

CHARACTERIZATION OF TWO COEXISTING PATHOGEN POPULATIONS OF *Leptosphaeria* spp., THE CAUSE OF STEM CANKER OF BRASSICAS

Joanna Kaczmarek, Małgorzata Jędrzycka

Institute of Plant Genetics, Polish Academy of Sciences, Strzeszyńska 34, 60-479 Poznań, Poland
e-mail: jkac@igr.poznan.pl

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Abstract

Stem canker of brassicas, also known as blackleg is the most damaging disease of many Brassicaceae. The disease is caused by *Leptosphaeria maculans* (Desm.) Ces et de Not. and *L. biglobosa* sp. nov., Shoemaker & Brun, which coexist in plants and resulting in disease symptoms and decreased yield, quantity and quality of cultivated vegetables and oilseed rape. The paper presents taxonomic relationships between these co-existing pathogen species, describes particular stages of their life cycles, summarizes the differences between the species, and reviews methods for their identification.

Key words: ascospore, stem canker, *Brassica napus*, *Leptosphaeria maculans*, *Leptosphaeria biglobosa*, oilseed rape, pseudothecium

INTRODUCTION

Stem canker or blackleg is one of the most destructive diseases of Brassicaceae worldwide. The disease attacks numerous forms of cabbage (*Brassica oleracea*), spring and winter forms of canola or oilseed rape (*B. napus* L. forma *annua* and f. *biennis*), as well as *Crambe* sp., *Eruca* sp., *Erysimum* sp., *Lepidium* sp., *Raphanus* sp., *Sisymbrium* sp., *Thlaspi* sp. and others (Jędrzycka, 2006). Disease symptoms are attributed to two pathogens: *Leptosphaeria maculans* (Desm.) Ces et de Not. and *L. biglobosa* sp. nov. (Shoemaker and Brun, 2001), with the former being more damaging than the latter (Petrie, 1978). The pathogenicity of the isolates of *L. maculans* may differ considerably (Kutcher et al. 2007). This is one of the reasons why *L. maculans* is becoming a model system for the study of genetic relationships between host and pathogen (Plummer et al. 1994;

Kutcher et al. 2010b). In numerous countries, including Poland, both pathogen populations co-exist, and they can jointly lead to severe disease symptoms as well as substantial yield losses of vegetable and agricultural crops (Brachaczek et al. 2010). Both species are found in numerous, but not all countries where stem canker has been reported (Rouxel et al. 2004). Moreover, in *L. maculans* the race composition of each local fungus population greatly depends on the specific resistance genes present in cultivated plants, such as canola (Kutcher et al. 2010a).

TAXONOMY OF *LEPTOSPHAERIA MACULANS* AND *L. BIGLOBOSA*

According to the latest taxonomy, the fungi *Leptosphaeria maculans* and *L. biglobosa* belong to the order Ascomycota, suborder Pezizomycotina, class Dothideomycetes and genus Pleosporales (www.index-fungorum.org, 2011).

The order Ascomycota contains the most numerous (>30,000 species) and also the most versatile group of fungi. In most cases they have septated mycelium, divide using asexual mechanisms such as budding, fragmentation of mycelium or vegetative spores such as conidia, and they produce resting spores such as chlamydospores. However, they mainly reproduce in a generative way, using sexual reproduction. The sexual spores are formed in asci, which is indicated by their classification into the taxonomic order, Ascomycota (Fiedorow et al. 2006).

Pezizomycotina forms the biggest suborder of Ascomycota. It contains endophytic fungi, lichens and the pathogens of plants and animals (Kirk et al. 2001). The majority of representatives of this group produce fruiting bodies in the form of asci that are

closed with the top, called operculum. The representatives of some genera of Pezizomycotina lost the ability to form such fruiting bodies and their phylogenetic relationship was established based on molecular studies (Spatafora et al. 2006).

Most fungi belonging to the class Dothideomycetes form pseudothecia with double layers of asci in the stroma. These two layers play different roles in ascospore release: the inner layer is stiff, whereas the outer layer is flexible, which allows high velocity ejection of spores. The asci are usually cylindrical and have thick cell walls. Ascospores are usually multicellular, with longitudinal and horizontal septa, and are transparent to dark brown. Members of the Dothideomycetes are mainly endo- or epiphytes of living plants; pathogens and saprophytes of plants or wood. The class contains twelve genera, including Pleosporales (Bisby et al. 2009).

Pseudothecia formed by Pleosporales are usually formed as single fruiting bodies, but sometimes can be produced in groups, usually on the substrate surface or shallowly submerged in mycelium. The asci are cylindrical and are separated by pseudoparaphyses. Ascospores of this class are usually hyaline and may be partly covered by slime. The fungi of this genus are mostly ubiquitous saprophytes living on dead fragments of plants or the pathogens of living plants (Kochman and Weber, 1997).

The family Leptosphaeriaceae usually forms transparent, hyaline ascospores with vertical septa. The most typical representatives of this family are fungi from the genus *Leptosphaeria*, including *L. maculans* and *L. biglobosa* (Fiedorow et al. 2006). These fungi commonly inhabit the ecosystems with moderate climates.

SIMILARITIES OF THE LIFE CYCLES OF *LEPTOSPHAERIA MACULANS* AND *L. BIGLOBOSA*

Both *L. maculans* and *L. biglobosa* have a similar life cycle (Figs 1, 2). In Australia, Canada and Europe ascospores are primarily responsible for plant infection. They are formed in pseudothecia that are produced on plant residues from the previous season (Petrie, 1995; West et al. 2001; Aubertot et al. 2006). The fruiting bodies of *L. maculans* and *L. biglobosa* are mainly formed on plants of the Brassicaceae family. It was found that pseudothecia on the stubble of oilseed rape can survive over five years, and for the first three years they can be a very efficient source of inoculum (Petrie, 1986). In this phase of saprophytic growth, the fungus *L. maculans* can produce phytotoxic metabolites from the sirodesmin family, such as sirodesmin PL (Ferezou et al. 1977). This metabolite is not produced by *L. biglobosa*, more-

over – sirodesmins can retard the growth of this fungal species, similar to several other microorganisms (Elliot et al. 2007).

The maturation of pseudothecia is influenced by air temperature and humidity (Toscano-Underwood et al. 2003). At temperatures lower than 10°C the fruiting bodies of *L. maculans* mature faster than those of *L. biglobosa*, which is why ascospores of *L. biglobosa* are observed later in the season (Fitt et al. 2006). The experiments done by Dawidziuk et al. (2010) did not prove differences in pseudothecia maturation of *L. maculans* and *L. biglobosa*, but the ratio between the species was favourable to the latter one, which could mask differences between these species. Monitoring of ascospores in air samples, combined with molecular detection of *L. maculans* and *L. biglobosa*, using Real-Time PCR method, showed no distinct pattern for time as concerns the earliness of spore release of these two pathogens.

The presence of spores in aeroplankton is related to meteorological factors (Grinn-Gofroń, 2009). Burge (1986) found more *Leptosphaeria* ascospores in rainy days. In Crete the ascospores of the genus *Leptosphaeria* were the most numerous among all spores of Ascomycotina, and they constituted nearly 7% of the air mycoflora and nearly half of all ascospores present in the air. It was suggested that such abundance of *Leptosphaeria* ascospores is connected with the microclimate of this island. The relationships between meteorological parameters and air spora can be studied with numerous methods, including neural-networks (Grinn-Gofroń and Strzelczak, 2008). In the case of the *L. maculans* and *L. biglobosa* species complex studies resulted in the elaboration of mathematical models (Salam et al. 2003 and 2007; Dawidziuk et al. 2011). To quantify the ratio between these fungal pathogens in air samples the technique of Real-Time PCR proved useful (Kaczmarek et al. 2009 and 2011). The genus *Leptosphaeria* showed allergenic properties and has been implicated in respiratory allergic diseases (Grinn-Gofroń, 2008), similar to the spores of *Alternaria* and *Cladosporium* (Stępańska et al. 1999; Kasprzyk and Worek 2006). In the UK, the spores of *Leptosphaeria* spp. produced positive skin-prick test reactions (Lacey, 1996). It means that the described fungal species are not only pathogenic to plants, but may also be allergenic to humans.

After their release, ascospores can survive dry conditions at 5°C to 20°C for as much as 30 days (Huang et al. 2003a) and they can be transmitted by air currents for 5 km (Hall, 1992). However, most spores are deposited on plants within 500 metres of the source (Aubertot et al. 2006). The spores

germinate on the cotyledons and young leaves of oilseed rape plants over a temperature range of 5°C to 20°C (Huang et al. 2003b) and they cause infection due to the penetration of plant tissues through stomata or directly through wounds caused by biotic (insects) or abiotic factors (Hammond et al. 1985, Chen and Howlett, 1996). Fungal infection results in large intercellular spaces between the mesophyll cells, but the mycelium of *L. biglobosa* grows much faster *in vitro* (Jędryczka, 2006) and *in planta* than

L. maculans (Huang et al. 2003b). As a result of infection, small lesions are formed on plants; they usually become paler and form larger symptoms as the disease develops. Symptoms can be seen even a few days after infection occurs (Sexton and Howlett, 2001). Experiments conducted under controlled environments indicated that temperature has an impact on the time of incubation. For example, this parameter requires 5 days at 20°C and 14 days at 8°C (Biddulph et al. 1999).

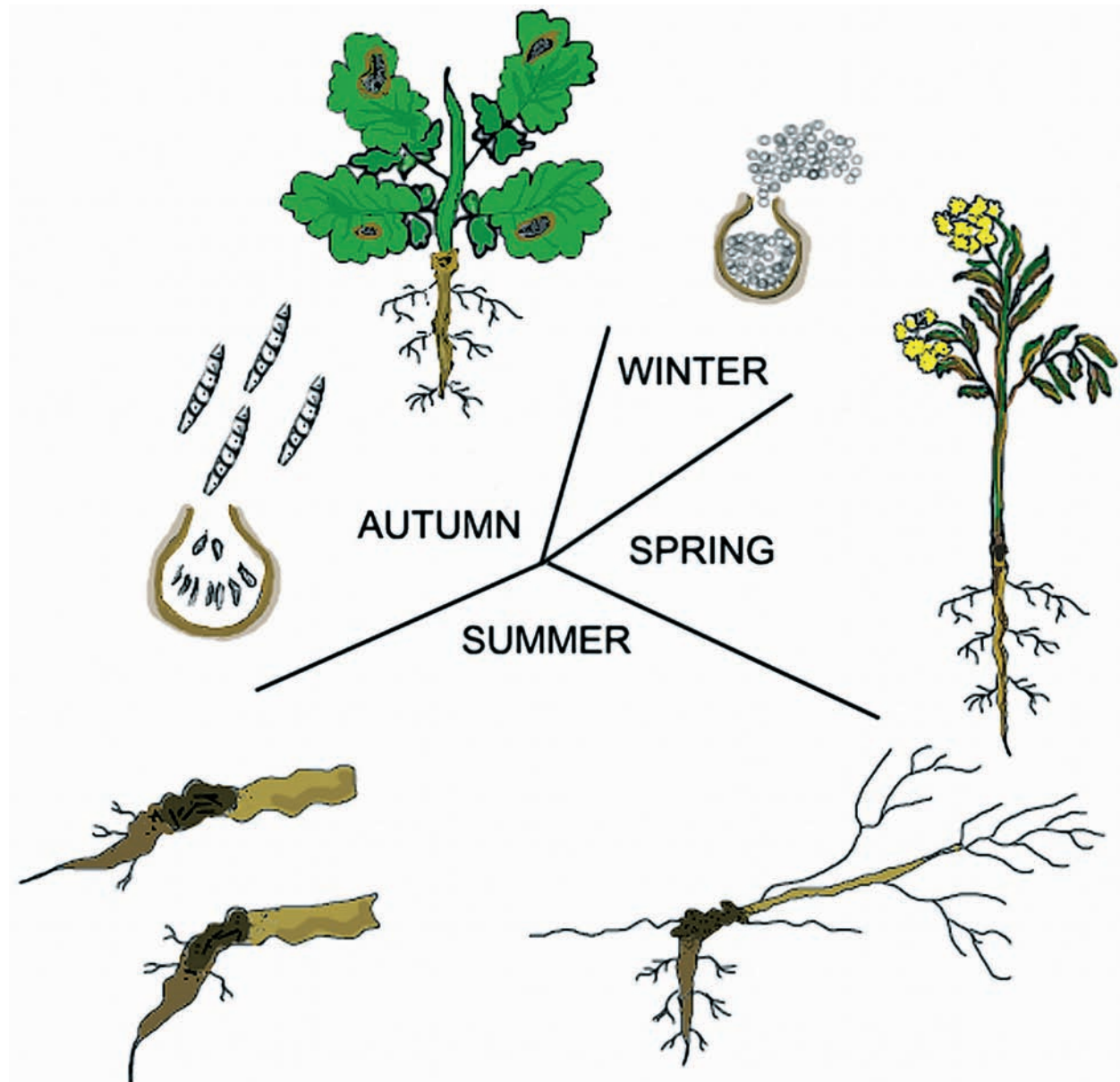


Fig. 1. The life cycle of *Leptosphaeria maculans* on winter oilseed rape in Europe.

Ascospores of *L. maculans* are formed in pseudothecia on infected stubble from the previous growing season(s). The spores land on leaves and cause leaf spots. The secondary infections are caused by pycnidiospores of *Phoma lingam*, formed in pycnidia. Over winter the fungus grows systemically in veins of leaf blades and petioles (latent phase). The growth rate is 2-3 times slower than this of *L. biglobosa*. The fungus invades the stem and causes severe stem cankers at root necks and stem bases.

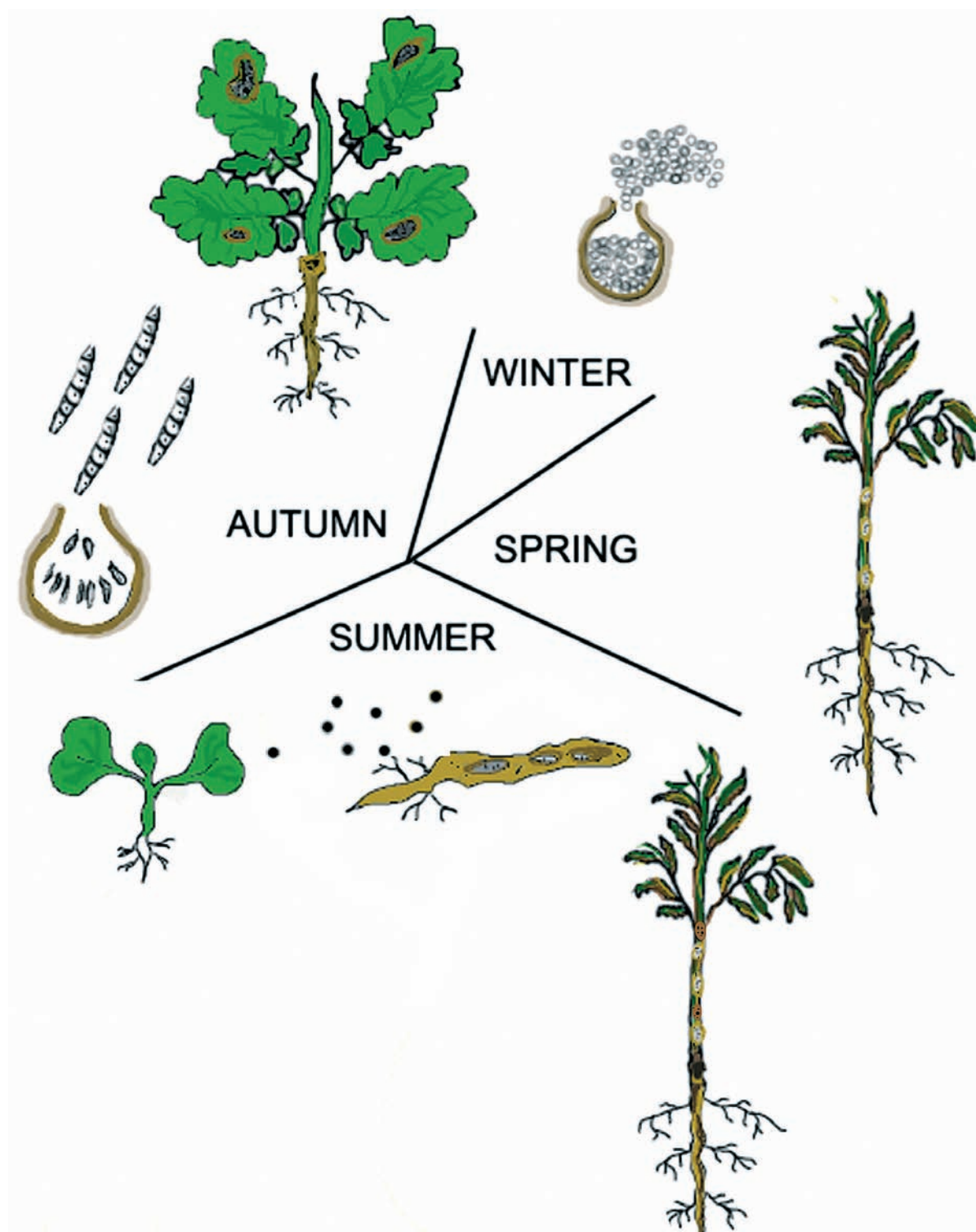


Fig. 2. The life cycle of *Leptosphaeria biglobosa* on winter oilseed rape in Europe.

Ascospores of *L. biglobosa* are formed in pseudothecia on infected stubble from the previous growing season(s). The spores land on leaves and cause leaf spots. The secondary infections are caused by pycnidiospores of *Phoma lingam*, formed in pycnidia. Over winter the fungus grows systemically in veins of leaf blades and petioles (latent phase). The growth rate is 2-3 times faster than this of *L. biglobosa*. The fungus invades the stem and causes profound but superficial upper stem lesions.

The symptoms on leaves depend on the fungal species causing infection. Generally symptoms caused by *L. biglobosa* are dark brown or grey, surrounded by a dark margin. In the case of *L. maculans*, most disease symptoms are light green to pale beige, with no margin. Jędrzycka (2006) reported that the identification of the particular species responsible for stem canker of brassicas from observation of disease symptoms can result in misdiagnosis because the symptoms are not as clear-cut as suggested in the literature (Brun et al. 1997, Fitt et al. 2006).

Small dark spots formed within leaf lesions are pycnidia – the fruiting bodies of the asexual stage of the pathogen. They contain pycnidiospores, which are transmitted by rain splash. In this anamorphic stage, the pathogen is referred to as *Phoma lingam* (Tode ex Fr.) Desm. (Desmazières, 1849), both for *L. maculans* and *L. biglobosa*, which is misleading, because it does not indicate which fungal species (*L. maculans* or *L. biglobosa*) is responsible for the disease. Pycnidiospores are numerous and regarded as the secondary inoculum of the pathogen. Compared to ascospores, pycnidiospores are transmitted in droplets of rain to short distances, usually from 2 to 40 cm (Traverson et al. 2007) and cause infection of the leaves, as do ascospores (Hall, 1992). It was reported, that pycnidiospores take longer to germinate than ascospores under the same environmental conditions (Li et al. 2004). According to West and Fitt (2005) pycnidiospores are much less important than ascospores in the epidemiology of stem canker of brassicas in Europe. A much greater role has been attributed to pycnidiospores in Australia (Barbetti, 1976; Howlett et al. 2001) and Canada (Guo and Fernando, 2005), although it is almost exclusively the spring form of oilseed rape (*Brassica napus* L. forma *annua*) that is grown in these countries.

The mycelium is at first restricted to a small leaf area, but then gradually expands and accesses the leaf petiole, through the veins. This phase of disease development is latent; there are no macroscopic symptoms on plants. With time, the symptoms of plant infection became visible on outer parts of plants. On stems dark spots surrounded by grey or brown margin are observed. Inside the plant, the fungus can partially or totally block the veins, which retards or inhibits the transport of water and nutrients. This destructive process results in premature ripening of oilseed rape plants (Hammond et al. 1985, Hammond and Lewis, 1987; West et al. 2001). The infection of stems may lead to the infection of siliques, which also results in pod spots with dark pycnidia inside. This process may result in direct contamination of the seeds. Infected seeds may lead to disease symp-

toms observed on plants that develop from infected seeds. In Australia, the transmission of the pathogen by infected seeds is regarded as an important source of plant infection (Salisbury et al. 1995; Li et al. 2003). It may bring new races of the pathogen to new area (Gugel and Petric, 1992). In Poland the movement of the pathogen on seed was reported to be negligible (Guoping, 1999, Gwiazdowski, 2004) and the spread of the pathogen was mainly by air and rainfall dissemination of spores produced on infected plant residues. Small plant parts of oilseed rape stubble, fragmented by harvesters, remain on the soil surface and give rise to the production of primary inoculum of these pathogens the following season. Gradually, *L. maculans* and *L. biglobosa* colonize previously uninfected stem fragments. With time, the fruiting bodies of the pathogen are found on many stubble fragments (Gladders and Musa, 1980). Infected stubble provides a good environment for the generative stage of pathogen development, particularly when the stubble is untilled (Salam et al. 2003).

GENERATIVE STAGES OF *LEPTOSPHAERIA MACULANS* AND *L. BIGLOBOSA*

Pseudothecia of *L. maculans* and *L. biglobosa* are round to oval and flattened at the bottom. The diameter of the fruiting bodies of *L. maculans* ranges from 300 to 400 µm, occasionally up to 500 µm, and are formed on the epidermis of the stem. The diameter of *L. biglobosa* pseudothecia ranges from 280 to 350 µm and are located under the epidermis of the stem (Toscano-Underwood et al. 2003). In both species the opening is in the centre of the fruiting body, although the size of this orifice is smaller in *L. maculans* (90 to 100 µm) than in *L. biglobosa*. It is formed from 5 to 8 layers of scleroplectenchymatic cells of 3-5(10) µm. Shoemaker and Brun (2001) observed that pseudothecia of *L. biglobosa* have a longer beak (200-400 µm tall by 200-300 µm wide) than *L. maculans*. It contains 8-10(15) layers of cells, of 5-8 µm diameter. The pseudothecial surface of both species is formed of cells with thick, melanised walls. The fruiting bodies of *L. biglobosa* contain paraphyses of 2-3 µm, distributed every 20-25 µm. The pseudothecia of *L. maculans* contain numerous, bitunicate asci of 100-120(150) x (12)18-21(22) µm, whereas asci of *L. biglobosa* are less numerous.

Similar to other ascomycetes, both species form asci with eight ascospores. These are long, straight or slightly curved cylindrically shaped spores. The size of *L. maculans* ascospores is on average 6-7 x (45)50-60(68) µm. The length to the width ratio is approximately 8:1. The spores of *L. biglobosa* are slightly smaller: 6-7 x 42-48(60) µm and the ratio of length to

width is in most cases 7:1. The ascospores of *L. maculans* usually contain 5 septa, and each cell has one to several droplets of fat. Ascospores of *L. biglobosa* have 3-5 septa and the biggest are their central cells, usually containing one or two droplets of fat. Ascospores formed by both species have a smooth surface (Williams, 1992; Shoemaker and Brun, 2001).

DESCRIPTION OF THE CONIDIAL STAGE – *PHOMA LINGAM*

Pycnidia of the fungus *P. lingam* are black, smooth and round (200 x 200 µm). The beak of a pycnidium is in the centre and is cylindrically shaped. These fruiting bodies contain thick walls (15-20 µm), composed of 6-8 layers of polygonal, pseudoparenchymatic cells with dimensions of 2-4 µm. Pycnidia do not contain paraphyses but are filled with pycnidiospores. In contrast to ascospores, which are composed of 6 cells of approximately 60 x 6,5 µm, pycnidiospores are composed of single cells of 4-5 x 1,5-2 µm. They are hyaline to light brown, cylindrical, but blunt at both ends. They contain two droplets of fat, which refract light. In contrast to ascospores, which are long and narrow, the length to width of a single pycnidiospore is 5:2. A detailed description of pycnidiospores was reported by Shoemaker and Brun (2001).

DIFFERENCES BETWEEN *LEPTOSPHAERIA MACULANS* AND *L. BIGLOBOSA* AND METHODS OF SPECIES IDENTIFICATION

Until 2001, *L. maculans* and *L. biglobosa* were classified as the same fungal species, in spite of numerous studies indicating substantial differences between these two pathotypes/groups. The first reports on the diversity of the population of the fungus *L. maculans* were published in 1927 in New Zealand. Cunningham (1927) described two separate forms of *L. maculans*, differing by morphological characteristics observed on agar media. Subsequent work confirmed these studies.

Slow growing isolates that could form abundant pycnidia were termed type 'A'. Type 'B' included fast growing isolates with more aerial mycelia and less pycnidia (Williams and Fitt, 1999). Due to differences in pathogenicity the isolates were also characterized as aggressive (A) and nonaggressive (NA) (Koch et al. 1989) or highly virulent (HV) and weakly virulent (WV) (Sippel and Hall, 1995). Isolates belonging to these two groups differed greatly in their ability to produce secondary metabolites, especially nonspecific phytotoxins called sirodesmins (Koch et al. 1989; Pedras and Seguin-Swartz, 1992). This is why isolates forming sirodesmins were also called Tox⁺ (Balesdent et al. 1992). Kachlicki

et al. (1996) demonstrated that although the isolates of type 'B' did not produce sirodesmins, they did produce other metabolites, some of which had phytotoxic properties. This is why Howlett et al. (2001) proposed the more appropriate term: Siro⁺ instead of Tox⁺ and Siro⁰ instead of Tox⁰. The common taxonomic affiliation of these two forms of *L. maculans* was questioned by many scientists. In 2001, Shoemaker and Brun, suggested the existence of two species *L. maculans* and *L. biglobosa*, based on morphological differences of pseudothecia. The morphology of the fruiting bodies is not the only feature differentiating the two pathogens, but usually is required to distinguish species diversity. Williams and Fitt (1999) reported that the fungi *L. maculans* and *L. biglobosa* differ in pathogenicity, with *L. maculans* responsible for the majority of yield loss. Disease symptoms caused by *Leptosphaeria* species are apparent at the early stages of plant development. Usually lesions form on leaves and subsequently necrosis may occur at the base of the stem or root collar. The symptoms at the soil level often result in disruption of the vascular system and decay of the adult plant may be observed as early as the flowering stage. *L. biglobosa* usually results in more superficial symptoms on the stem than *L. maculans* (Gladders and Musa, 1980; West et al. 2002). However, analysis of fungi present on the stem base of oilseed rape with symptoms of dry rot of brassica carried out in Poland by Jędrzycka (2006) has frequently demonstrated the presence of *L. biglobosa*. She also suggested that description of symptoms to differentiate the species of *Leptosphaeria* was oversimplified. The species *L. maculans* and *L. biglobosa* can be distinguished on the basis of growth rate and morphology of colonies cultured *in vitro*, as already described in 1927 by Cunningham. *Leptosphaeria maculans* is characterized by slow growth, less abundant mycelium and extensive sporulation on agar media. *Leptosphaeria biglobosa* produces abundant aerial mycelium, grows rapidly, but produces fewer pycnidia than *L. maculans* (Koch et al. 1989; Williams and Fitt, 1999).

Secondary metabolites are another criteria differentiating *L. maculans* and *L. biglobosa* (Koch et al. 1989). Chromatographic studies allow accurate diagnosis of chemotypes. Sirodesmin PL is the predominant metabolite produced in culture filtrate of *L. maculans* (Pedras and Seguin-Swartz, 1990; Kachlicki, 2004). The analysis of secondary metabolites produced by *L. biglobosa* revealed the presence of numerous compounds, with wasabi-dienon B prevalent (Pedras et al. 1995; Pedras and Biesenthal, 2001; Kachlicki, 2004). In the first phase of growth of *L. biglobosa* phomaligin A was produced (Pedras et al. 1995). Brown colored

culture filtrates or dark exudates present on mycelia grown on agar media indicate the development of melanins. Populations of isolates of *L. biglobosa* show considerable variation in the profiles of secondary metabolites (Jędryczka et al. 1999a). They may serve as chemotaxonomic markers, allowing identification of individual isolates of the fungus (Kachlicki, 2004).

Until recently, reports on the effect of fungicides on fungal growth of *L. maculans* and *L. biglobosa* were ambiguous. The research conducted by Eckert et al. (2004, 2010) and Karolewski (1998) indicated that inhibition of growth of *L. maculans* required lower doses of fungicides than *L. biglobosa*. In a study published by Gwiazdowski (2008) there were no statistically significant differences in the rate of fungal growth of *L. maculans* and *L. biglobosa* after treatment with fungicides, but the doses used were very high, which may have masked species-specific differences among isolates. Recently, Kaczmarek and Jędryczka (2010) clearly demonstrated differences in susceptibility of *L. maculans* and *L. biglobosa* to flusilazole and the flusilazole-containing fungicide, and they proved that *L. biglobosa* requires higher doses of these chemical compounds, to obtain fungal cultures comparable to *L. maculans*. The study took into account the different growth rate of both fungal species (Jędryczka, 2006).

Molecular studies provided further evidence of the existence of two biotypes or populations in the taxon identified as *L. maculans*. Isozyme diversity was high between these two sub-groups of isolates. Differences between the biotypes in the migration rate of phosphoglucisomerase (PGI) in starch gels was developed as a diagnostic tool (Sippel and Hall, 1995; Somda et al. 1996; Brun et al. 1997). Similarly, pulsed field gel electrophoresis, which separates whole chromosomes, also showed substantial differences between *L. maculans* and *L. biglobosa*. These species differed for the number and size of particular chromosomes (Morales et al. 1993). The isolates of *L. maculans* had a greater number of large chromosomes, the largest of which exceeded 3 Mbp, and *L. biglobosa* isolates possessed more small size chromosomes of about 1 Mbp (Jędryczka and Kachlicki, 1996; Batlińska, 2004; Białkowska, 2004).

Due to the significant genetic differences between *L. maculans* and *L. biglobosa*, identification of these species was also possible using methods that detect polymorphisms of nucleic acids. Restriction fragment length polymorphism (RFLP) analysis confirmed the existence of genetic differences between the species and showed more or greater variation among isolates of *L. biglobosa* than among isolates of *L. maculans* (Voigt et al. 1998). On the basis of RFLP analysis

and the profile of isoenzymes of the non-aggressive (NA) isolates, three subgroups were reported – NA1, NA2 and NA3 (Koch et al. 1991; Gall et al. 1994). Random Amplified Polymorphic DNA and rep-PCR methods also distinguished *L. maculans* from *L. biglobosa* and revealed the intraspecific differences of both groups (Plummer et al. 1994; Mahuku et al. 1997; Jędryczka et al. 1999b). Sequencing of the ITS ribosomal DNA fragment helped to illuminate the phylogenetic relationship of these species (Mendes-Pereira et al. 2003). The above-mentioned molecular methods gave rise to indirect proof of the distinctness of *L. maculans* and *L. biglobosa*.

In spite of numerous trials (Venn, 1979; Petrie and Lewis, 1985; Gall et al. 1994; Somda et al. 1997) it was not possible to obtain any fertile pseudothecia from matings between these two species. In contrast, the same research teams obtained hybrid isolates within *L. maculans* (Mengistu et al. 1993; Gall et al. 1994) and within *L. biglobosa* (Somda et al. 1997). Fertile pseudothecia of both species were frequently observed in natural environments (Johnson and Lewis, 1990).

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Charakterystyka populacji dwóch współistniejących patogenów rodzaju *Leptosphaeria* spp., powodujących suchą zgniliznę kapustnych

Streszczenie

Sucha zgnilizna, potocznie zwana czarną nóżką jest najgroźniejszą chorobą roślin rodziny Brassicaceae. Wywołują ją dwa patogeny *Leptosphaeria maculans* (Desm.) Ces et de Not. i *L. biglobosa* sp. nov. Shoemaker & Brun, które wspólnie kolonizują rośliny i prowadzą do powstania objawów chorobowych, utraty plonu oraz jakości warzyw kapustowatych i rzepaku. W niniejszym opracowaniu przedstawiono przynależność taksonomiczną i pokrewieństwo tych gatunków, szczegółowo opisano kolejne fazy rozwojowe obu patogenów, wskazano na różnice międzygatunkowe oraz metody identyfikacji opisanych gatunków.