interspecific competition on the population dynamics of migratory plant-parasitic nematodes with r and K survival strategies. *Revue de Nématologie*, **11**, 321–326.

Brzeski, M. W. (1998). Nematodes of Tylenchina in Poland and temperate Europe. Muzeum i Instytutu Zoologii, Polska Akademia Nauk (MiIZ PAN), 265-269.

Burger, Y., Katzir, N., Tzuri, G., Portnoy, V., Saar, U., Shriber, S., & Cohen, R. (2003). Variation in the response of melon genotypes to *Fusarium oxysporum* f. sp. *melonis* race 1 determined by inoculation. *Plant Pathology*, **52**, 204–211.

Carbone, I., & Kohn, L. M. (1999). A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia*, **91** (3), 553–556.

Carling, D. E., Baird, R. E., Gitaitis, R. D., Brainard, K. A., & Kuninaga, S. (2002a). Characterization of AG-13, a newly reported anastomosis group of *Rhizoctonia solani*. *Phytopathology*, **92** (8), 893–899.

Carling, D. E., Kuninaga, S., & Brainard, K. a. (2002b). Hyphal anastomosis reactions, rDNA-Internal transcribed spacer sequences, and virulence levels among subsets of *Rhizoctonia solani* anastomosis group-2 (AG-2) and AG-BI. *Phytopathology*, **92** (1), 43–50.

Carling, D. E., & Leiner, R. H. (1990). Effect of temperature on virulence of *Rhizoctonia solani* and other *Rhizoctonia* on potato. *Phytopathology*, **80** (10), 930–934.

Castillo, P. & Vovlas, N. (2007). *Pratylenchus* (Nematoda: Pratylenchidae): Diagnosis, Biology, Pathogenicity and Management. Nematology Monographs and Perspectives 6 (Series Editors: Hunt, D.J. & Perry, R.N.). Leiden, The Netherlands, Brill.

Chandler, D., Bailey, A. S., Tatchell, G. M., Davidson, G., Greaves, J., & Grant, W. P. (2011). The development, regulation and use of biopesticides for integrated pest management. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **366**, 1987–1998.

Chapman, J. (2004). Introducing systems thinking. Report. 1-25.

Chellemi, D. O. (2002). Nonchemical management of soilborne pests in fresh market vegetable production systems. *Phytopathology*, **92**, 1367–1372.

Chellemi, D. O., Gamliel, A., Katan, J., & Subbarao, K. V. (2016). Development and deployment of systems-based approaches for the management of soilborne plant pathogens. *Phytopathology*, **106**, 216–225.

Chen, S., Hendrickx, G. & Moens, M. (2000). The importance of organic matter when assaying *Meloidogyne chitwoodi* soil populations. *Russian Journal of Nematology,* **8**, 147-152.

Chen, D. Y., Ni, H. F., Tsay, T. T., & Yen, J. H. (2008). Identification of *Gracilacus bilineata* and *G. aculenta* (Nematoda: Criconematiodea, Tylenchulidae) among bamboo plantations in Taiwan. *Plant Pathology Bulletin*, **17**, 209–220.

Chen, D. Y., Ni, H. F., Yen, J. H., & Tsay, T. T. (2006). Identification of stunt nematode *Tylenchorhynchus annulatus* and a new recorded *Tylenchorhynchus leviterminalis* (Nematoda: Belonolaimidae) in Taiwan. *Plant Pathology Bulletin*, **15**, 251–262.

Chen, D. Y., Ni, H. F., Yen, J. H., & Tsay, T. T. (2007). Identification of a new recorded pin nematode, *Paratylenchus lepidus*, (Nematoda: Criconematoidea, Tylenchulidae) among tea plantations in Taiwan. *Plant Pathology*

Bulletin, **16**, 41–46.

Chen, D. Y., Ni, H. F., Yen, J. H., & Tsay, T. T. (2009). Identification of a new recorded pin nematode *Paratylenchus minutus* (Nematoda: criconematoidea, Tylenchulidae) in Taiwan. *Plant Pathology Bulletin*, **18**, 167–174.

Claerbout, J., Neukermans, J., Vandevelde, I., Decombel, A., de Sutter, N., Deeren, A.-M., Venneman, S., Bleyaert, P., Höfte, M., & Viaene, N. (in press). *Pratylenchus penetrans*, a potential risk in glasshouse-grown lettuce: population dynamics and damage threshold. *Nematology*.

Claerbout, J., Venneman, S., Vandevelde, I., Decombel, A., Bleyaert, P., Volckaert, A., Neukermans, J, & Höfte, M. (2018). First report of *Fusarium oxysporum* f. sp. *lactucae* race 4 on lettuce in Belgium. *Plant Disease*, **102** (5), 1037.

Cordero Lopez, M., Robbins, R. T., & Szalanski, A. L. (2013). Taxonomic and molecular identification of *Hemicaloosia, Hemicycliophora, Gracilacus* and *Paratylenchus* species. *Journal of Nematology*, **45**, 145–171.

Correl, J. C. (1991). The relationship between formae speciales, races, and vegetative compatibility groups in *Fusarium oxysporum*. *Phytopathology*, **81**, 1061–1064.

Coursen, B. W., Rhode, R. A., & Jenkins, W. R. (1958). Additions to the host lists of the nematodes *Paratylenchus projectus* and *Trichodorus christiei*. *Plant Disease Reporter*, **42**, 456–460.

Couteaudier, Y., & Alabouvette, C. (1990). Survival and inoculum potential of conidia and chlamydospores of *Fusarium oxysporum* f. sp. *lini* in soil. *Canadian Journal of Microbiology*, **36** (8), 551–556.

Cubeta, M. A., & Vilgalys, R. (1997). Population biology of the *Rhizoctonia solani* complex. *Phytopathology*, **87** (4), 480–484.

Darriba, D., Taboada, G. L., Doallo, R., & Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods,* **9**, 772–772.

Davis, R. M., Subbarao, K. V., Raid, N. R., & Kurtz, E. A. (Eds.). (1997). *Compendium of lettuce diseases*. St. Paul, Minesota: The American Phytopathological Society.

De Grisse, A. T. (1969). Redescription ou modifications de quelques techniques utilisées dans l'étude des nematodes phytoparasitaires. *Mededelingen Rijksfaculteit der Landbouwwetenschappen*, Gent 34, 351-369.

De Ley, P., Félix, M. A., Frisse, L. M., Nadler, S. A., Sternberg, P. W. & Thomas, W. K. (1999). Molecular and morphological characterisation of two reproductively species with mirror-image anatomy (Nematoda: Cephalobidae). *Nematology,* **2**, 591-612.

Decraemer, W. & Hunt (2006). Structure and classification. In Perry R. N. & Moens M. (Eds.*). Plant Nematology*. Wallingford, UK, CABI Publishing, 3-32.

Deltour, P., França, S. C., Liparini, O., Cardoso, I., De Neve, S., Debode, J., & Höfte, M. (2017). Agriculture, ecosystems and environment disease suppressiveness to Fusarium wilt of banana in an agroforestry system: Influence of soil characteristics and plant community. *Agriculture, Ecosystems and Environment*, **239**, 173–181.

Doornik, A. W. (1981). Temperature dependence of the pathogenicity of several isolates of *Rhizoctonia solani* in some bulb crops as an intrinsic property of the isolate. *Netherlands Journal of Plant Pathology*, **87** (4), 139–147.

Dunham, W., & Trimmer, M. (2018). Biological products around the world. Bio-products Industry Alliance Spring Meeting & International Symposium (BPIA.org).

Dunn, R. A. (1972). Importance of depth in soil, presence of host roots and role of eggs as compared to vermiform stages in overwintering of *Pratylenchus penetrans* at Ithaca, New York. *Journal of Nematology,* **4**, 221–222.

Elliott, A. P. & Bird, G. W. (1985). Pathogenicity of *Pratylenchus penetrans* to Navy Bean (*Phaseolus vulgaris* L.). *Journal of Nematology* 17, 81–85.

Esmaeili, M., Heydari, R., Castillo, P., Bidhendi, M. Z., & Palomares-Rius, J. E. (2016). Molecular characterisation of two known species of *Paratylenchus* Micoletzky, 1922 from Iran with notes on the validity of *Paratylenchus audriellus* Brown, 1959. *Nematology*, **18**, 591–604.

France, R. A. & Brodie, B. B. (1995). Differentiation of two New York isolates of *Pratylenchus penetrans* based on their reaction on potato. *Journal of Nematology*, **27**, 339–345.

Fujinaga, M., Ogiso, H., Shinohara, H., Tsushima, S., Nishimura, N., Togawa, M., Saito, H., & Nozue, M. (2005). Phylogenetic relationships between the lettuce root rot pathogen *Fusarium oxysporum* f. sp. *lactucae* races 1, 2, and 3 based on the sequence of the intergenic spacer region of its ribosomal DNA. *Journal of General Plant Pathology*, **71**, 402–407.

Fujinaga, M., Ogiso, H., Tuchiya, N., Saito, H., Yamanaka, S., Nozue, M., & Mineo, K. (2003). Race 3, a new race of *Fusarium oxysporum* f. sp. *lactucae* determined by a differential system with commercial cultivars. *Journal of General Plant Pathology*, **69**, 23–28.

Fytoweb (2019). https://fytoweb.be/nl accessed on 15th of August 2019.

Gamliel, A., & Van Bruggen, A. H. C. (2016). Maintaining soil health for crop production in organic greenhouses. *Scientia Horticulturae*, **208**, 120–130.

Garibaldi, A., Gilardi, G., & Gullino, M. L. (2002). First report of *Fusarium oxysporum* on lettuce in Europe. *Plant Disease*, **86** (9), 1052.

Garibaldi, A., Gilardi, G., & Gullino, M. L. (2004). Seed transmission of *Fusarium oxysporum* f. sp. *lactucae*. *Phytoparasitica*, **32** (1), 61–65.

Gaur, H. S. & Haque, M. M. (1987). Effect of irrigation and stage of moisture stress on the growth of nematode population under pea. *Indian Journal of Nematology,* **17**, 189–192.

Ghaderi, R. (2019). The damage potential of pin nematodes, *Paratylenchus* Micoletzky, 1922 *sensu lato* spp. (Nematoda: Tylenchulidae). *Journal of Crop Protection*, **8**, 243-257.

Ghaderi, R., Kashi, L., & Karegar, A. (2014). Contribution to the study of the genus *Paratylenchus* Micoletzky, 1922 sensu lato (Nematoda: Tylenchulidae). *Zootaxa*, **3841** (2), 151–187.

Gilardi, G., Demarchi, S., Lodovica, M., & Garibaldi, A. (2016). Evaluation of the short term effect of nursery treatments with phosphite-based products, acibenzolar-S-methyl, pelleted *Brassica carinata* and biocontrol agents, against lettuce and cultivated rocket fusarium wilt under artificial inoculation and greenhous. *Crop Protection*, **85**, 23–32.

Gilardi, G., Garibaldi, A., Matic, S., Senatore, M. T., Pipponzi, S., Prodi, A., & Gullino, M. L. (2019). First report of *Fusarium oxysporum* f. sp. *lactucae* race 4 on lettuce in Italy. *Plant Disease*. DOI: 10.1094/PDIS-05-19-0902-PDN

Gilardi, G., Gullino, M. L., & Garibaldi, A. (2018). Emerging foliar and soil-borne pathogens of leafy vegetable crops: a possible threat to Europe. *Bulletin OEPP/EPPO Bulletin*, **48** (1), 116–127.

Gilardi, G., Ortega, S. F., Rijswick, P. C. J. Van, Ortu, G., Gullino, M. L., & Garibaldi, A. (2016). A new race of *Fusarium oxysporum* f. sp. *lactucae* of lettuce. *Plant Pathology*, **66** (4), 677–688.

Gilardi, G., Pons, C., Gard, B., Franco-Ortega, S., & Gullino, M. L. (2017). Presence of Fusarium wilt, incited by *Fusarium oxysporum* f. sp. *lactucae*, on lettuce in France. *Plant Disease*, **101** (6), 1053.

Gill, J. S., Sivasithamparam, K., & Smettem, K. R. J. (2000). Soil types with different texture affects development of *Rhizoctonia* root rot of wheat seedlings. *Plant and Soil*, **221**, 113–120.

Glare, T., Caradus, J., Gelernter, W., Jackson, T., Keyhani, N., Köhl, J., Marrone, P., Morin, L., & Stewart, A. (2012). Have biopesticides come of age? *Trends in Biotechnology*, **30** (5), 250–258.

Goodey, J. B., M. T. Franklin, & D. J. Hooper. (1965). *T. Goodey's: The nematode parasites of plants catalogued under their hosts*. Commonwealth Agricultural Bureaux, Farnham Royal, Bucks, England. Third Edition.

Gordon, T. R. (2017). *Fusarium oxysporum* and the Fusarium wilt syndrome. *Annual Review of Phytopathology*, **55**, 23-39.

Gordon, T. R., & Koike, S. T. (2015). Management of Fusarium wilt of lettuce. *Crop Protection*, **73**, 45–49.

Grabau, Z. J., Maung, Z. T. Z., Noyes, D. C., Baas, D. G., Werling, B. P., Brainard, D. C. & Melakeberhan, H. (2017). Effects of cover crops on *Pratylenchus penetrans* and the nematode community in carrot production. *Journal of Nematology,* 49, 114–123.

Gracia, J.A., Reeleder, R.D. & Bélair, G. (1991). Interactions between *Pythium tracheiphilum*, *Meloidogyne hapla* and *Pratylenchus penetrans* on lettuce. *Phytoprotection,* **72**, 105–114.

Groenewald, J. Z., Nakashima, C., Nishikawa, J., Shin, H.-D., Park, J.-H., Jama, A. N., Groenewald, M., Braun, U., & Crous, P. W. (2003). Species concepts in *Cercospora*: spotting the weeds among the roses. *Studies in Mycology*, **75**, 115–170.

Grosch, R., & Kofoet, A. (2003). Influence of temperature, pH and inoculum densitiy on bottom rot on lettuce caused by *Rhizoctonia solani*. *Journal of Plant Diseases and Protection*, **110** (4), 366–378.

Grosch, R., Schneider, J. H. M., & Kofoet, A. (2004). Characterisation of *Rhizoctonia solani* anastomosis groups causing bottom rot in field-grown lettuce in Germany. *European Journal of Plant Pathology*, **110**, 53–62.

Grosch, G., Schneider, J. H. M., Kofoet, A., & Feller, C. (2010). Impact of continuous cropping of lettuce on the disease dynamics of bottom rot and genotypic diversity of *Rhizoctonia solani* AG 1-IB. *Journal of Phytopathology*, **159**, 35–44.

Gullino, M. L., Gilardi, G., & Garibaldi, A. (2019). The ready-to-eat salad crops: A plant pathogens's heaven. *Plant Diseases*, **103** (9), 2153–2170.

Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows95/98/NT. *Nucleic Acids Symposium Series*, **41**, 95-98.

Hansen, Z. R., Everts, K. L., Fry, W. E., Gevens, A. J., Grünwald, N. J., Gugino, B. K., Johnson, D. A., Johnson, S. B., Judelson, H. S., Knaus, B. J., McGrath, M. T., Myers, K. L., Ristaino, J. B., Roberts, P. D., Secor, G. A., & Smart, C. D. (2016). Genetic variation within clonal lineages of *Phytophthora infestans* revealed through genotyping-by-sequencing, and implications for late blight epidemiology. *PLoS ONE*, **11** (11), 1–22.

Harikrishnan, R., & Yang, X. B. (2004). Recovery of anastomosis groups of *Rhizoctonia solani* from different latitudinal positions and influence of temperatures on their growth and survival. *Plant Disease*, **88** (8), 817–823.

Harman, G. E. (2000). Myths and dogmas of biocontrol: Changes in perceptions derived from research on *Trichoderma harzianum* T-22. *Plant Disease*, **84** (4), 377–393.

Hassan, A. (1987). Interaction between *Pratylenchus coffea* and *Pythium aphanidermatum* and/or *Rhizoctonia solani* on chrysanthemum. *Journal of Phytopathology*, **123** (3), 227-232.

Haydock, P. P. J., Woods, S. R., Grove, I. G. & Hare, M. C. (2006). Chemical Control of Nematodes. In: Perry R. N. & Moens M. (Eds.). *Plant Nematology*. Wallingford, UK, CABI Publishing, 392-410.

Hendrickx, G. (1995). An automated apparatus for extracting free-living nematode stages from soil. *Nematologica*, **41**, 308. (Abstr.)

Herr, L. J. (1992). Characteristics of *Rhizoctonia* isolates associated with bottom rot of lettuce in organic soils in Ohio. *Phytopathology*, **82** (10), 1046–1050.

Heve, W. K., Been, T. H., Schomaker, C. H., & Teklu, M. G. (2015). Damage thresholds and population dynamics of *Meloidogyne chitwoodi* on carrot (*Daucus carota*) at different seed densities. *Nematology*, **17**, 501–514.

Hooks, C. R. R., Wang, K., Ploeg, A., & Mcsorley, R. (2010). Using marigold (*Tagetes* spp.) as a cover crop to protect crops from plant-parasitic nematodes. *Applied Soil Ecology*, **46** (3), 307–320.

Hua, G. K. H., & Höfte, M. (2015). The involvement of phenazines and cyclic lipopeptide sessilin in biocontrol of *Rhizoctonia* root rot on bean (*Phaseolus vulgaris*) by *Pseudomonas* sp. CMR12a is influenced by substrate composition. *Plant and Soil*, **388** (1-2), 243–253.

Huang, J. H., & Lo, C.T. (1998). Wilt of lettuce caused by *Fusarium oxysporum* in Taiwan. *Plant Pathology Bulletin*, **7**, 150–153.

Hubbard, J. C., & Gerik, J. S. (1993). A new wilt disease of lettuce incited by *Fusarium oxysporum* f.sp. *lactucum* forma specialis nov. *Plant Disease*, **77** (7), 750–754.

Ishibashi, N., Kondo, E., & Kashio, T. (1975). The induced molting of 4th-stage larvae of pin nematode, *Paratylenchus aciculus* brown (Nematoda: Paratylenchidae), by root exudate of host plant. *Applied Entomology and Zoology,* 10 (4), 275-283.

Jones, J. T., Haegeman, A., Danchin, E. G. J., Gaur, H. S., Helder, J., Jones, M. G. K., Kikuchi, T., Manzanilla-López, R., Palomares-Rius, J. E., Wesemael, W. M. L., & Perry, R. N. (2013). Top 10 plant-parasitic nematodes in molecular plant pathology. *Molecular Plant Pathology*, **14** (9), 946–961.

Joyce, S.A., Reid, A.P., Driver, F. & Curran, J. (1994). Application of polymerase chain reaction (PCR) methods to the identification of entomopathogenic nematodes. In: Burnell, A.M., Ehlers, R.-U. & Masson, J.P. (Eds). *Genetics of entomopathogenic nematode-bacterium complexes*. Luxembourg, E.C. DG XII, 178-187.

Katan, J. (2017). Diseases caused by soilborne pathogens: biology, managment and challenges. *Journal of Plant Pathology*, **99** (2), 305–315.

Kilpatrick, R. A., Chen, T.-A., Rich, A. E., & Rodrigues, L. (1963). Root symptoms and anatomical changes in clovers and lettuce resulting from injury by *Pratylenchus penetrans* and 2,4-dichlorophenoxyacetic acid. *Phytopathology,* **53**, 349.

Kim, D. H. (1992). Guidelines for drawing causal loop diagrams. *The Systems Thinker*, **3** (1), 5–6.

Kim, D., Hwang, H., Shim, J., Yang, J., Pak, J. H., & Lee, K. (2019). Molecular biodiversity assessment of plant-parasitic nematodes in Dokdo Island, Korea. *Nematologica*, **21**, 275–292.

Knudsen, I. M. B., Larsen, K. M., Jensen, D. F., & Hockenhull, J. (2002). Potential suppressiveness of different field soils to *Pythium* damping-off of sugar beet. *Applied Soil Ecology*, **21**, 119–129.

Kooistra, T. (1983). *Rhizoctonia solani as a component in the bottom rot complex of glasshouse lettuce*. PhD. University of Wageningen, The Netherlands, Wageningen.

Koot, P. & Kroonenbackbier, B. (1999). Creatief omgaan met wortellesie-aaltje binnen de rotatie. *PAV-Bulletin Vollegrondsgroenteteelt*, 38–40.

Korinsak, S., Tangphatsornruang, S., Pootakham, W., Wanchana, S., Plabpla, A., Jantasuriyarat, C., Patarapuwadol, S., Vanavichit, A., & Toojinda, T. (2019). Genomics genome-wide association mapping of virulence gene in rice blast fungus *Magnaporthe oryzae* using a genotyping by sequencing approach. *Genomics*, **111** (4), 661–668.

Kotcon, J. B., Rouse, D. I., & Mitchell, J. E. (1984). Interactions of *Verticillium dahlia*, *Colletotrichum coccodes*, *Rhizoctonia solani*, and *Pratylenchus penetrans* in the early dying syndrome of russet Burbank potatoes. *Ecology and Epidemiology*, **75**, 68-74.

Kumar, S., Sivasithamparam, K., Gill, J. S., & Sweetingham, M. W. (1999). Temperature and water potential effects on growth and pathogenicity of *Rhizoctonia solani* AG-11 to lupin. *Canadian Journal of Microbiology*, **45**, 389–395.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA 7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger datasets. *Molecular Biology and Evolution,* **33**, 1870-1874.

Kuninaga, S., Natsuaki, T., Takeuchi, T., & Yokosawa, R. (1997). Sequence variation of the rDNA ITS regions within and between anastomosis groups in *Rhizoctonia solani*. *Current Genetics*, **32** (3), 237–243.

Kuninaga, S., Yokosawa, R. & Ogoshi, A. (1979). Some properties of anastomosis group 6 and BI in *Rhizoctonia solani* Kühn. Annals of the phytopathology Society of Japan, **45**, 207–214.

Kuramae, E. E., Buzeto, A. L., Ciampi, M. B., & Souza, N. L. (2003). Identification of *Rhizoctonia solani* AG 1-IB in lettuce, AG 4 HG-I in tomato and melon, and AG 4 HG-III in broccoli and spinach, in Brazil. *European Journal of Plant Pathology*, **109**, 391–395.

Lambel, S., Lanini, B., Vivoda, E., Fauve, J., Wechter, W. P., Harris-Shultz, K. R., Massey, L., & Levi, A. (2014). A major QTL associated with *Fusarium oxysporum* race 1 resistance identified in genetic populations derived from closely related watermelon lines using selective genotyping and genotyping-by-sequencing for SNP discovery. *Theoretical and Applied Genetics*, **127**, 2105–2115.

LaMondia, J. A. (2003). Interaction of *Pratylenchus penetrans* and *Rhizoctonia fragariae* in strawberry black root rot. *Journal of Nematology*, **35** (1), 17-22.

Larkin, R. P., Hopkins, D. L., & Martin, F. N. (1996). Suppression of Fusarium wilt of watermelon by nonpathogenic *Fusarium oxysporum* and other microorganisms recovered from a disease-suppressive soil. *Phytopathology*, **86** (8), 812–819.

Latin, R. X., & Snell, S. J. (1986). Comparison of methods for inoculation of muskmelon with *Fusarium oxysporum* f. sp. *melonis*. *Plant Disease*, **70** (4), 297–300.

Leslie, J. F. (1993). Fungal vegetative compatibility. *Annual Review of Phytopathology*, **31** (1), 127–150.

Letunic, I., & Bork P., (2019). Interactive Tree Of Life (iTOL) v4: recent updates and new developments. *Nucleic Acids Research,* **47**, 256–259.

Li, G., Wang, D., Jiang, D., Huang, H. C., & Laroche, A. (2000). First report of *Sclerotinia nivalis* on lettuce in central China. *Mycological Research*, **104** (February), 232–237.

Lin, Y., Lai, P., & Chang, T. (2014). Genetic diversity and identification of race 3 of *Fusarium oxysporum* f. sp. *lactucae* in Taiwan, 721–733.

Liu, Y. J., Whelen, S., & Hall, B. D. (1999). Phylogenetic relationships among ascomycetes: evidence from an RNA polymerse II subunit. *Molecular Biology and Evolution*, **16** (12), 1799–1808.

Lombard, L., Sandoval-Denis, M. P., Lamprecht, S. C., & Crous, P. W. (2019). Epitypification of *Fusarium oxysporum* – clearing the taxonomic chaos. *Persoonia*, **43**, 1–47.

Malbrán, I., Mourelos, C. A., Mitidieri, M. S., Ronco, B. L., & Lori, G. A. (2014). Fusarium wilt of lettuce caused by *Fusarium oxysporum* f. sp. *lactucae* in Argentina. *Plant Disease*, **98** (9), 1281.

Mamiya, Y. (1971). Effect of temperature on the life cycle of *Pratylenchus penetrans* on Cryptomeria seedlings and observation on its reproduction. Nematologica, **17** (1), 82-92.

Mao, L., Wang, Q., Yan, D., Xie, H., Li, Y., Guo, M., & Cao, A. (2012). Evaluation of the combination of 1,3-dichloropropene and dazomet as an efficient alternative to methyl bromide for cucumber production in China. *Pest Management Science*, **68**, 602–609.

Marrone, P. G. (2019). Pesticidal natural products – status and future potential. *Pest Management Science*, **75**, 2325–2340.

Matheron, M. E., Street, W. E., Street, E. A., Tickes, B. R., & Porchas, M. (2005). Effect of planting date, cultivar, and stage of plant development on incidence of Fusarium Wilt of lettuce in desert production fields. *Plant Disease*, **89** (6), 565–570.

Matson, P. A., Parton, W. J., Power, A. G., & Swift, M. J. (1997). Agricultural intensification and ecosystem properties. *Science*, **277** (5325), 504–509.

Matuo, T., & Motohashi, S. (1967). On *Fusarium oxysporum* f. sp. *lactucae* n. f. causing root rot of lettuce. *Transactions of the Mycological Society of Japan*, **8**,13–15.

Mbofung, G. Y., Hong, S. G., & Pryor, B. M. (2007). Phylogeny of *Fusarium oxysporum* f. sp. *lactucae* inferred from mitochondrial small subunit, elongation factor 1-α, and nuclear ribosomal intergenic spacer sequence data. *Phytopathology*, **97** (1), 87–98.

Mbofung, G. C. Y., & Pryor, B. M. (2010). A PCR-based assay for detection of *Fusarium oxysporum* f. sp. *lactucae* in lettuce seed. *Plant Disease*, **94** (7), 860–866.

McDougall, P. (2016). The cost of new agrochemical product discovery, development and registration in 1995, 2000, 2005-8 and 2010-2014. R & D expenditure in 2014 and expectations for 2019. *Report*.

Millani M. J., Etebarian H. R., & Alizadeh A., (1999). Occurrence of Fusarium wilt of lettuce in Shahre-Ray, Varamin and Karj areas. *Iranian Journal of Plant Pathology*, **35**,44–45.

Miller, P. M. (1978). Reproduction, penetration, and pathogenicity of *Pratylenchus penetrans* on tobacco, vegetables, and cover crops. *Phytopathology,* **68**, 1502–1504.

Mizukubo, T., & Adachi, H. (1997). Effect of Temperature on *Pratylenchus penetrans* development. *Journal of*

Nematology, **29** (3), 306–314.

Mokrini, F., Waeyenberge, L., Viaene, N., Andaloussi, F. A. & Moens, M. (2016). Diversity of root-lesion nematodes (*Pratylenchus* spp.) associated with wheat (*Triticum aestivum* and *T . durum*) in Morocco. *Nematology,* **18**, 1–21.

Moretti, F., Cotroneo, A., & Mancini, G. (1981). The reproduction and pathology of *Pratylenchus penetrans* on some varieties of lettuce. *Revue de Nématologie*, **4** (2), 271–276.

Naiki, T., & Ui, T. (1978). Ecological and morphological characteristics of the sclerotia of *Rhizoctonia solani* Kühn produced in soil. *Soil Biology and Biochemistry*, **10**, 471–478.

Navas-cortés, J. A., Landa, B. B., Méndez-Rodríguez, M. A., & Jiménez-Díaz, R. M. (2006). Quantitative Modeling of the effects of temperature and inoculum density of *Fusarium oxysporum* f. sp. *ciceris* races 0 and 5 on development of Fusarium Wilt in chickpea cultivars. *Phytopathology*, **97**, 564–573.

Neher, D. A. & Olson, R. K. (1999). Nematode communities in soils of four farm cropping management systems. *Pedobiologia,* **43**, 430–438.

Nordskog, B., Eikemo, H., Gauslaa, E., Le, V., Warmington, R., & Clarkson, J. (2014). Distribution and prevalence of *Sclerotinia sclerotiorum* and *S. subarctica* in Norwegian lettuce. *Phytopathology*, **104** (11), 86.

O'Bannon, J.H. & Taylor, A.L. (1968). Migratory endoparasitic nematodes reared on carrot discs. *Phytopathology,* **58**, 385–386.

O'Donnell, K., Gueidan, C., Sink, S., Johnston, P. R., Crous, P. W., Glenn, A., Riley, r., Zitomer, N. C., Colyer, P., Waalwijk, C., van der Lee, T., Moretti, A., Kang, S., Kim, H.-S., Geiser, D. M., Juba, J. H., Baayen, R. P., Cromey, M. G., Bithell, S., Sutton, D. A., Skovgaard, K. Ploetz, R., Kistler, H. C., Elliot, M, Davis M, & Sarver, B. A. J. (2009). A two-locus DNA sequence database for typing plant and human pathogens within the *Fusarium oxysporum* species complex. *Fungal Genetics and Biology*, **46** (12), 936–948.

O'Donnell, K., Kistler, H. C., Cigelnik, E., & Ploetz, R. (1998). Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. *Proceedings of the National Academy of Sciences*, **95**, 2044–2049.

O'Neill, T. M., McPherson, M. (1996). The search for basal rot control. *The Grower*, 19-21.

Ogoshi, A. (1987). Ecology and pathogenicity of anastomosis and intraspecific groups of *Rhizoctonia solani* Kühn. *Annual Review of Phytopathology*, **25** (1), 125–143.

Olthof, T. H. A. (1968). Races of *Pratylenchus penetrans*, and their effect on black root rot resistance of tobacco. *Nematologica*, **14**, 482–488.

Olthof, T. H. A., & Potter, J. W. (1973). The relationship between population densities of *Pratylenchus penetrans* and crop losses in summer-maturing vegetables in Ontario. *Phytopathology,* **63**, 577–582.

Olthof, T. H. A. & Potter, J. W. (1974). Yield losses in fall-maturing vegetables relative to population densities of *Pratylenchus penetrans* and *Meloidogyne hapla*. *Phytopathology,* **64**, 1072-1075.

Oostenbrink, M., S'Jacob, J. J. & Kuiper, K. (1957). Over de waardplanten van *Pratylenchus penetrans*. *Tijdschrift over Plantenziekten,* **63**, 345–360.

Palomares-Rius, J. E., Vovlas, N., Subbotin, S. A., Troccoli, A., Cantalapiedra-Navarrete, C., Liébanas, G., Chizhov, V. N., Landa, B. B., & Castillo, P. (2010). Molecular and morphological characterisation of *Sphaeronema alni* Turkina & Chizhov, 1986 (Nematoda: Sphaeronematidae) from Spain compared with a topotype

population from Russia. *Nematology*, **12**, 649–659.

Pasquali, M., Dematheis, F., Gullino, M. L., & Garibaldi, A. (2007). Identification of race 1 of *Fusarium oxysporum* f. sp. *lactucae* on lettuce by inter-retrotransposon sequence-characterized amplified region technique. *Phytopathology*, **97** (8), 987–996.

Paugh, K. R., & Gordon, T. R. (2019). Effect of planting date and inoculum density on severity of Fusarium wilt of lettuce in California. *Plant Disease*, **103** (7), 1498–1506.

Pintore, I., Gilardi, G., Gullino, M. L., & Garibaldi, A. (2017). Analysis of vegetative compatibility groups of italian and dutch isolates of *Fusarium oxysporum* f. sp. *lactucae*. *Journal of Plant Pathology*, **99** (2), 517–521.

Pudasaini, M. P., Viaene, N., & Moens, M. (2006). Effect of marigold (*Tagetes patula*) on population dynamics of *Pratylenchus penetrans* in a field. *Nematology*, **8**, 477–484.

Pudasaini, M. P., Viaene, N., & Moens, M. (2008). Hatching of the root-lesion nematode, *Pratylenchus penetrans*, under the influence of temperature and host. *Nematology*, **10** (1), 47–54.

Quist, C. W., Schrama, M., de Haan, J. J., Smant, G., Bakker, J., van der Putten, W. H. & Helder, J. (2016). Organic farming practices result in compositional shifts in nematode communities that exceed crop-related changes. *Applied Soil Ecology*, **98**, 273–279.

Rahman, L., Chan, K. Y., & Heenan, D. P. (2007). Impact of tillage, stubble management and crop rotation on nematode populations in a long-term field experiment. *Soil & Tillage Research*, **95**, 110-119.

Rhoades, H. L., & Linford, M. B. (1959). Molting of preadult nematodes of the genus *Paratylenchus* stimulated by root diffusates. *Science*, **130**, 1476–1477.

Rhoades, H. L., & Linford, M. B. (1961a). A study of the parasitic habit of *Paratylenchus projectus* and *P. dianthus*. *Proceedings of the Helminthological Society of Washington*, **28** (2), 185–190.

Rhoades, H. L., & Linford, M. B. (1961b). Biological studies on some members of the genus *Paratylenchus* *. *Proceedings of the Helminthological Society of Washington*, **28** (1), 51–59.

Ritchie, E., Bain, R. A., & McQuilken, M. P., (2009). Effects of the nutrient status, temperature and pH on mycelial growth, sclerotial production and germination of *Rhizoctonia solani* from potato. *Journal of Plant Pathology*, **91** (3), 589-596.

Rojas, J. A., Jacobs, J. L., Napieralski, S., Karaj, B., Bradley, C. A., Chase, T., & Chilvers, M. I. (2017). Oomycete species associated with soybean seedlings in North America — Part I : Identification and pathogenicity characterization. *Phytopathology*, **107** (3), 280–292.

Runia, W. T., & Molendijk, L. P. G. (2010). Physical methods for soil disinfestation in intensive agriculture : old methods and new approaches. *Acta Horticulturae,* **883**, 249–258.

Savary, R., Masclaux, F. G., Wyss, T., Droh, G., Corella, J. C., Machado, A. P., Morton, J. B., & Sanders, I. R. (2018). A population genomics approach shows widespread geographical distribution of cryptic genomic forms of the symbiotic fungus *Rhizophagus irregularis*. *The ISME Journal*, **12**, 17–30.

Schneider, J. H. M., Schilder, M. T., & Dijst, G. (1997). Characterization of *Rhizoctonia solani* AG 2 isolates causing bare patch in field grown tulips in the Netherlands. *European Journal of Plant Pathology*, **103** (3), 265–279.

Scholte, K., & s'Jacob, J. J. (1989). Syngergistic interactions between *Rhizoctonia solani* Kühn, *Verticillium dahlia* Kleb., *Meloidogyne* spp. and *Pratylenchus neglectus* (Rensch) Chitwood & Oteifa, in potato. *Potato*

Research, **32**, 387-395.

Schomaker, C. H. & Been, T. H. (2013). Plant growth and population dynamics. In: Perry R. N. & Moens M. (Eds.). *Plant Nematology*. Wallingford, UK, CAB International, 275-301.

Scott, J., Gordon, T., Shaw, D., & Koike, S. (2010). Effect of temperature on severity of Fusarium wilt of lettuce caused by *Fusarium oxysporum* f. sp. *lactucae*. *Plant Disease*, **94** (1), 13–17.

Scott, J. C., Kirkpatrick, S. C., & Gordon, T. R. (2010). Variation in susceptibility of lettuce cultivars to fusarium wilt caused by *Fusarium oxysporum* f. sp. *lactucae*. *Plant Pathology*, **59**, 139–146.

Seinhorst, J. (1959). A rapid method for the transfer of nematodes from fixative to anhydrous glycerin. *Nematologica,* **4**, 67-69.

Seinhorst, J. W. (1966). The relationships between population increase and population density in plant parasitic nematodes. *Nematologica*. **12**, 157-169.

Seinhorst, J.W. (1986). Effect of nematode attack on the growth and yield of crop plants. In: Lamberti, F. & Taylor, C.E. (Eds). *Cyst nematodes*. Plenum Press, New York, NY, USA, 211-228.

Seinhorst, J.W. (1998). The common relation between population density and plant weight in pot and microplot experiments with various nematode plant combinations. *Fundamental and Applied Nematology,* **21**, 459–468.

Sharon, M., Kuninaga, S., Hyakumachi, M., Naito, S., & Sneh, B. (2008). Classification of *Rhizoctonia* spp. using rDNA-ITS sequence analysis supports the genetic basis of the classical anastomosis grouping. *Mycoscience*, **49** (2), 93–114.

Sharon, M., Kuninaga, S., Hyakumachi, M., & Sneh, B. (2006). The advancing identification and classification of *Rhizoctonia* spp. using molecular and biotechnological methods compared with the classical anastomosis grouping. *Mycoscience*, **47** (6), 299–316.

Shimazu, J., Yamauchi, N., Hibi, T., Satou, M., Horiuchi, S., & Shirakawa, T. (2005). Development of sequence tagged site markers to identify races of *Fusarium oxysporum* f. sp. *lactucae*. *Journal of General Plant Pathology*, **71**, 183–189.

Siddiqi, M. R. (2000). *Tylenchida parasites of plants and insects*, 2nd edition. Wallingford, UK, CABI Publishing.

Smith, L. J. A., Smith, M. K. B., Tree, D. A., Keefe, D. O. C., & Galea, V. J. D. (2008). Development of a small-plant bioassay to assess banana grown from tissue culture for consistent infection by *Fusarium oxysporum* f. sp. *cubense*. *Australasian Plant Pathology*, **37**, 171–179.

Smith, S. N., & Snyder, W. C. (1971). Relationship of inoculum density and soil types to severity of Fusarium wilt of sweet potato. *Phytopathology*, **61**, 1049–1052.

Sneh, B., Jaboji-Hare, S., Neate, S., & Dijst, G. (1996). *Rhizoctonia species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control*. Dordrecht: Kluwer academic Publishers.

Sowley, E. N. K., Dewey, F. M., & Shaw, M. W. (2010). Persistent, symptomless, systemic, and seed-borne infection of lettuce by *Botrytis cinerea*. *European Journal of Plant Pathology*, **126**, 61–71.

Srinivasan, K., Spadaro, D., Poli, A., Gilardi, G., Gullino, M. L., & Garibaldi, A. (2012). Genetic diversity and pathogenicity of *Fusarium oxysporum* isolated from wilted rocket plants in Italy. *Phytoparasitica*, **40**, 157–170.

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics,* **30**, 1312–1313.

StatBel (2019). "Landbouwgegevens: https://statbel.fgov.be/nl/themas/landbouw-visserij/land-en-tuinbouwbedrijven#figures".

Subbarao, K. V. (1998). Progress toward integrated management of lettuce drop. *Plant Disease*, **82** (10), 1068–1078.

Subbotin, S. A., Sturhan, D., Chizhov, V. N., Vovlas, N., & Baldwin, J. G. (2006). Phylogenetic analysis of *Tylenchida Thorne*, 1949 as inferred from D2 and D3 expansion fragments of the 28S rRNA gene sequences. *Nematology*, **8**, 455–474.

Subbotin, S. A., Vovlas, N., Crozzoli, R., Sturhan, D., Lamberti, F., Moens, M., & Baldwin, J. G. (2005). Phylogeny of *Criconematina* Siddiqi, 1980 (Nematod : Tylenchida) based on morphology and D2-D3 expansion segments of the 28S-rRNA gene sequences with application of a secondary structure model. *Nematology*, **7**, 927–944.

Sung, G., Sung, J., Hywel-Jones, N. L., & Spatafora, J. W. (2007). A multi-gene phylogeny of *Clavicipitaceae* (Ascomycota , Fungi): Identification of localized incongruence using a combinational bootstrap approach. *Molecular Phylogenetics and Evolution*, **44**, 1204–1223.

Sutton, B. C., & Holderness, M. (1986). *Microdochium panattonianum* IMI Descriptions of Fungi and bacteria. Set 104. *Transactions of the British Mycological Society*, **86**, 620.

Szczygiel, A. (1981). The ectoparasitic nematode, *Merlinius microdorus*, harmful to lettuce and strawberry. *Zeszyty Problemowe Postepow Nauk Rolniczych,* **249**, 87-91.

Taheri, A., Hollamby, G. J., Vanstone, V. A., & Neate, S. M. (2016). Interaction between root lesion nematode, *Pratylenchus neglectus* (Rensch 1924) Chitwood and Oteifa 1952, and root rotting fungi of wheat. *New Zealand Journal of Crop and Horticultural Science*, **22** (2), 181-185.

Talas, F., & McDonald, B. A. (2015). Genome-wide analysis of *Fusarium graminearum* field populations reveals hotspots of recombination. *BMC Genomics*, **16**, 1–12.

Taylor, A., & Clarkson, J. (2018). Technical review on lettuce Fusarium wilt, caused by Fusarium oxysporum f. sp. lactucae. *Report.*

Taylor, A., Jackson, A. C., & Clarkson, J. P. (2019). First report of *Fusarium oxysporum* f. sp. *lactucae* race 4 causing lettuce wilt in England and Ireland. *Plant Disease*, **103** (5), 1033.

Teklu, M.G., Meressa, B.H., Radtke, E., Been, T.H. & Hallmann, J. (2016). Damage thresholds and population dynamics of *Pratylenchus penetrans* on carrot (*Daucus carota* L. cv. Nerac) at three different seed densities. *European Journal of Plant Pathology,* **146**, 117–127.

Thornton, C. R., Neill, T. M. O., Hilton, G., & Gilligan, C. A. (1999). Detection and recovery of *Rhizoctonia solani* in naturally infested glasshouse soils using a combined baiting, double monoclonal antibody ELISA. *Plant Pathology*, **48**, 627–634.

Timper, P., Davis, R., Jagdale, G. & Herbert, J. (2012). Resiliency of a nematode community and suppressive service to tillage and nematicide application. *Applied Soil Ecology,* **59**, 48–59.

Tomprefa, N., Hill, R., Whipps, J. M., & McQuilken, M. (2011). Some environmental factors affect growth and antibiotic production by the mycoparasite *Coniothyrium minitans*. *Biocontrol Science and Technology*, *21*,

721–731.

UNEP. (2011). Montreal protocol on substances that deplete the ozone layer. *2010 Report of the Halons Technical Options Committee.*

Upadhaya, A., Yan, G., Pasche, J., & Kalil, A. (2019). Occurrence and distribution of vermiform plant-parasitic nematodes and the relationship with soil factors in field pea (*Pisum sativum*) in North Dakota, USA. *Nematology*, **21**, 445–457.

Vallad, G. E., Bhat, R. G., Koike, S. T., Ryder, E. J., & Subbarao, K. V. (2005). Weedborne reservoirs and seed transmission of *Verticillium dahliae* in lettuce. *Plant Disease*, **89** (3), 317–324.

Van Beneden, S., Leenknegt, I., França, S. C., & Höfte, M. (2010). Improved control of lettuce drop caused by *Sclerotinia sclerotiorum* using Contans combined with lignin or a reduced fungicide application. *Crop Protection*, **29** (2), 168–174.

Van Beneden, S., Pannecoucque, J., Debode, J., De Backer, G., & Höfte, M. (2009). Characterisation of fungal pathogens causing basal rot of lettuce in Belgian greenhouses. *European Journal of Plant Pathology*, **124**, 9–19.

Van den Berg, E., Tiedt, L. R., & Subbotin, S. A. (2014). Morphological and molecular characterisation of several *Paratylenchus* Micoletzky, 1922 (Tylenchida: Paratylenchidae) species from South Africa and USA, together with some taxonomic notes. *Nematology*, **16**, 323–358.

van Diepeningen, A.D., de Vos, O.J., Korthals, G.W. & van Bruggen, A.H.C. (2006). Effects of organic versus conventional management on chemical and biological parameters in agricultural soils. *Applied Soil Ecology,* **31**, 120–135. DOI: 10.1016/j.apsoil.2005.03.003.

Van Poucke, K., Haegeman, A., Goedefroit, T., Focquet, F., Leus, L., Horta Jung, M., Junker, C., Redondo, M. A., Husson, C., Kostov, K., Lyubenova, A., Christova, P., Chandelier, A., Slavov, S., de Cock, A., Bonants, P., Werres, S., Palau, J.O., Marçais, B., Jung, T., Stenlid, J., Ruttink, T., & Heungens, K. (submitted). Unravelling hybridization in the genus *Phytophthora* using phylogenomics and genome size estimation. *Systematic Biology*.

Vandevelde, I., Höfte, M., Claerbout, J., Venneman, S., Neukermans, J., Volckaert, A., Bleyeart, P., Decombel, A., & Leenknegt, I. (2019). Hygiëneprotocol Fusarium in sla. *Report.*

VBT (2015). Verbond van Belgische Tuinbouwcoöperaties: Jaarverslag 2015. Leuven, VBT.

VBT (2016). Verbond van Belgische Tuinbouwcoöperaties: Jaarverslag 2016. Leuven, VBT.

VBT (2017). Verbond van Belgische Tuinbouwcoöperaties: Jaarverslag 2017. Leuven, VBT.

VBT (2018). Verbond van Belgische Tuinbouwcoöperaties: Jaarverslag 2018. Leuven, VBT.

VLAM Marketingdienst (2019). Belgische export van verse groenten per groentesoort (in 1.000 euro), groentebarometer 2019, 1-2.

Ventura, J. A., & Costa, H. (2008). Fusarium wilt caused by *Fusarium oxysporum* on lettuce in Espirito Santo, Brazil. *Plant Disease*, **92** (6), 976.

Verschoor, B. C., de Goede, R. G. M., de Hoop, J., & de Vries, F. W. (2001). Seasonal dynamics and vertical distribution of plant-feeding nematode communities in grasslands. *Pedobiologia*, **45**, 213–233.

Viaene, N. M., & Abawi, G. S. (1996). Damage threshold of *Meloidogyne hapla* to lettuce in organic soil. *Journal of*

Nematology, **28**, 537–545.

Viaene, N., Coyne, D. L. & Kerry, B. R. (2006). Biological and cultural management. In Perry R. N. & Moens M. (Eds.). *Plant Nematology*. Wallingford, UK, CAB Interenational, 346-369.

Wareing, P. W., Wang, Z.-N., Coley-Smith, J. R., & Jeves, T. M. (1986). Fungal pathogens in rotted basal leaves in lettuce in Humberside and Lancashire with particular reference to *Rhizoctonia solani*. *Plant Pathology*, **35**, 390–395.

Wei, L., Xue, A. G., Cober, E. R., Babcock, C., Zhang, J., Zhang, S., & Li, W. (2011). Pathogenicity of *Pythium* species causing seed rot and damping-off in soybean under controlled conditions. *Phytoprotection*, **91**, 3–10.

Whipps, J. M., Sreenivasaprasad, S., Muthumeenakshi, S., Rogers, C. W., & Challen, M. P. (2008). Use of *Coniothyrium minitans* as a biocontrol agent and some molecular aspects of sclerotial mycoparasitism. *European Journal of Plant Pathology*, **121** (3), 323–330.

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. A. Innis, D. H. Gefland, J. Sninsky, & T. J. White (Eds.), *PCR protocols: A guide to methods and applications*, San Diego: Academic, 315–322

Williamson, B., Tudzynski, B., Tudzynski, P., & Van Kan, J. A. L. (2007). *Botrytis cinerea* : the cause of grey mould disease. *Molecular Plant Pathology*, **8** (5), 561–580.

Willis, C. B. (1972). Effects of soil pH on reproduction of *Pratylenchus penetrans* and forage yield of alfalfa I. *Journal of Nematology*, **4** (4), 291–295.

Winfield, A. L. (1985). Observations on the pin nematode *Paratylenchus nanus*, a possible pest of glasshouse lettuce, *Lactuca sativa*. *Crop Research*, **25**, 3–12.

Wobbrock, J. O., Findlater, L., Gergle, D., & Higgins, J. J. (2011). The aligned rank transform for nonparametric factorial analyses using only ANOVA procedures. *CHI '11 Proceedings of the SIGCHI Conference on Human Factors in Computing Systems*, 143–146.

Wood, F. H. (1973). Biology and host range of *Paratylenchus* projectus Jenkins, 1956 (Nematoda: Criconematidae) from a sub-alpine tussock grassland. *New Zealand Journal of Agricultural Research*, **16** (3), 381–384.

Wu, H. Y., Tsay, T. T., & Lin, Y. Y. (2002). Indentification and biological study of *Pratylenchus* spp. isolated from the crops in Taiwan. *Plant Pathology Bulletin*, **11**, 123–136.

Xiao, X., Ohm, H. W., Hunt, G. J., Poland, J. A., Kong, L., Nemacheck, J. A., & Williams, C. E. (2016). Genotyping-by-sequencing to remap QTL for type II Fusarium head blight and leaf rust resistance in a wheat – tall wheatgrass introgression recombinant inbred population. *Molecular Breeding*, **36** (51), 1–11.

Zasada, I. A., Halbrendt, J. M., Kokalis-Burelle, N., LaMondia, J., Mckenry, M.V. & Noling, J. W. (2010). Managing nematodes without methyl bromide. *Annual Review of Phytopathology*, **48**, 311–328.

Zeng, Y., Abdo, Z., Charkowski, A., Stewart, J. E. & Frost, K. (2019). Responses of bacterial and fungal community structure to different rates of 1,3-dichloropropene fumigation. *Phytobiomes Journal*, **3**, 212-223.

Zhan, J., Thrall, P. H., Papaïx, J., Xie, L., & Burdon, J. J. (2015). Playing on a pathogen's weakness: using evolution to guide sustainable plant disease control strategies. *Annual Review of Phytopathology*, **53**, 19–43.

Zhou, X. G., Everts, K. L., & Bruton, B. D. (2010). Race 3, a new and highly virulent race of *Fusarium oxysporum* f. sp. *niveum* causing Fusarium wilt in watermelon. *Plant Disease*, **94** (1), 92–98.

Supplementary I

First Report of *Fusarium oxysporum* f. sp. *lactucae* Race 4 on Lettuce in Belgium

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In Belgium, lettuce (*Lactuca sativa* L.) is an important crop that is mainly grown in soil in glasshouses. During autumn 2015, wilting symptoms on butterhead lettuce 'Halewyn' (Rijk Zwaan, the Netherlands) and an unknown cultivar were observed in two different commercial glasshouses in the Province of Antwerp, Belgium. The disease incidence was around 10 and 20%, respectively, with a disease severity of 9 and 18%. Since 2015 the disease has spread very fast; already 15% of the glasshouse lettuce production area in Flanders (northern part of Belgium) is infested. Dwarf growth and yellowing of the outer leaves were noticed on affected plants, followed by complete wilting and death. The vascular tissue showed a brown to red discoloration. Affected root and leaf tissues were surface-sterilized with 1% NaOCl for 30 s and washed three times with sterile water. The plant tissues were cut into 1 cm^2 pieces and plated on potato dextrose agar amended with streptomycin sulfate (100 mg/liter) and incubated at room temperature (19 to 22°C). Consistently dense fungal colonies with pale cream to purplish mycelia grew out of the plant tissues. Microconidia, macroconidia, and chlamydospores typical for *Fusarium oxysporum* were observed. Microconidia from isolates Fus1.01 and Fus1.02, coming from the two different glasshouses, measured respectively 5.99 to 8.64 (mean 6.98) × 2.75 to 4.39 (mean 3.32) µm and 6.75 to 11.50 (mean 8.42) × 2.75 to 4.59 (mean 3.61) µm. Chlamydospores were terminal and intercalary, rough walled, and measured 6.86 to 10.72 (mean 8.25) µm for Fus1.01 and 6.13 to 10.80 (mean 8.55) µm for Fus1.02. Macroconidia were straight to slightly curved with three septa and measured 24.49 to 31.27 (mean 27.27) × 2.93 to 4.42 (mean 3.84) µm for Fus1.01 and 20.91 to 26.09 (mean 22.58) × 3.42 to 4.70 (mean 3.98) µm for Fus1.02. Subsequently, DNA from single-spore cultures (Fus1.01 and Fus1.02) was extracted using the Invisorb Spin Plant Mini Kit (Stratec Molecular). The translation elongation factor 1-α (EF1-α) gene was amplified using primers EF1/EF2 (O'Donnel *et al.*, 1998) and sequenced in both directions by LGC Genomics (Berlin) using Sanger sequencing technology. The EF1-α sequences of both isolates showed 100% similarity with the EF1-α sequence of *F. oxysporum* f. sp. *lactucae* strain S1 (accession no. DQ837657) (Mbofung *et al.*, 2007) and were deposited (MG599512 and MG599513). By using specific primers FPUF and FPUR (Gilardi *et al.*, 2016), we could show that both isolates belong to race 4. Moreover, pathogenicity tests with three different lettuce cultivars ('Costa Rica No. 4', 'Banchu Red Fire', and 'Romana Romabella 30CN') provided by Rijk Zwaan (the Netherlands) were conducted to confirm the positive result with the primers FPUF and FPUR and to complete Koch's postulates. Roots of 2-week-old lettuce plants were dipped in a 5×10^5 spores/ml suspension, and five plants per cultivar were used. The experiment was carried out twice. Inoculated lettuce seedlings were planted in 100 g of steamed potting substrate and were maintained in

a climate room at 24°C. In both experiments, wilting was observed after 4 weeks for the cultivars Costa Rica No. 4 and Romana Romabella 30CN, but no symptoms could be seen on the cultivar Banchu Red Fire. *F. oxysporum* was consistently reisolated from all inoculated cultivars. These results are consistent with pathogenicity tests carried out before with two isolates of *F. oxysporum* f. sp. *lactucae* race 4 from the Netherlands (Gilardi *et al.*, 2016) and indicate that this new race is also the causal agent of Fusarium wilt on lettuce in Belgium. This report shows that race 4 is spreading fast and imposes a serious risk to other lettuce production areas in Europe.

Curriculum vitae

Personal information

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Education

2010-2013: Bachelor degree in Bioscience engineering, Ghent University

2013-2015: Master degree in Bioscience engineering, Agriculture, Ghent University
Dissertation: 'Biologische bestrijding van *Rhizoctonia solani* in de slateelt met behulp van *Trichoderma'*
Promotor: Prof. M. Höfte
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Career

September 2015 – August 2019: IWT-project 140984 entitled: 'Geïntegreerde beheersingsstrategie voor grondgebonden schimmels en nematoden in bladgroenten onder glas'

Ad hoc reviewer for BioControl

November 2019 - present : Applied agricultural researcher on strawberry at Inagro

Teaching experience

Practical courses 'Praktijkstudies gewasbeschadigers' (2016-2019) and 'Gewasbeschadigers' (2018)

Supervision of undergraduate students

Andrew Kigozi (2016-2017). The pin nematode *Paratylenchus* sp. on lettuce. Promotors: Prof. N. Viaene and Prof. W. Bert. Thesis to obtain the degree of International Master of Science in Agro- and Environmental Nematology.

Jens De Busscher (2016-2017). 'Biologische bestrijding en karakterisatie van *Fusarium oxysporum* f. sp. *lactucae* in de slateelt'. Promotor: Prof. M. Höfte. Copromotor: Dr. ir. S. França. Thesis to obtain the degree of Bio-Engineer, master Agriculture.

Bart De Cock (2017-2018). 'Resistentiescreening van bladrammenas voor *Meloidogyne* spp. en overleving van *Ditylenchus destructor* in grond'. Thesis to obtain the degree of Bachelor in Biomedical laboratory technology.

Binash Ajmal (2018-2019). The effect of shell-derived bioactive compounds and *Pseudomonas* CMR12a on *Pratylenchus penetrans*. Promotor: Prof. N. Viaene. Thesis to obtain the degree of International Master of Science in Agro- and Environmental Nematology.

Publications

Peer-reviewed

Claerbout J, Venneman S, Vandevelde I, Decombel A, Bleyaert P, Volckaert A, Neukermans J and Höfte M (2018). First report of *Fusarium oxysporum* f. sp. *lactucae* race 4 on lettuce in Belgium. Plant Disease 105: 1037.

Claerbout J, Decombel A, Volckaert A, Venneman S, Vandevelde I, Bleyaert P, Neukermans J, Viaene N and Höfte M (2019). Glasshouse-specific occurrence of basal rot pathogens and the seasonal shift of *Rhizoctonia solani* anastomosis groups in lettuce. European Journal of Plant Pathology, 155 (3), 841-858.

Claerbout J, Neukermans J, Vandevelde I, Decombel A, de Sutter N, Deeren A-M, Venneman S, Bleyaert P, Höfte M and Viaene N (2019). *Pratylenchus penetrans*, a potential risk in glasshouse-grown lettuce: population dynamics and damage threshold. Nematology. DOI: 10.1163/15685411-00003324.

Claerbout J, Vandevelde I, Venneman S, Kigozi A, de Sutter N, Neukermans J, Bleyaert P, Bert W, Höfte M and Viaene N (Accepted pending minor revisions). A thorough study of a *Paratylenchus* sp. in glasshouse-grown lettuce: characterization, population dynamics, host plants and damage threshold as keys to its integrated management. Annals of Applied biology.

Not-peer reviewed

Leenknegt I, Venneman S, Vandevelde I, Claerbout J, Viaene N, Höfte M, Decombel A, Bleyaert P, Volckaert A and Beyers T (2016). 'Belangrijke ziekten en plagen in sla'. Proeftuinnieuws 10: 17-19.

Claerbout J, Viaene N, Höfte M, Decombel A, Bleyaert P, Venneman S, Vandevelde I, Volckaert A and Beyers T (2016). '*Paratylenchus* spp. en *Pratylenchus penetrans* in bladgroenten onder glas'. Proeftuinnieuws 10: 20-21.

Vandevelde I, Arnouts T, Venneman S, Claerbout J and Viaene N (2016). 'Aaltjes aanpakken met stomen via stoomdrainage'. Proeftuinnieuws 19: 16-18.

Vandevelde I, Venneman S, Claerbout J, Höfte M, Viaene N, Neukermans J, Decombel A and Bleyaert P (2017). 'Fusarium, een grote bedreiging voor de teelt van serresla'. Proeftuinnieuws 5: 34-36.

De Busscher J, Claerbout J, Höfte M, França S, Vandevelde I and Venneman S (2017). 'Grondstomen kan de Fusariumdruk sterk verminderen in de teelt van serresla'. Proeftuinnieuws 9: 12-13.

Claerbout J, Viaene N, Höfte M, Decombel A, Bleyaert P, Venneman S, Vandevelde I, Volckaert A and Neukermans J (2017). 'Het speldaaltje, geen gevaar voor veldsla'. Proeftuinnieuws 9: 14-15.

Vandevelde I, Venneman S, Leenknegt I, Claerbout J, Höfte M, Viaene N, Neukermans J, Volckaert A, Decombel A and Bleyaert P (2018). 'Fusarium in serresla breidt heel snel uit'. Management&Techniek 21: 46-47.

Claerbout J, Höfte M, Neukermans J, Vandevelde I, Venneman S, Decombel A, Bleyaert P and Viaene N (2018). 'De FUNSLA-app helpt je bij het herkennen en bestrijden van ziekten in sla'. Proeftuinnieuws 9: 20-21.

Neukermans J, Decombel A, Bleyaert P, Claerbout J, Höfte M, Vandevelde I, Venneman S and Viaene N (2018). 'Reportage: Telers aan het woord over Fusarium in kropsla'. Proeftuinnieuws 19: 32-34.

Vandevelde I, Venneman S, Claerbout J, Höfte M, Neukermans J, Lippens L, Bleyaert P and Decombel A (2019). 'Wondermiddel tegen Fusarium bestaat niet, maatregelen combineren is de boodschap'. Proeftuinnieuws 3: 33-35.

Moeneclaey B, Pauwelyn E, Decombel A, Bleyaert P, Neukermans J, Claerbout J, Höfte M, Vandevelde I and Venneman S (2019). 'Ontsmetting materialen beperkt verspreiding sla-Fusarium'. Proeftuinnieuws 15: 12-13.

Attended conferences and symposia with oral presentation

Claerbout J, Volckaert A, Neukermans J, Decombel A, Bleyaert P, Venneman S, Vandevelde I and Höfte M (2017). *Fusarium oxysporum* f. sp. *lactucae* a new emerging disease in Belgian lettuce. KNPV working group Fusarium, October 25, 2017, Utrecht, the Netherlands.

Claerbout J, Volckaert A, Neukermans J, Decombel A, Bleyaert P, Venneman S, Vandevelde I and Viaene N (2017). 'Schade en populatieontwikkeling van *Paratylenchus* sp. in sla'. KNPV working group Nematodes, November 17, 2017, Lelystad, the Netherlands.

Claerbout J, Volckaert A, Neukermans J, Decombel A, Bleyaert P, Venneman S, Vandevelde I, Viaene N and Höfte M (2018). The seasonal pattern of *Rhizoctonia solani* causing basal rot in Belgian lettuce. 70th International Symposium on Crop Protection, May 22, 2018, Ghent, Belgium.

Claerbout J, Kigozi A, Volckaert A, Neukermans J, Decombel A, Bleyaert P, Venneman S, Vandevelde I, Höfte M and Viaene N (2018). *Paratylenchus* sp. in intensive lettuce production in Flanders: prevalence, damage threshold and host status. 33rd European Society of Nematologists conference, September 9 –13, 2018, Ghent, Belgium.

Claerbout J, Neukermans J, Decombel A, Bleyaert P, Venneman S, Vandevelde I and Höfte M (2019). Fusarium wilt threatens Belgian lettuce production. 14th International IUPAC Congress on Crop Protection, 71st International Symposium on Crop Protection, May 21, 2019, Ghent, Belgium.

Attended conferences and symposia with poster presentation

Claerbout J, Volckaert A, Decombel A, Bleyaert P, Venneman S, Vandevelde I, Höfte M and Viaene N (2016). Population dynamics of *Paratylenchus* spp. and *Pratylenchus penetrans* in glasshouse lettuce. 32nd European Society of Nematologists conference, August 29 – September 2, 2016, Braga, Portugal.

Claerbout J, Volckaert A, Neukermans J, Decombel A, Bleyaert P, Venneman S, Vandevelde I, Viaene N and Höfte M (2016). Lettuce in Belgium has fun(gal and nematode problems). 12th Conference of the European Foundation for Plant Pathology (EFPP), May 29 – June 2, 2017, Dunkerque, France.

Attended local meetings with oral presentation

Infovergadering Tuinderskring Roeselare & Omgeving. 'Populatieontwikkeling en schade door het speldaaltje in sla'. December 12, 2017, Reo veiling, Roeselare, Belgium.

Infoavond duurzaam stomen. 'Fusariumverwelking in sla'. March 18, 2019, Belorta, Sint-Katelijne-Waver, Belgium.