Stellingen

1. Ringvlekkenziekte wordt als een typische herfstziekte in volgroeide koolgewassen gekarakteriseerd. Dit zegt veel over de onbekendheid met de epidemiologie van de veroorzaker.

   dit proefschrift

2. Infectie van sluitkoolplanten door *M. brassicicola* treedt op bij kortere bladnatperioden dan algemeen wordt aangenomen.

   dit proefschrift
   Wicks, T.J. and B. Vogelzang, 1988. Effect of fungicides applied after infection on the control of Mycosphaerella brassicicola on brussels sprouts. Australian Journal of Experimental Agriculture 28: 411-416.

3. De door Götz en Boyle (1993) beschreven toetsmethode voor de bepaling van resistentie in koolgewassen met behulp van door *M. brassicicola in vitro* geproduceerde antibiotische metabolieten is onbetrouwbaar en negeert eerder gepubliceerde eenvoudiger methoden.

   dit proefschrift

4. Het huidige waarschuwingssysteem voor *Mycosphaerella* bestrijding in koolgewassen laat de lange incubatie- en latentie-periode van de ringvlekkenziekte onder veldomstandigheden buiten beschouwing. Dit leidt tot overbodige gewasbespuitingen.

   dit proefschrift

5. "It can be argued that simple models handled in an enlightened way can yield at least as good results in practical disease predictions as complex models with inflexible characteristics, apart perhaps from the satisfaction, in difficult seasons, of being wrong for more sophisticated reasons"

   Bourke, P.M.A., 1970. Use of weather information in the prediction of plant diseases epiphytotics. Annual Review of Phytopathology 8: 345-370.
   Bastiaansen, C., A.Th.J. Koster, L.J. van der Meer, J.E. van den Ende, M.G. Pennock and F.M.P. Buurman, 1997. A disease-forecasting system of Botrytis blight ('fire') in lily. Acta Horticulturae 430:657-660.

6. De toenemende afhankelijkheid van additionele financiering van het landbouwkundig onderzoek werkt probleemgericht onderzoek in de hand, daar waar systeemgericht onderzoek noodzakelijk is voor een verdere stimulering van duurzame landbouw in Nederland.

7. Biologische landbouw is niet per definitie goed voor het milieu. De milieu- en natuurbeweging en politieke partijen zullen in hun streven naar een beter milieu meer effect sorteren wanneer ze niet eenzijdig de biologische landbouw propageren, maar ook milieu-vriendelijke maatregelen uit andere landbouwvormen en -stijlen erkennen en integreren tot een duurzame vorm van landbouw.

   Van Leeuwen, T. en J. Dekker, 1998. Gok niet alleen op de biologische landbouw. Milieudefensie 27: 18-19.

8. Het gebruik van de term "synergiewinst" om de beoogde samenwerking van landbouwkundige onderzoeksinstellingen binnen het Wageningse Universiteits en Research Centrum te karakteriseren is misleidend. In het beste geval kan er sprake zijn van antagonismeverlies.

   Peper, B., 1996. Duurzame kennis, duurzame landbouw. Een advies aan de Minister van Landbouw, Natuurbeheer en Visserij over de kennisinfrastructuur van de landbouw in 2010. 43 pp.

9. Internationale financiële instellingen als het IMF en de Wereldbank zijn opgericht om ontwikkeling te bevorderen en armoede te bestrijden. Door van ontwikkelingslanden te eisen dat ze jaarlijks ten minste een kwart van hun exportinkomsten aan rente en aflossing van hun schuld betalen, bereiken ze eerder het tegendeel.

   Anonymous, 1998. Making debt relief work: a test of political will. Oxfam International Position Paper: 12 pp.

10. De Veluweloop hoort autoloos te zijn.

11. Promoveren op de elfde van de elfde is ernstig.

Stellingen behorend bij het proefschrift:

Ring spot disease of brassica crops: resistance and epidemiology

Wageningen, 11 november 1998 Ernst van den Ende

Ring spot disease of brassica crops: resistance and epidemiology

Promotor:	Dr. J.C. Zadoks Emeritus Hoogleraar in de Ecologische Fytopathologie
Co-promotor:	Ir. H.D. Frinking Oud Universitair Hoofddocent Vakgroep Fytopathologie

J.E. van den Ende

Ring spot disease of brassica crops: resistance and epidemiology

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Landbouwuniversiteit te Wageningen,
Dr. C.M. Karssen,
in het openbaar te verdedigen
op woensdag 11 november 1998
des namiddags te vier uur in de Aula.

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Van den Ende, J.E.

Ring spot disease of brassica crops: resistance and epidemiology

Thesis Landbouwuniversiteit Wageningen - With ref. - With summary in Dutch

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Subject headings: *Brassica oleracea / Mycosphaerella brassicicola / control*

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Author's abstract

Van den Ende, J.E., 1998. Ring spot disease of brassica crops: resistance and epidemiology. PhD. Thesis, Wageningen Agricultural University, The Netherlands, 123 pp., 21 tables, 30 figures, English and Dutch summary.

Ring spot disease of brassica crops is caused by the fungus *Mycosphaerella brassicicola*. The disease can cause severe qualitative and quantitative losses, depending on the initial inoculum, the environmental conditions during the growing season and the susceptibility of the cultivar. A greenhouse screening method for resistance to ring spot in *Brassica oleracea* was developed. Young plants were screened by using mycelial inoculum enriched with 3% sucrose. Resistance was expressed both in cotyledons and true leaves by a lower number of lesions than the susceptible control and/or by hypersensitive reactions, and reflected adult plant resistance known from field studies. Studies with isolates of *M. brassicicola*, originating from various locations in Europe, indicated a differential host-pathogen interaction. Several aspects of the epidemiology of the disease were studied. Infection of cabbage plants under controlled conditions showed that ascospores of *M. brassicicola* are able to infect cabbage plants within shorter wetness periods (< 1 day) than generally assumed. The pathogen is well adapted to exploit leaf wetness interrupted by dry periods, which makes the definition of an infection period for *M. brassicicola* in terms of continous leaf wetness or high humidity unwarranted. From field studies it is concluded that the fungus has long incubation and latent periods, which should be taken into consideration when applying fungicides to control the disease.

Additional keywords: *Brassica oleracea,* Brussels sprouts, cabbage, cauliflower, control, host range, incubation period, inoculation method, latent period, *Mycosphaerella brassicicola*, screening method.

Voorwoord

Een voorwoord wordt vaak gebruikt om dank te betuigen aan de grote hoeveelheid mensen die vreugde hebben ontvangen, dan wel last hebben gehad van het geploeter van de promovendus. Hoe langer je over het afronden van een proefschrift doet, hoe meer mensen betrokken worden bij het wel en wee van de schrijver, en hoe langer dus het voorwoord wordt.

Herman Frinking heeft aan de wieg gestaan van dit onderzoek. Dankzij zijn inzet en enthousiasme werd het *Mycosphaerella* project gefinancieerd, en kon ik al voordat ik was afgestudeerd beneden in de kelder toe treden tot het groepje bovengrondse pathogenen.

Professor Zadoks heeft mij met zijn rode pen gedurende een groot aantal jaren bestookt. De vele discsussies over de manuscripten waren voor mij bijzonder leerzaam. Ik ben hem erkentelijk voor het geduld dat hij ongewenst moest opbrengen bij het afronden van mijn proefschrift.

De eerste fase van het onderzoek werd gefinancieerd door het bedrijfsleven: Bert Schrijvers (Bejo Zaden), Frans van den Bosch (Royal Sluis), Bart van den Bulk en Ad van Nieuwenhuizen (Rijk Zwaan), Hans Bongers (Nunhems Zaden), Anja van Herp en Jan-Leendert Harrewijn (Nickerson Zwaan), Peter Hermans en Walter de Milliano (Novartis Seeds) hebben mij sturing gegeven bij het tot stand komen van de resistentietoets.

Corrie Geerds en Wout Hoogkamer hebben mij menig maal geassisteerd wanneer de hoeveelheid werk de pan uit rees. Dankzij hen is ook mijn liefde voor sterke koffie en cryptogrammen met sprongen toegenomen.

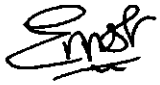
Gedurende de jaren dat ik aan *Mycosphaerella* heb gewerkt zijn er een groot aantal studenten geweest die hun steentjes en rotsblokken hebben bijgedragen aan het onderzoek: Bert Evenhuis, Henk Tiggelaar, Jannie Atzema, Marieke Bonhof, Jeroen Sytsma, Jan-Kees Goud, Jan-Eelco Jansma en Marian Folkers. Bedankt (8x).

Proeven doen met planten kan niet zonder steun van kas en tuinpersoneel. Bedankt voor de goede verzorging en het assisteren bij aanleg van veldproeven.

Op de vakgroep was het goed toeven, en dat was niet in de laatste plaats te danken aan de collega's. Henk Schouten leerde mij de kneepjes van het Zadoks-AIO zijn. Diederik Smilde was een prima kamergenoot, en Marjan Verhaar tot op het laatst een prettige praatpaal.

Tja, en dan de years after. De collega's in Wilhelminadorp, de Schuilenburg clan en de medewerkers van het LBO zijn in meer of mindere mate betrokken geweest bij mijn zwoegen. Gegeven het feit dat de laatste loodjes het zwaarst wegen dank ik met name Jan van Aartrijk van het LBO voor de ruimte die mij is gegeven om het proefschrift af te ronden. Ineke, bedankt voor het vele bijspringen, ik heb straks eindelijk weer tijd voor Botrytis en whisky.

Tot slot prettig nieuws voor de bruine ogen en al mijn naasten: hèhè, 't is af,



... je moet wel respect hebben voor iemand die DINSDAG kan spellen, ook al is het dan niet helemaal goed; maar spellen is uiteindelijk niet de hele wereld. Sommige dagen is het helemaal niets waard of je dinsdag kan spellen of niet.

(Winnie-the-Pooh)

Contents

Chapter 1

Ring spot disease (*Mycosphaerella brassicicola*) of brassica crops: an introduction

1.1 Disease

In the Netherlands, Brussels sprouts (*Brassica oleracea* L. var. *gemmifera* Zenker), cabbage (*B. oleracea* var. *capitata* L.) and cauliflower (*B. oleracea* var. *botrytis* L.) are important vegetable crops, grown on more than 10.000 ha (Anonymous, 1992). Ring spot disease, caused by *Mycosphaerella brassicicola*, has long been considered of minor importance to these crops. Since the early eighties, however, severe epidemics of ring spot disease in cabbage and Brussels sprouts in the Netherlands, Germany and the UK changed this view drastically (Frinking and Geerds, 1987; Humpherson-Jones and O'Brien, 1986; Zornbach, 1990).

Ring spot disease, usually indicated briefly as 'ring spot', is not restricted to Western Europe. Yield losses caused by the disease have been reported in other cool and moist regions of the world. In South Australia, growers have to spray every 10-14 days to control ring spot in Brussels sprouts (Wicks *et al.*, 1987; Wicks and Vogelzang, 1988). Ring spot was reported in the USA, where it caused serious losses in cabbage seed yields in western Washington (Gabrielson, 1981) and damaged Brussels sprouts and cauliflower production in coastal regions of California (Nelson and Pound, 1959; Osmun and Anderson, 1915; Weimer, 1926; Welch *et al.*, 1969). In the mountains of eastern-Java (Indonesia), ring spot is the most important disease in areas where cabbage is grown yearround (Van den Ende, unpublished). The occurrence of ring spot on *B. oleracea* is also known from Chile (Gonzalez and Montealegre, 1987), Venezuela (Tortolero and Carrasco, 1982), Russia, Peru and Ecuador (Chupp and Sherf, 1960).

1.2 Fungus

Mycosphaerella brassicicola (Duby) Lind. (Ascomycetes, Dothideaceae) was first described by Chevallier (1826) in France. Osmun and Anderson (1915) and Weimer (1926) described the imperfect stage of the fungus. Snyder (1946) showed that the 'pycnidiospores' were not able to germinate. The imperfect stage of the fungus does not represent a conidial but a spermatial stage, which should be placed in the form genus *Asteromella*: *Asteromella brassicicola* (Chev.) Boerema & v. Kesteren (Boerema and Van Kesteren, 1964). The fungus sometimes forms 'chlamydospore like' structures (Dring, 1961; Van den Ende *et al.*, 1986). According to Zornbach (1990) these structures do not play a role in the life cycle of the fungus. For dispersal and infection the fungus depends on the production of ascospores within pseudothecia.

No physiological specialisation has been reported for *M. brassicicola.* The fungus is homothallic (Punithalingam and Holliday, 1975).

1.3 Symptoms

Characteristic symptoms of ring spot disease are shown in Fig. 1.1A. Lesions appear on all aerial plant parts, but usually mature foliage is most heavily affected. Two types of lesions are formed. Ring spot derives its name from the most commonly formed type which is circular in outline with a definite margin, while the other is more irregular both in shape and outline (Weimer, 1926). Spermogonia and pseudothecia may be arranged in concentric rings or scattered over the lesion. The pseudothecia occur on both sides of the leaves, but more abundantly on the adaxial side. Lesions on sprouts, stems, and cabbage heads are often black, irregularly shaped and without sporulation (Rudnick, 1986; Zornbach, 1990) (Fig 1.1B).

Symptoms of ring spot on *B. oleracea* are often confused with symptoms of *Alternaria brassicicola* (Schw.)Wiltsch., *A. brassicae* Berk. and *Phoma lingam* (Tode:Fr.)Desm.. Full grown lesions of *Alternaria* show dark-brown or black conidiophores with spores, in contrast to the black fruiting bodies of *M. brassicicola*. Young lesions of *Alternaria* are often darker and less granulated than ring spot lesions (Fig. 1.2). *Phoma* leaf spots have more widely spaced brown pycnidia which produce a pink cirrhus under humid conditions. Ring spot symptoms on oilseed have been confused with white leaf spots caused by *Pseudocercosporella capsellae* Ell. & Ev., although lesions of the latter lack the presence of spermogonia and pseudothecia (Inman *et al.*, 1991; Petrie and Vanterpool, 1978; Vanterpool, 1960; 1968).

Severe infestations lead to early leaf senescence and defoliation (Rudnick, 1986). Serious economic losses occur as a result of both quantitative and qualitative damage at harvest time (Jouan *et al.*, 1972; Long, 1986; Everaarts and De Moel, 1991). Severe infestations can reduce cabbage seed yields (McKay, 1956; Gabrielson, 1981). *M. brassicicola* is a serious storage pathogen of cabbage. Lesions provide a point of entry for secondary infection by *Botrytis* spp., causing losses during storage of cabbage (Geeson and Robinson, 1975; Geeson, 1978). Moreover, ring spot lesions grow at low temperatures (0-5 °C) resulting in an increase of disease severity during storage (Zornbach, 1988).

1.4 Host range

Most brassica crops are susceptible to *M. brassicicola* (Table 1.1). All well-known *B. oleracea* varieties are reported as host plants. Data on *B. campestris* varieties are contradictory. Chinese cabbage (*B. campestris* var. *pekinensis* Rupr.) is considered a host by some authors (Nelson and Pound, 1959), but others disagree (Zornbach, 1988). In Germany, oilseed rape (*B. napus* Mill.) plays an important role in the epidemiology of ring spot as a winter host (Zornbach, 1990). Until 1988, oilseed rape was not considered to be a host for ring spot in the Netherlands (R. Meier, personal communication).

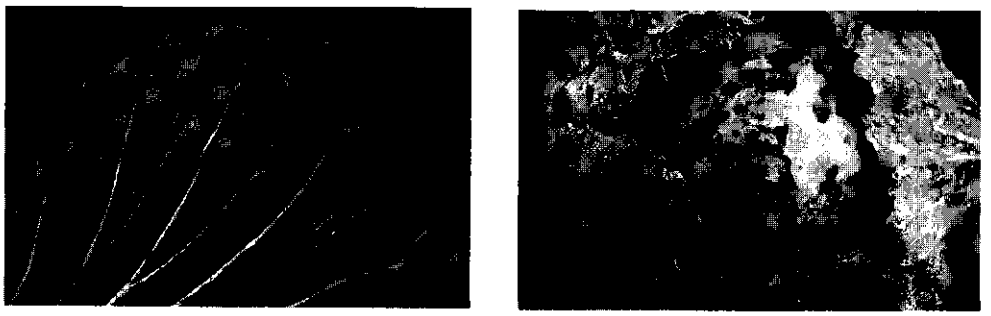


Figure 1.1 *Symptoms of Mycosphaerella brassicicola on brassica. Characteristic lesions on leaves (A) and on cabbage heads (B).*

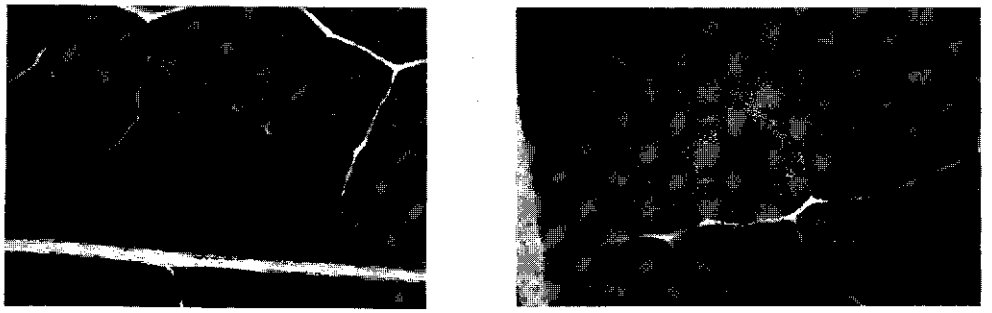


Figure 1.2 *Young lesions of Mycosphaerella brassicicola (A) and Alternaria sp. (B) on brassica leaves.*

Table 1.1 *Overview of reported hosts of Mycosphaerella brassicicola.*

Brassica campestris L.	var. *chinensis* L.	pak choi
	var. *pekinensis* Rupr.	chinese cabbage
Brassica carinata Braun		ethiopian mustard
Brassica juncea (L.)Czern.		mustard
Brassica napus Mill.		rape
Brassica nigra L.		black mustard
Brassica oleracea L.	var. *acephale* D.C.	kale
	var. *botrytis* L.	cauliflower
	var. *capitata* L.	cabbage
	var. *gemmifera* Zenke	Brussels sprouts
	var. *gongylodes* L.	kohl rabi
	var. *italica* Plenck.	broccoli
	var. *sabbelica* L.	collard
Raphanus sativus L.		radish
Cruciferous weeds	*Hirschfeldia incana* (Lagreze)Fossat	
	Matthiola incana L.	
	Sisymbrium officinale (L.)Scop.	
	Thlapsi arvense L.	

Four cruciferous weeds have been reported as host plants for ring spot (Dingley, 1969), but in infection studies only one of these weeds showed symptoms of ring spot, *Thlapsi arvense* L.(Zornbach, 1990).

1.5 Life cycle

Primary infection

M. brassicicola is able to penetrate seed coats and to produce lesions on the underlying cotyledons (Huber and Gould, 1949; McKay, 1956; Weimer, 1926). Huber and Gould (1949) and Snyder (1943) derived their idea that seed infection plays a major role in the epidemiology of the fungus from field data. Others showed evidence to the contrary and stated that ring spot is not effectively seedborne (Pound *et al.*, 1951; Jouan *et al.*, 1972).

Usually, disease originates from infected plant debris in and on the soil, or from mature infected crops growing near to the younger ones (Dixon, 1981). Pseudothecia are able to overwinter in the soil (Zornbach, 1990). The practice of continuous cropping of host plants yearround leads to build-up of inoculum in the field (Weimer, 1926; Welch *et al.*, 1969,

Zornbach, 1990). Infected transplants can spread the disease over a large area (Huber and Gould, 1949).

Spore discharge

Under field conditions ascospore discharge of *M. brassicicola* takes place over a wide range of temperatures, from 0-26 °C (Butler and Jones, 1955; Quak, 1957; Weimer, 1926). *In vitro* studies with Dutch isolates showed a lower maximum temperature of spore discharge, 22 °C (Frinking and Geerds, 1987). Spore discharge is stimulated by rainfall and mainly occurs under daylight (Hartill, 1977; Hartill and Sutton, 1980; Humpherson-Jones and O'Brien, 1986; Staunton and Ryan, 1977). Zornbach (1990) trapped spores after dew periods and concluded that rainfall was not an absolute prerequisite for spore discharge. Fruiting bodies of *M. brassicicola* on dry leaves need 24-48 hours after wetting to discharge ascospores (Zornbach, 1990).

After discharge, ascospores are dispersed by wind (Weimer, 1926). In the coastal regions of north-west Europa the ascospores can be found yearround (Jouan *et al.*, 1972; Quak, 1957).

Spore germination and penetration

Germination of ascospores of *M. brassicicola* strongly depends on wetness. Weimer (1926) showed that some ascospores germinated in the presence of free water within 24 hours, but that the majority of spores needed 48 hours at temperatures of 18-22 °C. At low temperature (7 °C) the majority of spores needed 96 hours to germinate. This result contradicts that of Snyder (1946) who found 100% germination within 24 h in free water in an outdoor experiment during winter. Although free moisture seems to be required for germination, continuous availability of free water is not necessary. At 11 °C ascospores can support a 5 days delay between inoculation and moist incubation (Staunton and Ryan, 1977).

M. brassicicola ascospores do not germinate, or germinate poorly, on expanding cabbage and cauliflower leaves (Hartill and Sutton, 1980). The effect is probably due to a volatile metabolite (allyl-isothiocyanate) present in younger leaves.

Penetration of the host takes place through stomata. No direct penetration of epidermal cells has been observed (Dring, 1961; Zornbach, 1990). Hyphae become established between and within host cells throughout the leaf, giving rise to the appearance of lesions on both leaf surfaces (Dring, 1961). Penetration of the host takes place after long periods of high humidity or leaf wetness. Reports on the required length of the wetness period are contradictory. Nelson and Pound (1959) mention a one week period of (near-)saturated atmosphere. Meier (1985) assumed that the fungus needs 4-6 days of high relative humidity (>90 %) to infect Brussels sprouts. Zornbach (1990) showed that infection on cabbage can occur after 3-4 days of high relative humidity. Staunton and Ryan (1977) and Wicks and Vogelzang (1988) reported even shorter periods, and stated that the length of the required wetness period depended on

temperature.

Incubation period

The period of time between the start of host infection and when the first disease symptoms become visible is called the incubation period. Many studies have been carried out to determine the incubation period, but data were contradictory again. Some field data were no more than rough estimations under various climatical circumstances. Data from infection studies resulted from inoculations with either ascospores or mycelial fragments. Inoculation of cabbage plants with mycelial inoculum under controlled conditions at temperatures between 12 and 20 °C led to incubation periods between 24 and 12 days (Nelson, 1958). Zornbach (1990) used ascospore inoculum and showed that the incubation period on young cabbage plants was 10-14 days at 20 °C.

According to most observations in the field, the incubation period varied between 3-4 weeks (Dring, 1961; Frinking and Geerds, 1987; Quak, 1957; Weimer; 1926, Wicks and Vogelzang, 1988; Zornbach, 1988). In Australia, Hartill (1977) reported a much shorter incubation period of 1 week after heavy rainfall during summer.

Spermogonia and pseudothecia formation

The fungus forms spermogonia within a broad temperature range (0-22 °C) independent of wetness (Weimer, 1926). Ripening and spread of spermatia probably take place under wet conditions, as shown with other *Mycosphaerella* species (Higgins, 1920, 1929, 1936).

Pseudothecia with ripe ascospores are mainly formed on old, already yellowing leaves (Osmun, 1915; Dring, 1961). At least 4 days of high relative humidity (>98%) are necessary for formation of the pseudothecia (Dring, 1961; Nelson and Pound, 1959). Pseudothecia containing ascospores were produced in a temperature range of 8-20 °C. Fruiting bodies were also produced at 4, 24 and 28 °C but these did not produce mature ascospores. At 20°C ripe ascospores were found 7 days after exposure of full grown ring spot lesions to a 7 days moisture period. At lower temperatures (8 °C) the formation of ripe ascospores took at least 3 weeks (Nelson and Pound, 1959). Nelson and Pound (1959) found an optimum temperature for ascospore formation between 15-22 °C, but Van den Ende *et al.* (1986) observed most ripe ascospores at temperatures between 10-15 °C. Apparently, light is not required for fruiting, since pseudothecia were formed abundantly on lesions in the dark (Nelson and Pound, 1959). Ripening of ascospores in fruiting bodies can be influenced by short periods of UV light (Zornbach, 1990; Götz *et al.*, 1993).

Latency period

The period of time between the start of host infection and when the lesion becomes infectious (e.g., begins to sporulate) is called the latency period. No accurate data are available on the length of the latency period. Rough estimations based on field obervations are given by

Frinking and Geerds (1987), who found pseudothecia 4 weeks after infection. Using field observations Zornbach (1990) estimated the latency period to be 5-6 weeks.

Infectious period

The period of time a ring spot lesion with pseudothecia is able to sporulate and cause more disease is called the infectious period. A single pseudothecium will produce ascospores for only a few days (Dring, 1961). However, the successive formation of pseudothecia within one ring spot lesion results in an infectious period of at least four weeks (Frinking and Geerds, 1987).

1.6 Control

In the Netherlands, epidemics of ring spot occur under wet conditions during late summer and autumn (Van den Ende *et al.*, 1986; Frinking and Geerds, 1987). Dutch growers use benzimidazoles (benomyl, carbendazim) and, more recently, pyrifenox to control the disease. Under Dutch legislation the number of fungicide applications against ring spot is restricted to two times benzimidazoles and/or one time pyrifenox per growing season (Mandersloot, 1993). Guidelines based on the duration of wetness periods are used to determine the timing of control (De Moel *et al.*, 1991; Van Oeveren, 1991). However, the lack of proper data about the effect of wetness on infection by *M. brassicicola* and the lack of knowledge about the life cycle of the fungus make these guidelines questionable. The use of fungicides to control ring spot is not without risk. In several countries, repeated applications of benzimidazoles resulted in resistance of the fungus against these fungicides (Gladders *et al.*, 1992; Wicks *et al.*, 1987). Breeding for resistance seems to be a promising strategy to control ring spot. Resistance to *M. brassicicola* is claimed to exist in Roscoff-type cauliflowers which have been selected for centuries in the UK and France. Similarly, selection of Brussels sprouts in south-west England is reputed to have produced cultivars, for example Moases, with ring spot resistance (Dixon, 1981). Differences in susceptibility between cabbage cultivars are reported since the first epidemics in the Netherlands (Mulder, 1985; Rijbroek, 1985, 1989). Unfortunately, systematic screening of cultivars in the field is hampered by variability in the occurrence of ring spot. Before 1988, screening tests under controlled conditions were not successful. Poor results were mainly due to poor growth of the fungus and lack of sporulation on artificial media, and to poor knowledge of the infection cycle of *M. brassicicola*. Therefore, six seed companies (Bejo Zaden, Nickerson-Zwaan, Nunhems Zaden, Rijk Zwaan, Royal Sluis and Novartis Seeds) financed a project, coordinated by the Dutch Horticultural Seed Trade Association (NVZP, former NTZ), to develop a screening test for ring spot resistance in *Brassica* and to study the infection cycle of ring spot in order to optimize disease control and selection for resistance. The present thesis describes the major results of this project.

1.7 Outline of this thesis

During the first phase of the project, research focused on the development of a screening method for resistance to ring spot in young plants of *B. oleracea*. Young cabbage seedlings with different levels of resistance to *M. brassicicola* were tested under field conditions, and the effect of early infection on the epidemiology of ring spot was quantified (Chapter 2). Practicability and reliability of a new method of screening under controlled conditions were evaluated on different locations by potential users (Chapter 3). Because few data were available about the variability of the fungus, isolates of *M. brassicicola*, originating from various locations in Europe, were tested on a differential set of brassica cultivars (Chapter 4).

During the second phase of the project experiments were carried out to determine the temperature and moisture requirements for infection more precisely, in order to optimize disease control in the field. Most infection studies in the past were carried out with mycelial inoculum, as production of the ascospores *in vitro* was poor and unreliable. It was not known whether data from inoculation studies with mycelial fragments were representative for infection of plants by ascospores in the field. Chapter 5 describes a selective method to collect ascospores from lesions of *M. brassicicola* on cabbage. Effects of temperature and leaf wetness on the infection of cabbage plants after inoculation by ascospores and by mycelial fragments of *M. brassicicola* were compared. Effects of temperature, relative humidity and leaf wetness on infection by ascospores were studied more thoroughly in order to find simple guidelines for infection conditions applicable in the field (Chapter 6). Data from field studies with ascospores of *M. brassicicola* were used to describe the epidemiology of ring spot in the field, and to evaluate the present disease control strategy (Chapter 7). A general discussion of the results obtained in this study concludes this thesis (Chapter 8).

Chapter 2

Seedbed infection of cabbage by *Mycosphaerella brassicicola*[1]

[1] J.E. van den Ende, 1993. Netherlands Journal of Plant Pathology 99: 139-148

Abstract

Three cultivars of cabbage with different levels of resistance to *Mycosphaerella brassicicola* were tested for seedbed infection. Seedlings grown in seedbeds, to which infected plant debris was added as an inoculum, showed typical ring spot lesions on the cotyledons and first two leaves before seedlings reached the transplanting stage, whereas non-inoculated controls had few lesions only. Differences in levels of resistance between cultivars were present in seedlings grown under field conditions. Disease severity of transplants at the end of the season reflected disease severity of seedlings before transplanting in each cultivar. To lower the risk of a severe epidemic of ring spot at the end of the growing season, the seedbed should be protected from infection by *M. brassicicola*.

2.1 Introduction

In the Netherlands, ring spot disease of brassica species, caused by *Mycosphaerella brassicicola* (Duby) Lindau, is a major disease in cabbage. Under conditions of rainfall, high humidity (2 days of 18 hours RLV>90%) and moderate temperatures (5-20 °C), the disease can cause severe damage to the host (Hartill, 1977; Staunton and Ryan, 1978; Ryan and Staunton, 1983; Frinking and Geerds, 1987; Zornbach, 1990; Van den Ende, 1992a). During severe epidemics most of the exposed, older leaves become extensively spotted. Young leaves of cabbage are usually without symptoms and they seem to become susceptible only shortly before they finish expansion (Hartill, 1977, 1978, 1980). Therefore, seedlings of cabbage are usually thought to be uninfected, though circumstances in spring can be favourable for disease development. Several authors presumed the importance of seedbed sanitation in the control of ring spot (Weimer, 1926; Chupp and Sherf, 1960), but experimental data on seedbed infection are not available. Usually, infection of *M. brassicicola* is considered to occur after the transplanting stage, that is at the age of 8 to 10 weeks (Frinking and Geerds, 1987; Meier, 1985; Zornbach, 1990). By the time the plants reach this stage of susceptibility, conditions usually no longer favour disease development, as temperatures rise (>20 °C) and long periods of high humidity become rare during the Dutch summer. In late summer and autumn plants are fully developed and climatic conditions can be optimal for disease development. Accordingly, ring spot is considered to be a problem in the Netherlands at the end of the growing season only, and little attention is paid to early infection in spring (Van den Ende *et al.*, 1986).

In greenhouse studies, severe infection by *M. brassicicola* was found on inoculated cotyledons and on the first two leaves of inoculated three-weeks old seedlings of cabbage, cauliflower and Brussels sprouts. Clear differences in resistance of seedlings to *M. brassicicola* were found between the brassica cultivars (Van den Ende, 1992b). This result

indicated the possibility of seedbed infection of young plants by *M. brassicicola*. If under field conditions seedlings of cabbage are susceptible to *M. brassicicola*, occasional infection in spring may result in a build-up of the disease to damaging amounts by harvest time at the end of the growing season.

As no quantative data were available about infection of young plants before the transplanting stage under field conditions, three field experiments were conducted (1989, 1990, 1991) to study seedbed infection and its effect on the severity of ring spot disease at the end of the growing season. As no information was available on the resistance of seedlings to *M. brassicicola* between different brassica cultivars under field conditions, seedbed experiments were carried out with several cabbage cultivars, with known and different responses to *M. brassicicola* under greenhouse conditions.

2.2 Materials and methods

Seedbed infection

Three cabbage cultivars with different levels of resistance to *M. brassicicola* (CA01: susceptible, CA02: resistant, CA03: partially resistant) were sown in two seedbeds on 24 April, 1989, in Wageningen. Seedbeds (1.5 x 1.5 m) were surrounded by 0.5 m high walls and located at 10 m distance to avoid cross infection between the seedbeds. Per seedbed each of the three cultivars was sown in two rows of 150 seeds per row. Row distance was 15 cm. In a second experiment seeds of the same three cabbage cultivars were sown on 3 May, 1990, in two seedbeds. The seedbeds were surrounded by 0.8 m high walls and separated by 20 m distance to avoid cross infection between the seedbeds. Each seedbed (6 x 6 m) was divided in three blocks (1 x 1 m), at 1.5 m distance between the blocks. Per block each of the three cultivars was sown in one row of 150 seeds, at 25 cm row distance.

In both experiments inoculum was placed in one seedbed by strewing crushed dried cabbage leaves collected during the previous season between the rows. The dried cabbage leaves were for 50 to 60% of their surface covered by lesions of *M. brassicicola*. In the other seedbed seeds were sown in untreated soil on which no cabbage had grown for years.

On 7 May, 1991, CA01, CA02 and CA03 were sown in four seedbeds. Three seedbeds were treated with inoculum similar to the previous experiments, one seedbed remained untreated. The seedbeds were surrounded by 0.8 m high walls. Distance between seedbeds treated with inoculum was 5 m, the control seedbed without inoculum was located 20 m away from the treated seedbeds to avoid cross infection. Each seedbed (6 x 6 m) was divided in three blocks (1 x 1 m), with 1.5 m distance between the blocks. Per block each cultivar was sown in one row of 150 seeds, at 25 cm row distance.

Disease in transplants

In the 1989 experiment, a sample of 200 seedlings per cultivar was taken from every seedbed ten weeks after sowing. For every seedling, the numbers of leaves and of ring spot lesions per leaf were counted. In addition, 50 seedlings per cultivar were transplanted to two experimental plots, one for seedlings from the treated seedbed and the other for seedlings from the control seedbed. The experimental plots, measuring 9 by 5 m, were divided into three subplots of 2.5 by 5 m, with 0.5 m spacing between the subplots. Every subplot contained one cultivar of which the seedlings were planted in five rows of ten plants. Distance between plants and between rows was 50 cm. The experimental plots were separated from each other by maize over a distance of 60 m, to avoid cross infection. Within each sub-plot the resistant cultivar was planted between the susceptible and the partially resistant cultivar so that cross infection between the sub-plots was minimized. The rows were planted perpendicular to the direction of the prevailing wind. At the end of the growing season, on 19 October, 1989, plants were harvested. Per plant, the numbers of leaves and of lesions per leaf caused by *M. brassicicola* were recorded.

In the 1990 and 1991 experiment a sample of 100 seedlings per cultivar was taken from every seedbed ten weeks after sowing. For every seedling, the numbers of leaves and of ring spot lesions per leaf were counted.

Lesion type

To gain more insight in differences in resistance between cultivars to ring spot under field circumstances, the lesion size of *M. brassicicola* on young seedlings was measured in the 1989 experiment. Lesions were either classified as a hypersensitive reaction, small (0-0.5 cm), medium (0.5-1.0 cm) or large (1.0-1.5 cm).

Weather data

In all seedbed experiments temperature and relative humidity were recorded by use of termohygrographs (Thies, Göttingen). Data on daily precipitation were obtained from a weather station on a distance of 500 m from the experimental plots.

Statistical analysis

Data were analyzed using the Statgraphics computer software package (Statgraphics, release 4.0). Analysis of the raw data indicated a heterogeneous error. Therefore, data of three succeeding years were subjected to an analysis of variance after logarithmic transformation of the number of lesions per cultivar. Because the data set involved small values, $\log_{10}(X + 1)$ was used instead of $\log_{10} X$, where X is the number of lesions per plant (Gomez and Gomez, 1984). Differences between treatments and between cultivars were tested for significance at 95% probability with LSD.

2.3 Results

In all three years rainfall together with long periods (≥ 2 days) of high humidity (daily average > 80%) and moderate temperatures (5 < T < 20 °C) were recorded during the period that plants were growing in seedbeds (Fig. 2.1).

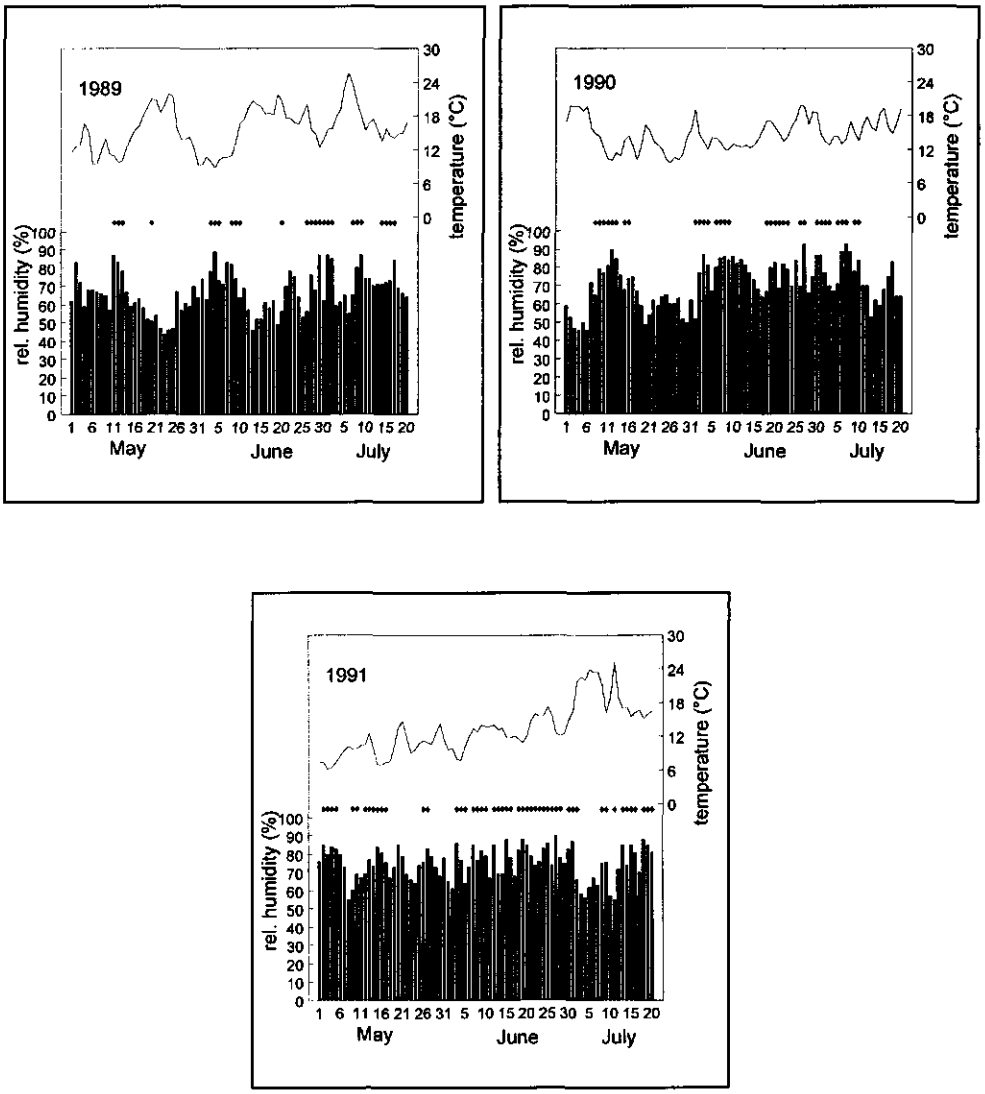


Figure 2.1 *Weather conditions during seedbed experiments in 1989, 1990 and 1991. Bars represent daily average relative humidity (%), dotted lines represent daily average temperature (°C) and diamonds represent rain days.*

In 1989 and 1990, the first lesions of *M. brassicicola* appeared on the susceptible cultivar (CA01) in the treated seedbed five and six weeks after sowing, respectively. In the 1991 experiment lesion development began 9 weeks after sowing. Results of lesion counts on seedlings ten weeks after sowing are presented in Fig 2.2. Data are expressed as the logarithm of the average number of lesions per leaf plus 1.

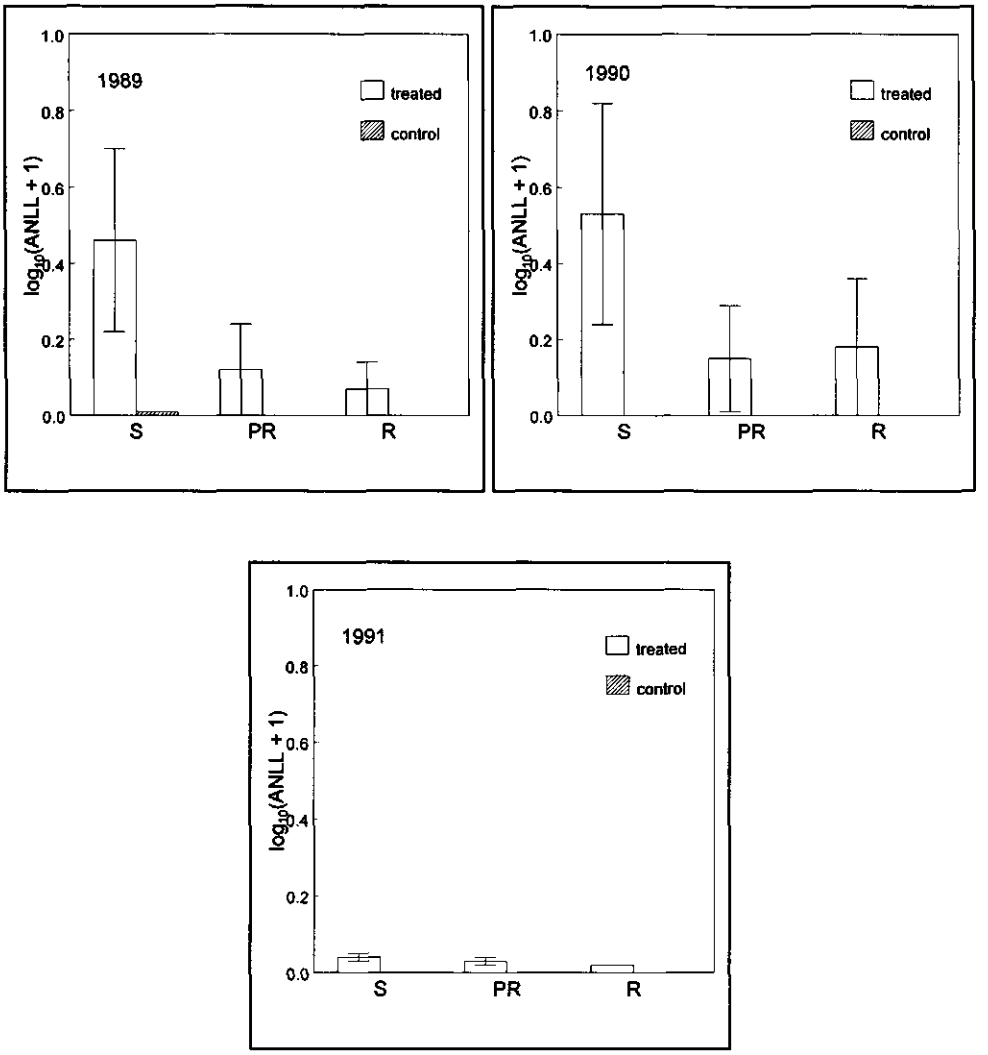


Figure 2.2 *The effect of seedbed inoculation in 1989, 1990 and 1991 by Mycosphaerella brassicicola on seedlings of three different cabbage cultivars: susceptible (S), partially resistant (PR), and resistant (R). The vertical bar represent the logarithm of the average number of lesions per leaf per cultivar (ANLL) added with 1.*

Figure 2.3 *Lesions of Mycosphaerella brassicicola on a susceptible cultivar seedling.*

Figure 2.4 *Hypersensitive reaction of a cultivar seedling resistant to Mycosphaerella brassicicola.*

High numbers of lesions were found on the susceptible cultivar in the treated seedbeds of 1989 and 1990. Seedlings of CA01 from the treated seedbeds showed typical ring spot lesions, predominantly on the cotyledons and the first two leaves (Fig 2.3). The resistant cultivar (CA02) showed a clear hypersensitive reaction on the green leaves of the young plants (Fig. 2.4), whereas on the older yellowed leaves some lesion development took place. In the control seedbeds only very few lesions were found on the susceptible cultivar and no lesions were found on the partially resistant and resistant cultivars. In the 1991 experiment disease severity on seedlings of the susceptible cultivar was very low. The

analysis of variance on the $\log_{10}$ transformed data of all three experiments showed significant effects for seedbed treatment and cultivar ($P < 0.01$). No significant interaction effects were present. Disease severity in the treated seedbed was significant higher than in the control seedbed (LSD, $\alpha = 0.05$). The number of lesions of the susceptible cultivar was significant higher than that of the partially resistant and resistant cultivar (LSD, $\alpha = 0.05$).

If the lesion type is taken into account the differences in susceptibility to *M. brassicicola* between the three cultivars become clearer. Fig. 2.5 shows data from the 1989 seedbed experiment. Most lesions on the susceptible cultivar tend to be large, whereas on the resistant cultivar most infections result in a hypersensitive response or in small lesions.

At the end of the 1989 growing season the effect of the seedbed-inoculation was clear in all three cultivars (Fig 2.6). Transplants of the susceptible cultivar from the treated seedbed showed severe infection by *M. brassicicola* in contrast to the transplants of the same cultivar from the control seedbed. Though relatively few lesions were counted on seedlings of the partially resistant and resistant cultivars, the seedbed inoculation nevertheless had effects on disease severity of both cultivars at the end of the growing season. Differences in disease severity between cultivars of the treated plot correspond to the difference in resistance to *M. brassicicola* of these cultivars.

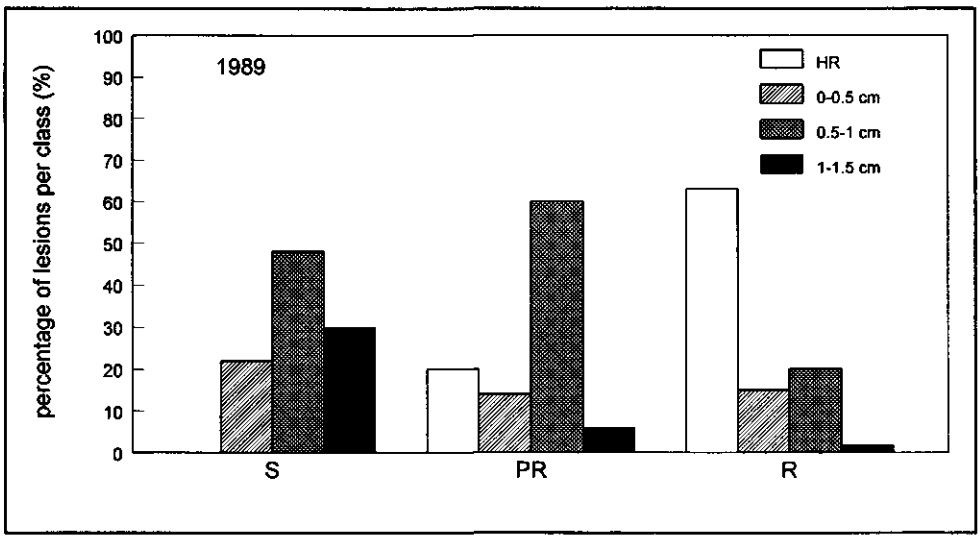


Figure 2.5 *Frequency distribution of lesion types of Mycosphaerella brassicicola on a susceptible (S), partially resistant (PR) and resistant (R) cultivar. Lesion are classified as not present (hypersensitive reaction, HR), small (0-0.5 cm), medium (0.5-1) or large (1-1.5).*

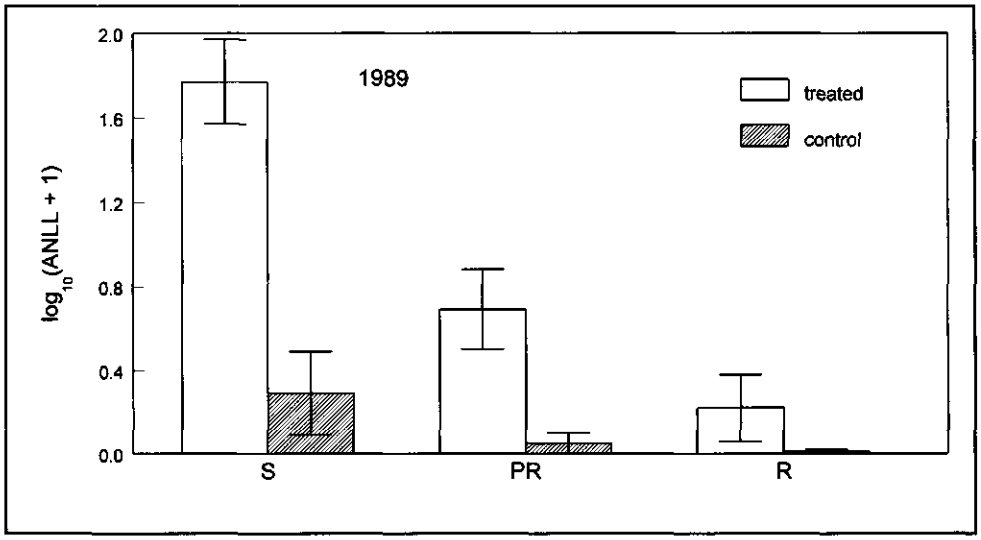


Figure 2.6 *The effect of seedbed inoculation of Mycosphaerella brassicicola on transplanted, fully grown plants of three different cabbage cultivars: susceptible (S), partially resistant (PR) and resistant (R). The vertical bar represent the logarithm of the average number of lesions per leaf per cultivar (ANLL) added with 1.*

2.4 Discussion

The seedbed experiments of 1989 and 1990 show that infection of young seedlings is likely to occur before transplanting if a source of inoculum is available and if weather conditions are suitable for infection. If an incubation period of approximately 20 days (Van den Ende, 1992b) is taken into account, the results show that in the field seedling infection can already occur at a plant age of 2-3 weeks. Although conditions for infection seemed to be optimal in all three years, disease severity levels in the 1991 experiment were very low. This is probably caused by a low quality of the inoculum used in this experiment. Sporulation of the particular dried leaf material was very poor, when tested in vitro according to the method of Van den Ende and Frinking (1993b).

Infection by *M. brassicicola* is not restricted to the fully grown plants as suggested by Osmun and Anderson (1915) and Butler and Jones (1949). Cotyledons and the first two leaves of cabbage seedlings are also susceptible to *M. brassicicola*. This result does not contradict the conclusion of Hartill (1977, 1980) that symptoms rarely develop until leaf expansion is complete, and never develop on rapidly expanding leaves. Two or three weeks after sowing, cotyledons and the first two leaves of cabbage are likely to be fully expanded. Therefore, infection of *M. brassicicola* is possible well before transplantation,

which is at an earlier stage than suggested by Frinking and Geerds (1987) and Zornbach (1988).

Although statistical reliable conclusions cannot be drawn, the results of the 1989 experiment after transplantation indicated that infected seedlings can have a strong impact on the severity of infection at the end of the growing season. As shown in studies with transplants older than 8 weeks, infection of plants early in the growing season will especially occur in regions where large scale cultivation of *Brassica* is a regular procedure and host plants are grown yearround (Jouan, 1972; Zornbach, 1990). In the spring, ascospores from infested wintercrops can cause a high inoculum pressure for transplants, but also for plants in seedbeds. The possibility of infection of young plants in seedbeds in spring will certainly lead to a change in the current view on the control of ring spot during the growing season of cabbage in the Netherlands. The experimental results of this study support the recommendations by Weimer (1926) and Sherf and MacNab (1986) to locate seedbeds far from infected fields, to keep the seedbeds free from infected plant debris, and to have the seedbeds protected from the prevailing wind which might carry ascospores over a considerable distance.

Under field conditions differences in resistance of cultivars are present even when plants are in a very young stage of development, which confirms results found in greenhouse studies (Van den Ende, 1992b). Resistance is expressed as a lower number of lesions, a reduced lesion growth or as a hypersensitive response. The results of seedbed infection in the partially resistant and resistant cultivars may have been influenced by cross contamination from the severely diseased plants of the susceptible cultivar. Further research should exclude this possibility by a better experimental design.

The present study suggests the possibility to select for resistance in cabbage seedlings in the field. Under field conditions, seedling resistance seemed to be correlated to adult plant resistance against *M. brassicicola*. This information may be useful to the plant breeders, who presently select for resistance in late fall when cabbage plants are fully grown.

Chapter 3

A screening test for *Mycosphaerella brassicicola* on *Brassica oleracea*[1].

[1] J.E. van den Ende, 1991. Netherlands Journal of Plant Pathology 98: 227-236.

Abstract

A greenhouse screening method for resistance to ring spot (*Mycosphaerella brassicicola*) in *Brassica oleracea* is described. High infection levels were achieved by spraying young plants by mycelial inoculum enriched with 3% sucrose. The screening method was tested on 9 cultivars, 3 Brussels sprouts, 3 cabbage and 3 cauliflower respectively, with known reactions to ring spot in the field. Resistance was expressed both in cotyledons and true leaves by a lower number of lesions than the susceptible control and/or by hypersensitive reactions. Results of the seedling tests reflected differences in resistance in the field. Under controlled conditions the new test can be applied year-round to young plants thus accelerating selection procedures.

3.1 Introduction

In the Netherlands severe epidemics of ring spot in cabbage and Brussels sprouts in 1984 and 1988, caused by *Mycosphaerella brassicicola* (Duby) Lindau, emphasized the need for resistant cultivars. Sources of resistance are available (Dixon, 1981; Mulder, 1985; Zornbach, 1990), but progress is hampered by the time-consuming procedure of the current resistance screening method. A test requires a complete growing season because fully grown brassica crops have to be tested in the field and disease symptoms develop slowly. Screening plants in the field is time-consuming and expensive, and results are confounded by many factors, including weather conditions and uneven distribution of the disease. For some pathogens, cabbage is screened in an early growth stage (Bansal *et al.*, 1990; Braverman, 1977; Natti *et al.*, 1967; Greenhalgh and Dickinson, 1975; Sjödin and Glimelius, 1988; Williams, 1985). The use of seedlings or young plants has an advantage since large populations of plants can be screened under controlled conditions, in a short period, economizing growth chamber or greenhouse space. The results of screening tests on seedlings or young plants are only reliable under the condition that resistance found in an early growth stage of the host is correlated with resistance in its adult stage (Sjödin and Glimelius, 1988).

Inoculations with *M. brassicicola* are mostly carried out with suspensions of mycelial fragments (Nelson and Pound, 1959: Zornbach, 1990), because mass production of spores *in vitro* is hardly possible. It is not known whether inoculations with mycelium give the same reactions on cultivars with different susceptibility levels as natural infection by ascospores (field situation). Temperature and leaf wetness are crucial factors in infection studies with *M. brassicicola* on cabbage. Optimum values of these parameters have been determined in other studies (Weimer, 1926; Nelson and Pound, 1959; Van den Ende, 1993b). From studies with *M. citri* it is known that sucrose increases the level of disease of citrus leaves after inoculation with ascospores (Whiteside, 1974). Glucose was found to be a weak stimulant for lesion formation of *Botrytis squamosa* and *B. cinerea* on onion (Clark

and Lorbeer, 1977). Comparable results for *M. brassicicola* are not available.

The objective of the present study was to develop an inoculation technique on young plants of *B. oleracea* as part of a routine screening method for resistance against *M. brassicicola*.

3.2 Materials and methods

Inoculum preparation

M. brassicicola was isolated from diseased leaves, collected in cabbage fields in the northern part of the Netherlands. Isolates were grown on V8 agar at 17 °C under alternating light: 12 h UV (380 nm) - 12 h dark. Four weeks before inoculation small pieces of mycelium (1-2 mm^2) were transferred to fresh V8 agar to start new colonies. After four weeks eight of these colonies were suspended in 250 ml of distilled water by use of a microblender. An estimation of the number of units in the suspension, which can possibly act as infection units, was determined by counting individual mycelial fragments with a hemocytometer.

For good results isolates should not be used when they are older than six months, or have been transferred more than four times (Van den Ende, unpublished). Therefore, every six months *M. brassicicola* was reisolated from diseased plant material.

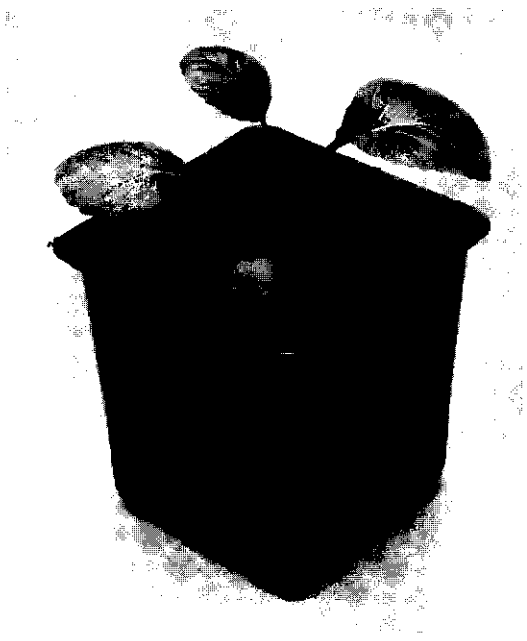


Figure 3.1. *Third-leaf stage of a cabbage plant.*

Host preparation

Inoculations were carried out on cotyledons (CO) and young plants in the third leaf stage (YP). Plants are in the third leaf stage when the third leaf is unfolded and the fourth leaf is just visible (Fig 3.1).

Plants were grown on a potting mixture consisting of decomposed sphagnum peat to which some clay and marl were added (TRIO 17: pH 5.4; organic matter 74%). For the inoculation of cotyledons, plants were grown in small polyethylene pots (7·7·6 cm) in the greenhouse at 17 to 20 °C in daylight, supplemented with artificial light (Philips, HPIT, 400 W) when necessary. Cotyledons were inoculated

at an age of ten days. For inoculation of young plants, plants were grown in bigger polyethylene pots (10·10·12 cm). Starting when the two first leaves of the plants in the greenhouse were visible, fertilizer was added weekly (Kristalon blauw: 19%N, 6%P, 20%K, 3%Mg). Plants were inoculated in the third leaf stage.

Inoculation technique

Young plants were inoculated by spraying a mycelial suspension with a micro ulva (Micron Sprayers LTD, Bromyard, England), approximately 3 ml per plant. For the inoculation of cotyledons 3 ml per 10 seedlings was used.

After inoculation, plants were transferred to a growth chamber with a constant temperature (15 °C) and low light intensity (1000 lux) (16 h light; 8 h dark). Plants inoculated in the cotyledon stage (CO) were kept in closed plastic containers to maintain a high humidity. Plants inoculated in the third leaf stage were covered with plastic bags to ensure a high humidity. After a six days period of high humidity plants were transferred to the greenhouse (17 to 20 °C, daylight only). Symptoms could be read 18 to 24 days after inoculation. Isolations were made from lesions on the cotyledons and leaves to confirm the presence of *M. brassicicola*.

Effect of sugar

To test a possible influence of sucrose on the disease level of *M. brassicicola* on cabbage cotyledons, sucrose was added to standard mycelial inoculum ($1.6 \cdot 10^5$ infection units per ml) in order to obtain three concentrations and a control (0, 2, 4, 6 g 100 ml^{-1}). Each concentration was applied to 10 pots with 5 plants each, in two replications. Inoculation of cotyledons was carried out according to the standard procedures.

The effect of sucrose and glucose added to the mycelial inoculum of *M. brassicicola* when used in inoculation studies on young plants (third-leaf stage) was tested on 8 cultivars with different levels of susceptibility to ring spot. Standard mycelial inoculum ($1.4 \cdot 10^5$ infection units per ml) was divided in five parts. To each part different amounts of glucose or sucrose were added to obtain the following concentrations: 0% sugar (C), 1% sucrose (S1), 3% sucrose (S3), 1% glucose (G1) and 3% glucose (G3). Per treatment, two plants were inoculated in four replications according to the standard procedures.

Screening of cultivars

Cultivars of cabbage, Brussels sprouts and cauliflower which showed resistance (R), partial resistance (PR) or susceptibility (S) in previous field trials were selected for the screening tests. The screening test was carried out on cotyledons (CO) and on plants in the third leaf stage (YP). In the CO test 12 pots of 5 plants each were used per cultivar in three replications. Cotyledons were inoculated with mycelial suspension ($1.6 \cdot 10^5$ infection units per ml) to which 3 g sucrose per 100 ml was added. As a control 12 pots with 5 plants each were used, of which cotyledons were treated with a 3% sucrose solution. In the YP

test two plants per cultivar were inoculated in four replications by standard procedures. The inoculum consisted of standard mycelial inoculum ($1.4 \cdot 10^5$ infection units per ml) to which 3 g sucrose per 100 ml was added. As a control two plants of each cultivar were treated with a 3% sucrose solution.

Screening of cultivars on location

To evaluate the practical use of the screening tests, both the CO test and the YP test were carried out on different locations. Six breeding companies (A, B, C, D, E, F) used the same nine cultivars from the preceding screening test.

In both tests plants were grown according to the standard procedures of the companies, using there own potting mixtures and fertilizers. Circumstances in the greenhouse could vary per location due to differences in construction of the greenhouses. Inoculations were carried out after a fixed number of days from sowing. The different inoculations at the six locations took place within a three day's period to minimise a possible time effect. Preparation of the inoculum was standardized, using the same isolate for all locations.

The CO test was carried out in April-May 1989. On each location 12 pots with 5 plants each were used per cultivar in three replications. Cotyledons were inoculated ten days after sowing, with an inoculum containing $1.7 \cdot 10^5$ infection units per ml to which 3 g sucrose per 100 ml was added. As a control 12 pots with 5 plants each were used, of which cotyledons were treated with a 3% sucrose solution. After inoculation plants were kept at high humidity in closed plastic containers at 15 °C in the dark during 6 days. After this incubation period they were transferred to the greenhouse.

The YP test was carried out in April-May 1990. On each location two plants of each cultivar were inoculated in nine replications. Plants were inoculated 28 days after sowing. The inoculum consisted of $1.4 \cdot 10^5$ infection units per ml to which 3 g sucrose per 100 ml was added. As a control two plants of each cultivar were treated with a 3% sucrose solution. Before transfer to the greenhouse, plants were kept at high humidity in closed plastic containers at 15 ° C under low light intensity (± 800 lux) for 6 days.

3.3 Results

Effect of sucrose

After 24 days the number of lesions per cotyledon was counted, and the average number of lesions per cotyledon was determined for each sucrose level (Fig. 3.2). The results were analyzed with ANOVA (LSD, α=0.05). The inoculum without sucrose resulted in a significantly lower number of lesions than the inocula with sucrose. A concentration of 2% sucrose in the inoculum gave a significantly higher number of lesions per cotyledon than the control (0% sucrose), but a significantly lower one than the concentrations of 4 and 6% sucrose.

The number of lesions per leaf on young plants was counted 26 days after inoculation.

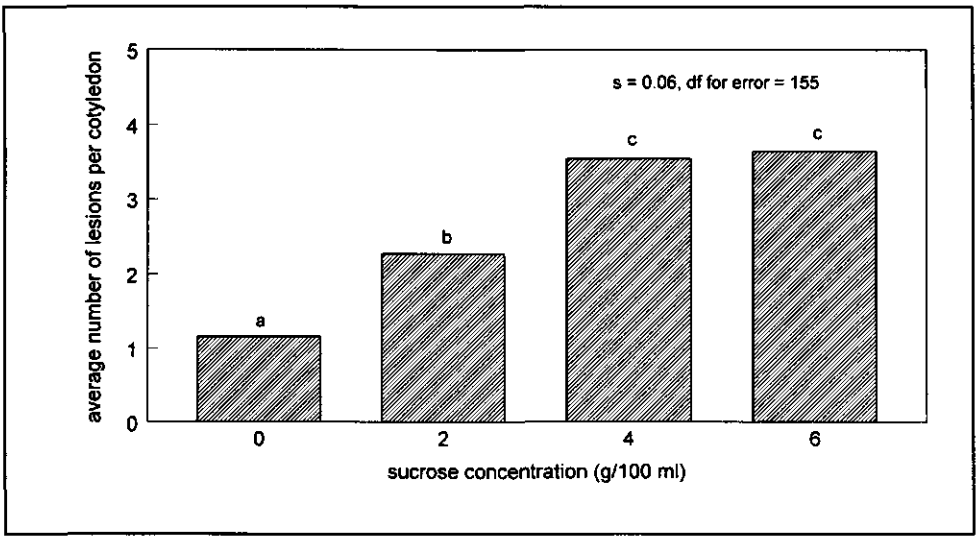


Figure 3.2. *Average number of lesions per cotyledon (CO test) per concentration of sucrose (0, 2, 4, 6 g 100ml^{-1}). Bars with different letters are significantly different (LSD, α = 0.05)*

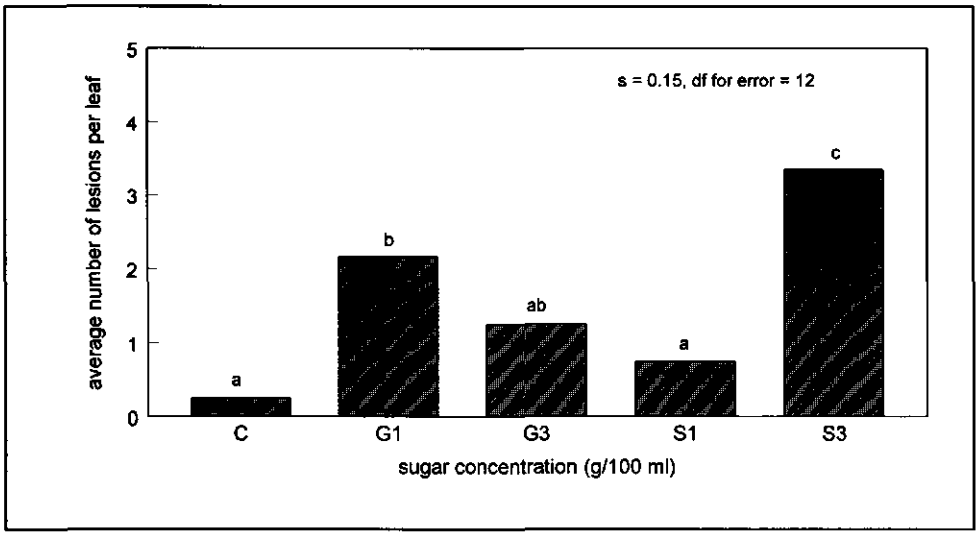


Figure 3.3. *Average number of lesions per leaf (YP test) in relation to sugar concentration in mycelial inoculum. 0 = sugar-free control, G1 = 1 g glucose 100 ml^{-1}, G3 = 3 g glucose 100 ml^{-1}, S1 = 1 g sucrose 100 ml^{-1}, S3 = 3 g sucrose 100 ml^{-1}. Bars with different letters are significantly different (LSD, α = 0.05).*

Per treatment the average number of lesions per leaf per plant was determined. The results were analyzed by means of ANOVA (LSD, $\alpha = 0.05$). Fig. 3.3 shows the results on the most susceptible cultivar, CA01. Inoculation with the standard mycelial inoculum enriched with 3% sucrose (S3) resulted in a significantly higher number of lesions per leaf than the other treatments (Fig. 3.3). This was true for all the susceptible cultivars tested. A low concentration of sucrose (S1) resulted in a low number of lesions per leaf not significantly different from the control (C). A low concentration of glucose (G1) resulted in a significantly higher number of lesions per leaf than the control (C) and the S1 treatment. Increase of the glucose concentration (G3) in the inoculum led to a low number of lesions per leaf which was not significantly different from the control (C).

Results of the screening test

In both tests observations were made 26 days after inoculation. In the screening test on cotyledons the average number of lesions per cotyledon was used as a measure for susceptibility. In the screening test on young plants the average number of lesions on the second leaf was used as a measure for susceptibility, because the second leaf of the plants in the third-leaf stage is the most susceptible one (Van den Ende, unpublished). Data were tested by means of ANOVA. Considering the fact that the data from these experiments were used to gain insight in slight differences between cultivars and not to select cultivars, a rather discriminative multiple range test was chosen (LSD, $\alpha = 0.05$).

Table 3.1 shows the results of the screening tests on cotyledons (CO) and young plants (YP). No symptoms were found in the control treatments. The second column of Table 3.1 shows the field observations according to the breeding companies. No quantative data on the resistance under field conditions are available.

In the screening tests resistance was either expressed as a lower number of lesions per leaf than the susceptible cultivar and/or as hypersensitive reactions. On cotyledons (CO) high levels of resistance were identified in CA02 and BS05, a result corresponding with the field observations. CA03 showed a high level of resistance, corresponding with the high level of partial resistance found in the field. No significant difference was found between the cauliflower cultivars (CF), which disagrees with the field observations (CF07).

Results of the screening test on young plants (YP) were in agreement with field observations. No symptoms were found on the second leaf in the cultivars CA02, CA03 and BS05. Even in cauliflower (CF07) some resistance could be distinguished. Differences between the partially resistant and resistant cultivars were not significant. As in the seedling test the partially resistant cabbage cultivar (CA03) showed a high level of resistance.

Table 3.1 *Differences in susceptibility to M. brassicicola between nine cultivars of Brassica oleracea, cabbage (CA01, CA02, CA03), Brussels sprouts (BS04, BS05, BS06) and cauliflower (CF07, CF08, CF09).*
Field data are rendered as S (susceptible), PR (partially resistant), and R (resistant). The average number of lesions (ANL) per cotyledon (CO) and per leaf (second leaf in YP) are shown. Means followed by the same letter are not significantly different (LSD, $\alpha = 0.05$).

cultivar	field data	ANL CO	ANL YP
CA01	S	3.4 a	3.3 a
CA02	R	0.2 c	0.0 d
CA03	PR	0.3 c	0.0 d
BS04	PR	1.4 b	0.3 cd
BS05	R	0.5 c	0.0 d
BS06	S	1.9 b	1.0 b
CF07	R	1.2 b	0.1 d
CF08	S	1.7 b	0.7 bc
CF09	PR	1.2 b	0.4 bcd

Results of the screening test on location

At four locations (A, B, C, D) the CO test resulted in satisfactory disease levels on cotyledons of the susceptible cultivar (85-100% of the cotyledons of the susceptible cultivar were infected). At one location plants died before observations could take place, while at the sixth location disease levels were too low for analysis (10% of the cotyledons of the susceptible cultivar were infected).

Considering the four locations as replications of one experiment, data can be analyzed by ANOVA. Although the results show high variability in numbers of lesions per cotyledon between the four locations (Fig. 3.4), no significant difference in overall disease level could be determined between the locations. Analysis of variance showed interaction between cultivars and locations. Three statistically different reaction types could be distinguished on each location: resistant or low susceptibility (CA02, CA03, BS05), moderate susceptibility (CF07, BS04, CF09) and high susceptibility (BS06, CF08, CA01).

At two locations the instructions for the YP test were not followed. This resulted in poor plant growth. At one location plants died because of drought, while at the other location (the same as in the CO test) the disease level was too low for analysis. The remaining two locations can be considered as replications, and were statistically analyzed with ANOVA.

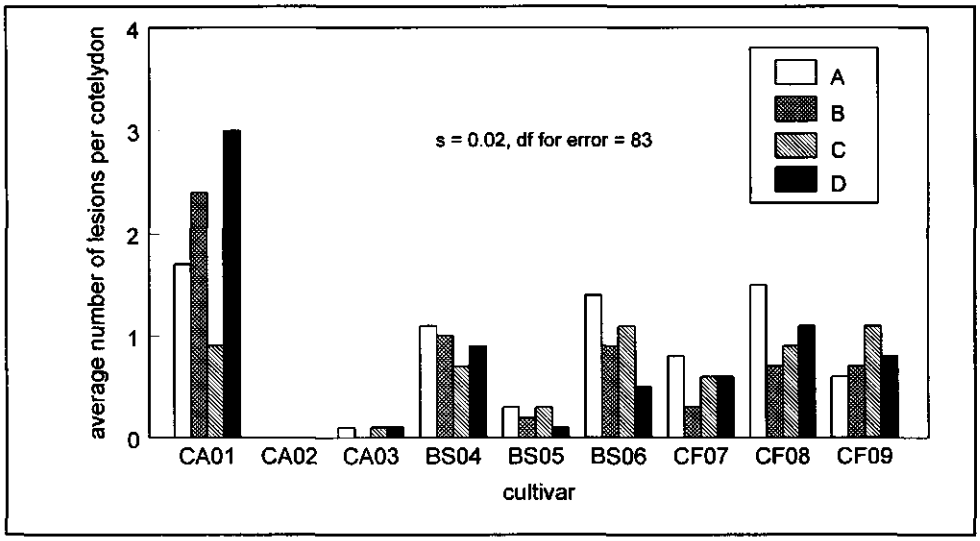


Figure 3.4 *Results of the CO test carried out at four locations (A, B, C, D). Differences in susceptibility to M. brassicicola between nine cultivars of Brassica oleracea, abbage (CA01, CA02, CA03), Brussels sprouts (BS04, BS05, BS06) and cauliflower (CF07, CF08, CF09). Bars represent average numbers of lesions per cotyledon.*

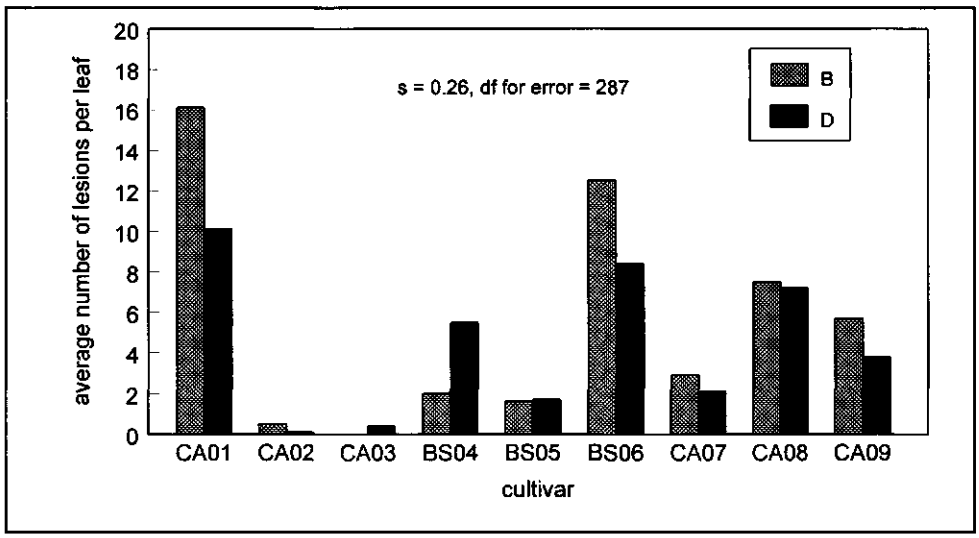


Figure 3.5 *Results of the YP test carried out at two locations (B, D). Differences in susceptibility to M. brassicicola between nine cultivars of Brassica oleracea, cabbage (CA01, CA02, CA03), Brussels sprouts (BS04, BS05, BS06) and cauliflower (CF07, CF08, CF09). Bars represent average numbers of lesions per leaf.*

Because of the rather high number of cultivars in the experiment a conservative multiple range test (Scheffé, α = 0.05) was used to discriminate between levels of susceptibility. Variability in numbers of lesions per leaf (Fig. 3.5) was not as high as in the CO test. No interaction between location and cultivar was found. CA02, CA03, BS05 and CF07 showed a significantly lower number of lesions per leaf than CF08, BS06 and CA01. Moderate susceptibility was found on the cultivars BS04 and CF09, which showed a significantly lower number of lesions per leaf than CA01, but significantly higher than CA02 and CA03.

3.4 Discussion

For most diseases of cabbage, screening tests on plants are carried out by application of spore suspensions of the pathogen (Williams, 1985). Due to the difficulty of producing high numbers of ascospores of *M. brassicicola* in vitro, screening of cultivars by means of inoculation with ascospores is hard to accomplish at a large scale. In 1958, Nelson found no difference in disease level after inoculation of 8 to 16 weeks old plants of a susceptible cultivar with mycelial and ascospore suspensions. The results of present inoculation studies on much younger plants of different cultivars indicated that inoculation with a mycelial suspension can be used to screen cultivars under controlled conditions. Data from the indoor tests, obtained with mycelial inoculum, corresponded to field data, resulting from natural infection by ascospores.

Adult plant resistance against *M. brassicicola* could already be detected in the cotyledons of seedlings. Resistance expressed at all developmental stages of the host was also found for *Phoma lingam* on *Brassica oleracea* (Sjödin and Glimelius, 1988; Mithen and Lewis, 1988). Results from cauliflower, were not as clear as from cabbage and Brussels sprouts (hybrids), probably because the cauliflower cultivars were not genetically uniform.

The standard inoculum resulted in rather low numbers of lesions on leaves of susceptible cultivars. To increase the number of lesions per leaf the mycelial suspension can be enriched with a sugar, which stimulates leaf penetration partly because of nutritional value and partly because of hygroscopic properties (Whiteside, 1974). A concentration of 2% sucrose in the mycelial inoculum increased the number of lesions on cotyledons significantly. If mycelial inoculum was enriched with 3% sucrose and applied on plants in the third leaf stage, the number of lesions was over 6 times that of the control. Enrichment of the inoculum by glucose can have a similar effect as by sucrose, but only when glucose is used in a lower concentration (1%). A 3% glucose treatment decreases the number of lesions per leaf in comparison to the sucrose 3% treatment. Whether differences in disease level might be explained by differences in osmotic value between sugar concentrations is not known.

Development of a screening test by research workers should be followed by evaluation of the test on different locations carried out by potential users. Unexpected variability and other problems will provide more insight in the practical use of the test. High variability in

the results of the CO test on the four locations demonstrated the importance of standardized circumstances during growth of the cotyledons. Differences in potting soil, fertilizer use, temperature and radiation can affect the growth of the plant. The developmental stage of the plant can affect the susceptibility for ring spot as shown in other studies (Van den Ende, 1993b). It is important to screen cultivars uniform in growth stage at the time of inoculation. Although much variability existed in disease level at the different locations, resistant cultivars and highly susceptible cultivars could be easily distinguished. Results of the young plant (YP) test also showed clear differences between resistant cultivars and highly susceptible cultivars. Failures of the YP test at the different companies were caused in three cases by lack of standardization in plant handling during growth. The low disease levels in both tests at location E cannot be explained.
High temperatures in May 1990 may have had an effect on the results of the YP test. Optimum growth of the fungus occurs between 15 and 22 °C (Nelson and Pound, 1959). When temperatures rise above 25 °C, the fungus cannot survive in leaf tissue.

In the present study inoculation of plants with *M. brassicicola* was followed by a 5 day's period of high humidity. Longer periods of high humidity gave higher numbers of lesions as shown in a previous study (Van den Ende, unpublished). However, results obtained from experiments with very long periods of high humidity may not be representative for natural conditions. Plants in a young stage of development cannot survive a long period of high humidity, which is a disadvantage for screening seedlings. Seedlings of cauliflower could not withstand a period of 6 days of high humidity, and died before the symptoms of ring spot could develop. Such problems do not arise when plants are tested in the third leaf stage.

Testing young plants in the third leaf stage will give the best results. With a YP test, resistant and highly susceptible cultivars can be distinguished, partial resistance is still difficult to assess. Screening *B. oleracea* in the greenhouse for resistance against *M. brassicicola* should therefore be considered a preliminary step to field screening. The ability to screen *B. oleracea* for resistance to *M. brassicicola* under controlled conditions offers the potential for rapid determination of suitability of *B. oleracea* selections for inclusion in a breeding and selection program.

Physiological specialization in *M. brassicicola* (Dixon, 1981) is not (yet) known. The present study was carried out with isolates of *M. brassicicola* from the northern part of the Netherlands. The existence of more races of the fungus would complicate the interpretation of the results of the screening test.

Chapter 4

Differential interaction of *Mycosphaerella brassicicola* and brassica cultivars[1]

[1]J.E. van den Ende, 1993. Netherlands Journal of Plant Pathology 99: 149-162.

Abstract

Isolates of *Mycosphaerella brassicicola*, originating from various locations in Europe, differed in their virulence on a differential set of brassica cultivars, as measured by the number of lesions per leaf. Hypersensitivity and significant cultivar-isolate effects were observed, indicating a differential host-pathogen interaction. Although expression of resistance depends on plant development, the differential host-pathogen interaction was found in all plant stages tested. This is the first report on the existence of physiological specialization of *M. brassicicola*.

4.1 Introduction

The ring spot disease of brassica crops is restricted to areas with high humidity and moderate temperatures. The disease is not only found in coastal areas, as in north-west Europe and Australia (Nelson and Pound, 1959), but also in certain mountain areas in more tropical climates (Punithalingam and Holliday, 1975; Frinking and Geerds, 1987; Gonzalez and Montealegre, 1987).

Ring spot of brassica crops is caused by *Mycosphaerella brassicicola* (Duby) Lindau. For dispersal and infection the fungus depends on ascospores. No imperfect stage of *M. brassicicola* is known (Dring, 1961; Zornbach, 1990). The presence of ring spot in isolated geographical regions with specific climatic conditions for the development of the disease, and the dependence of the fungus on its sexual stage, may have resulted in the development of different populations of the fungus with specific genetic characteristics.

The host range of *M. brassicicola* is not restricted to varieties (in the botanical sense) of *B. oleracea* L. only. Varieties of *B. campestris* L. and *B. napus* L. are also susceptible to ring spot disease (Nelson and Pound, 1959). It is not known whether isolates of *M. brassicicola* differ in their adaption to different varieties within one species. Previous studies showed that differences in levels of resistance between cultivars of one variety may occur (Zornbach, 1990; Van den Ende, 1992b), but physiological specialization of the fungus has not yet been reported (Dixon, 1981).

Specificity implies that genetic variation in the host and the pathogen are correlated (Vanderplank, 1982). When pathogenic races can attack some but not all cultivars of the host, resistance is expressed in a qualitative way and distinct races can easily be identified. If all relevant races of the pathogen can attack all relevant cultivars of the host, physiological specialization may be based on quantitative differences in disease expression. Several factors influence disease expression, and therefore affect conclusions with respect to specificity. Disease development of ring spot on brassica plants depends on leaf age (Van den Ende, unpublished). If fully developed leaves or cotyledons are present on susceptible plants of *Brassica* spp., plants can be infected by *M. brassicicola*

(Hartill and Sutton, 1980). Testing of plants in different stages of development can reveal an age-cultivar-isolate interaction.

Differential adaptation of pathogen isolates to certain host genotypes can complicate screening strategies for the selection of disease-resistant host varieties. Knowledge of the virulence structure of the pathogen population will therefore be of value in developing effective strategies for breeding for resistance to this disease. To test the hypothesis that physiological specialization of *M. brassicicola* does exist, several isolates of the fungus obtained from different European regions were tested on cotyledons and on plants in the third and seventh leaf stage of brassica cultivars.

4.2 Materials and methods

Biological material

Isolates evaluated in these experiments were taken from different geographic regions and from different cultivars of *B. oleracea* (Table 4.1). Eight single spore isolates of *M. brassicicola* were obtained from infected leaves from the field. To collect the spores lesions were cut out of infected leaves and soaked in demineralized water. After 30 minutes lesions were placed on wet filter paper in the lid of a petri dish. The bottom of the petri dish was filled with a thin layer of water agar. After sealing the petri dish with parafilm, it was placed upside down under light (8000 lux) at 15 °C. Within a few days (1-2) the ascospores were shot into the water agar and could be transferred to growing media. Isolates were grown on V8 agar (Miller, 1955) at 17 °C under alternating light (12 h UV (380 nm) - 12 h dark) conditions.

Brassica cultivars were provided by Dutch breeding companies. Susceptibility levels for *M. brassicicola* under field conditions in the Netherlands are known for most cultivars (Table 4.2). Plants were grown in polyethylene pots in the greenhouse at 17-20 °C in daylight, supplemented with artificial light (Philips, HPIT, 400 W). A potting mixture was used consisting of a decomposed sphagnum peat to which some clay and marl were added (TRIO 17; pH 5.4; organic matter 74%). Beginning at the 2-leaves stage, fertilizer was added weekly (Kristalon blue: 19%N, 6%P, 20%K, 3%Mg).

Inoculum preparation

Inoculations were carried out with suspensions of mycelium according to Van den Ende (1992). Isolates were collected over a period of 6 months. Therefore, some reduction in virulence due to prolonged storage of some of the isolates could have occurred. To minimize variation caused by age differences of the cultures, fresh cultures were started prior to inoculation. After a growth period of 3-4 weeks, inoculum was produced by grinding mycelium of *M. brassicicola* in distilled water for four minutes in a micro blender. To ensure

Table 4.1 *Identity of isolates used in the study of physiological specialization in Mycosphaerella brassicicola.*

Isolate	Region	Host
NH	The Netherlands	*B. oleracea* var. *capitata*
OP	The Netherlands	*B. oleracea* var. *botrytis*
FR	France	*B. oleracea* var. *botrytis*
GE	Germany	*B. oleracea* var. *capitata*
DK	Denmark	*B. oleracea* var. *capitata*
UK	United Kingdom	*B. oleracea* var. *gemmifera*

Table 4.2 *Cultivars of Brassica species with known field resistance against the Dutch NH isolate, used in the study on physiological specialization in Mycosphaerella brassicicola.*

species and varieties	cultivar	
B. oleracea var. *botrytis*	CF02	susceptible
	CF07	resistant
	CF08	susceptible
	CF09	susceptible
B. oleracea var. *gemmifera*	BS01	partially resistant
	BS04	susceptible
	BS05	resistant
	BS06	susceptible
B. oleracea var. *capitata*	CA01	susceptible
	CA02	resistant
	CA03	partially resistant
	CA04	partially resistant
	CA05	partially resistant
B. pekinensis	CC01	unknown
B. napus	OR01	unknown

a high disease severity 3 g sucrose per 100 ml was added to the inoculum. A hemocytometer was used to estimate the density of infection units per ml.

Inoculation procedure

The inoculum was divided into parts, according to the number of replications in the experiment. Each part was sprayed separately onto a set of differential cultivars with a micro ulva (Micron Sprayers, LTD, Bromyard, England). One can argue about the validity of the replications because of the similarity of inoculum used in each replication. However, preparation of different inocula for each replication would create too much variability in the results, because standardization of mycelial suspensions is hardly possible. The experimental design was of a split-plot nature with individual isolates of *M. brassicicola* assigned to flats with plants as main plots, and cultivars randomly assigned to cells (one plant or pot per cell) within flats as subplots. Main plots were replicated.

After inoculation, plants were transferred to a growth chamber with constant temperature (15 °C) and low light intensity (1000 lux) (16 h light; 8 h dark). Pots with plants were covered with plastic bags to ensure a high humidity. After six days of high humidity plants were transferred to the greenhouse (17 to 20 °C). Light conditions were not standardized, as light does not influence symptom development after penetration of the fungus (Van den Ende, 1992a). The first symptoms could be read 18 to 24 days after inoculation. The optimal moment for disease asssessment is when the most susceptible cultivar approaches its maximum score (Parlevliet, 1989). At 28 or 29 days after inoculation disease severity was assessed as the number of lesions per leaf.

Experiments

Cotyledons (CO). For the inoculation of cotyledons, seedlings of cauliflower (CF02, CF07, CF08, CF09), cabbage (CA01, CA02, CA03, CA04, CA05), Brussels sprouts (BS04, BS05, BS06), Chinese cabbage (CC01) and oilseed rape (OR01) were grown in small polyethylene pots (7·7·6 cm). Each pot contained five seedlings. Ten days after sowing cotyledons were inoculated with mycelial suspensions of two isolates (NH and FR, Table 4.1). Per isolate mycelial suspensions were prepared by grinding eight colonies of 28 days old in 250 ml distilled water (NH: $1.4 \cdot 10^5$ infection units per ml, FR: $1.5 \cdot 10^5$ infection units per ml). Approximately 2 ml inoculum was applied per pot. Each cultivar was inoculated in six replications. As a control treatment one pot of each cultivar was treated with a 3% sucrose solution in distilled water. The average number of lesions per cotyledon per pot was assessed 29 days after inoculation.

Third leaf stage (TL). Plants of cauliflower (CF02, CF07, CF08, CF09), cabbage (CA01, CA02, CA03, CA04, CA05), Brussels sprouts (BS04, BS05, BS06) and Chinese cabbage (CC01) were grown in small polyethylene pots (7·7·6 cm). Each pot contained 1 plant. Plants in the third leaf stage (third leaf unfolded and the fourth leaf just visible) were

inoculated with mycelial suspensions of the isolates NH and FR. Per isolate mycelial suspensions were prepared by grinding 10 colonies of 20 days old in 250 ml distilled water (NH and FR: $0.7 \cdot 10^5$ infection units per ml). Approximately 3 ml inoculum was applied per plant. Each cultivar was inoculated in five replications. As a control treatment one plant of each cultivar was treated with water. After 29 days lesions on the first two leaves of each plant were counted.

Seventh leaf stage (SL). Plants of cauliflower (CF07, CF08, CF09), cabbage (CA01, CA02, CA03) and Brussels sprouts (BS04, BS06, BS07) were grown in polyethylene pots (10·10·12 cm). Each pot contained 1 plant. Plants in the seventh leaf stage (seventh leaf unfolded and eighth leaf just visible) were inoculated with mycelial suspensions of six isolates, NH, DK, FR, GE, OP, UK (Table 4.1). Per isolate mycelial suspensions were made by grinding 8 colonies of 28 days old in 100 ml distilled water. No estimations of infection units per ml were made. After 28 days lesions were counted on each of the leaves 4 to 7.

Statistical analyses

Data were analyzed using the Genstat computer software package (Genstat 5, release 2.1). Analysis of the raw data indicated a heterogeneous error. Therefore, disease severity means of each isolate-cultivar combination were subjected to an analysis of variance after logarithmic transformation of the data. The Genstat procedure for a split-plot design was used.

To study a possible leaf age effect on the isolate-cultivar interaction the log transformed data of the SL experiment were subjected to an analysis of variance using the Genstat procedure for a split-split-plot experiment.

4.3 Results

Cotyledons

In Fig. 4.1 data are represented as the average number of lesions per cotyledon. Statistical analysis on the log transformed data showed a significant cultivar-isolate interaction effect (Table 4.3). Main effect (isolate) and subplot effect (cultivar) are significant too. Considerable differences in susceptibility to the Dutch isolate (NH) of *M. brassicicola* existed between cultivars. These differences were not always in agreement with the field data of the cultivars as provided by the breeding companies (Table 4.2). A striking difference in susceptibility to the Dutch (NH) and the French (FR) isolate existed between the cabbage cultivars (CA01 to CA05). Cultivars which are known to be resistant or partially resistant against the Dutch isolate of *M. brassicicola* (CA02, CA03, CA04 and CA05), showed high disease severity levels when tested with the French isolate.

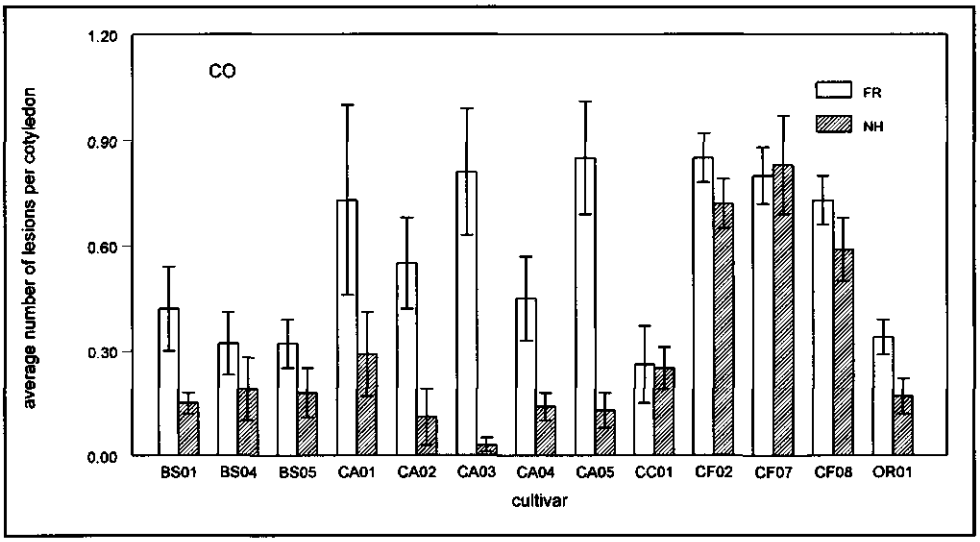


Figure 4.1 *Average number of lesions per cotyledon (CO) for two isolates of M. brassicicola (NH and FR) and 13 cultivars of Brussels sprouts (BS01, BS04, BS05), cabbage (CA01, CA02, CA03, CA04, CA05), chinese cabbage (CC01), cauliflower (CF02, CF07, CF08) and oilseed rape (OR01). Per bar standard errors are indicated.*

Table 4.3 *Analysis of variance of the log transformed average numbers of lesions per leaf on several Brassica cultivars of three plant developmental stages caused by two isolates of M. brassicicola (NH and FR). Cotyledons (CO): 13 Brassica cultivars. Third leaf stage (TL): 12 Brassica cultivars. Seventh leaf stage (SL): 9 Brassica cultivars.*

Source of variation	CO		TL		SL	
	df[a]	MS[b]	df[a]	MS[b]	df[a]	MS[b]
Replication	5	0.0060^{ns}	4	0.076^{ns}	3	0.18^{ns}
Isolate	1	0.29**	1	5.49**	1	0.60^{ns}
Main plot error	5	0.0036	4	0.21	3	0.071
Cultivar	12	0.045**	11	0.44**	8	1.49**
Cultivar-isolate	12	0.017**	11	0.28**	8	0.68**
Subplot error	109(11)	0.0043	85(2)	0.082	40(8)	0.093
Total	145(11)		117(2)		63(8)	

df degrees of freedom
[a] (..) = number of missing values
MS Mean Square
[b] ns *F*-value not significant
* *F*-value significant for $P < 0.05$
** *F*-value significant for $P < 0.01$

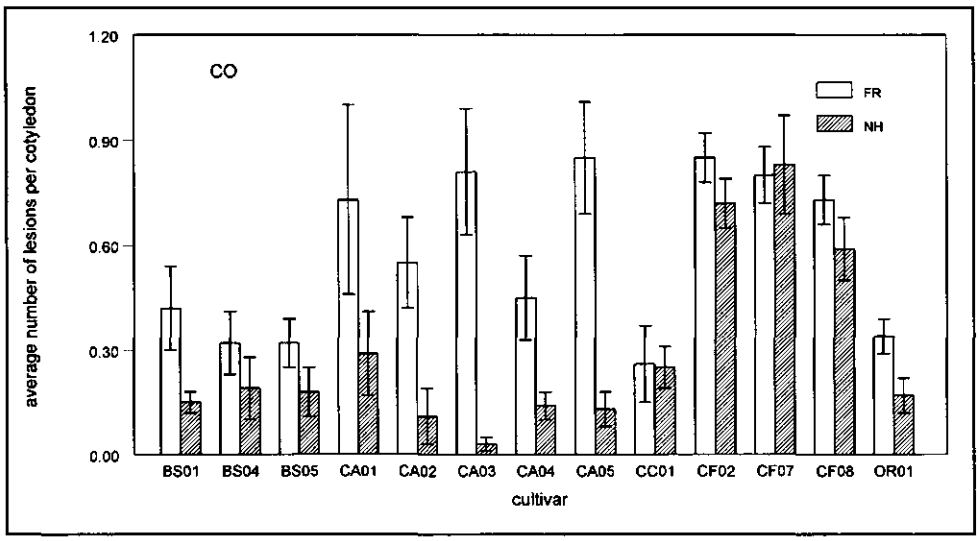


Figure 4.2 *Average number of lesions per leaf (TL) for two isolates of M. brassicicola (NH and FR) and 12 cultivars of Brussels sprouts (BS01, BS04, BS05), cabbage (CA01, CA02, CA03, CA04, CA05), chinese cabbage (CC01) and cauliflower (CF02, CF07, CF08). Per average number of lesions standard errors are indicated.*

The cotyledons of Brussels sprouts (BS01, BS04, BS05) and cauliflower (CF07, CF08, CF09) did not show much difference in susceptibility for the two isolates. Cotyledons of chinese cabbage (CC01) and oilseed-rape (OR01) were susceptible to both the NH and FR isolates.

Third leaf stage

The number of lesions on the first two leaves per plant were averaged. In Fig. 4.2 the average number of lesions per leaf is shown. Statistical analysis on the log transformed data showed a significant cultivar-isolate interaction effect (Table 4.3). Main effect (isolate) and subplot effect (cultivar) were significant too. Differences in susceptibility between cultivars to the Dutch isolate (NH) were in agreement with the known field data (Table 4.2). Hypersensitive reactions as a result of infection with the NH isolate were found on the cabbage cultivars CA02 and CA03 and the Brussels sprouts cultivar BS05. Hypersensitive reactions were not found in the same cultivars when tested with the FR isolate, resulting in high numbers of lesions per leaf especially on the cabbage cultivars CA02 and CA03. Even in cauliflower, resistance to the NH isolate seemed to be not effective against the FR isolate, as shown by the relatively high number of lesions on CF07 when tested with the FR isolate. Not all cultivars showed differences in disease severity levels when tested with the NH and FR isolates. The partially resistant cultivars CA04 and BS01, which did not

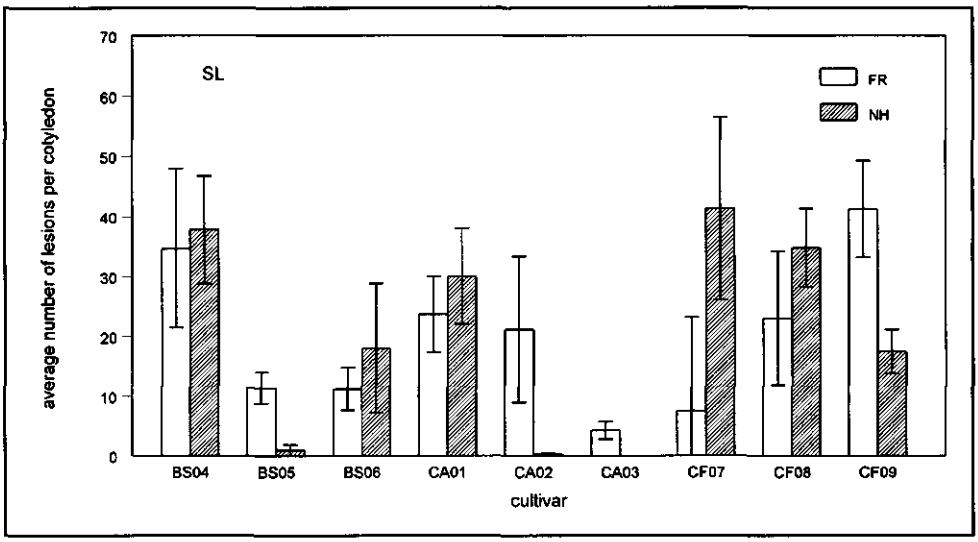


Figure 4.3 *Average number of lesions per leaf (SL) for two isolates of M. brassicicola (NH and FR) and 9 cultivars of Brussels sprouts (BS04, BS05, BS06), cabbage (CA01, CA02, CA03) and cauliflower (CF07, CF08, CF09). Per average number of lesions standard errors are indicated.*

show hypersensitive reactions against the NH isolate but a relatively low number of lesions per plant, showed no significant differences between the NH and FR isolates.

Seventh leaf stage

Many of the lower leaves of the plants had withered or were dead at the time of assessment. Therefore, only the lesion numbers of leaves 4 to 6 were averaged. In Fig. 4.3 the average number of lesions per leaf is shown for the Dutch (NH) and French (FR) isolates. Statistical analysis of the log transformed data showed a significant cultivar-isolate interaction effect (Table 4.3). The main effect of isolates was not significant. Cultivars were significantly different. For the cabbage and Brussels sprouts cultivars differences in susceptibility between cultivars to the NH isolate were more pronounced than in the two previous experiments. These data were in agreement with the known field data (Table 4.2). Hypersensitive reactions were found in BS05, CA02 and CA03, but were absent when the cultivars were tested with the FR isolate. Results on the cauliflower cultivars showed a striking difference with the results found in the previous experiment (TL). The NH isolate showed a high number of lesions on the resistant CF07, whereas the inoculation with the FR isolate resulted in a low number of lesions per leaf. In younger plant stages no big differences could be found in disease severity of the cabbage cultivars when tested with

Table 4.4. *Analysis of variance of the log transformed average numbers of lesions per leaf on nine Brassica cultivars in the seventh leaf stage caused by six isolates (NH, FR, DK, GE, OP, UK) of M. brassicicola.*

Source of variation	df[a]	MS[b]
Replication	3	0.080[ns]
Isolate	5	1.62[*]
Residual	15	0.094
Cultivar	8	5.98[**]
Isolate·cultivar	40	0.40[**]
Residual	122(22)	0.070
Total	193(22)	

df degrees of freedom
[a] (..) = number of missing values
MS Mean Square
[b] ns = *F*-value is not significant
* = *F*-value significant for $P<0.05$
** = *F*-value significant for $P<0.01$

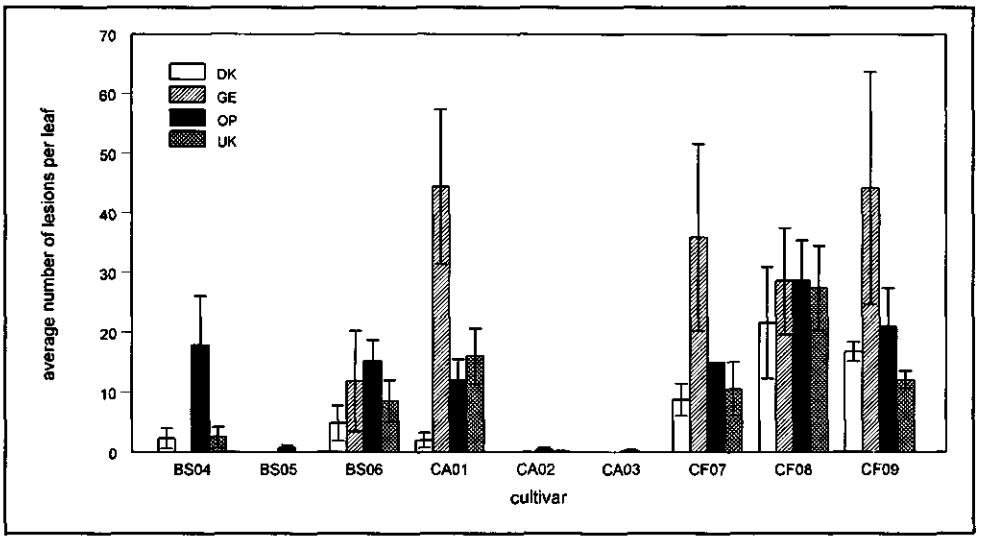


Figure 4.4 *Average number of lesions per leaf (SL) for four isolates of M. brassicicola (DK, GE, OP, UK) and 9 cultivars of Brussels sprouts (BS04, BS05, BS06), cabbage (CA01, CA02, CA03) and cauliflower (CF07, CF08, CF09). Per average number of lesions standard errors are indicated.*

Table 4.5 *Analysis of variance for the log transformed numbers of lesions per leaf (5 different leaf ages per cultivar) on nine Brassica cultivars caused by six isolates (NH, FR, DK, GE, OP, UK) of M. brassicicola.*

Source of variation	df[a]	MS[b]
Replication	3	0.83[ns]
Isolate	5	3.56[**]
Main plot error	15	0.23
Cultivar	8	14.19[**]
Isolate·cultivar	40	1.013[**]
Subplot error	143(1)	0.30
Leaf age	4	18.47[**]
Isolate·leaf age	20	0.22[**]
Cultivar·leaf age	32	1.57[**]
Isolate·cultivar·leaf age	156(4)	0.17[**]
Sub-sub plot error	527(121)	0.085
Total	953(126)	

df degrees of freedom
[a] (..) = number of missing values
MS Mean Squares
[b] ns *F*-value not significant
* *F*-value significant for $P<0.05$
** *F*-value significant for $P<0.01$

the FR isolate (Fig. 4.1 and 4.2), but disease severity on the cabbage cultivars of older plant stages did differ (Fig. 4.3).

In the same experiment four other isolates (DK, GE, OP, UK) of *M. brassicicola* showed differential responses on the brassica cultivars too (Fig. 4.4). The analysis of all the transformed data of this experiment resulted in a significant cultivar-isolate interaction effect. Main effect (isolate) and subplot effect (cultivar) were also significant (Table 4.4). Except for the FR isolate, CA02, CA03 and BS05 showed hypersensitive reactions against all isolates. The cultivars in this experiment were all susceptible for the FR isolate, although differences in disease severity exists. The two isolates from the Netherlands (NH and OP) and the French isolate (FR) gave high disease severity levels on BS04, which was not the case for isolates from Denmark (DE), Germany (GE) and the United Kingdom (UK).

Influence of plant stage

The influence of leaf age on a possible isolate-cultivar interaction could be determined using the data from the experiment with plants in the seventh leaf stage at the time of inoculation. Transformed data of the average numbers of lesions per leaf from leaves 4 to

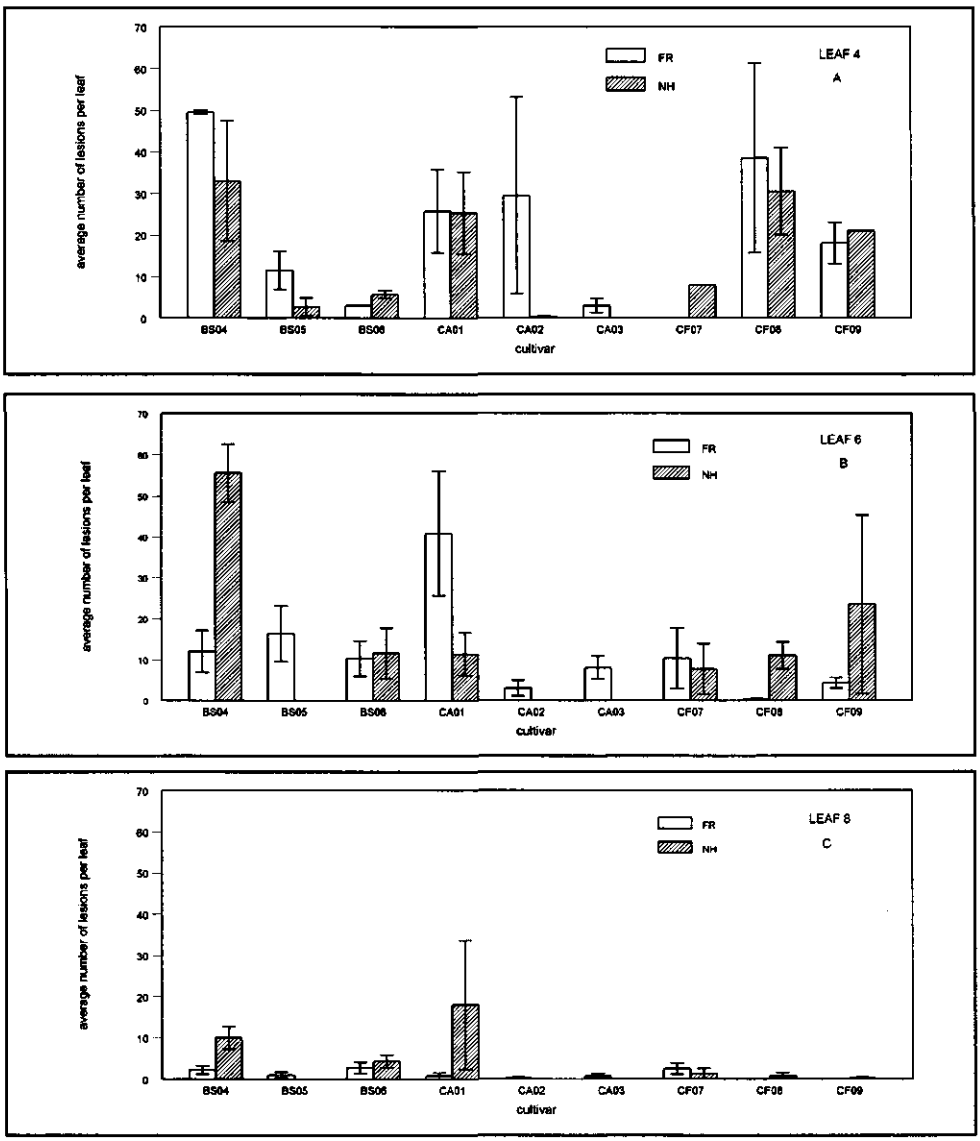


Figure 4.5 *Average number of lesions per leaf (SL) for two isolates of M. brassicicola (NH and FR) and 9 cultivars of Brussels sprouts (BS04, BS05, BS06), cabbage (CA01, CA02, CA03) and cauliflower (CF07, CF08, CF09) in relation to leaf number. Leaf age decreases with increasing leaf number. A = leaf number 4, B = leaf number 6, C = leaf number 8. Per average number of lesions standard errors are indicated.*

8 were subjected to a split-split-plot analysis of variance (Table 4.5). Main effects of isolate, cultivar and leaf age were present, and account for a high percentage of the total variance, as can be seen from the high magnitude of the respective mean sums of squares. Interaction effects of isolate-cultivar, isolate-age, age-cultivar and isolate-age-cultivar were significant.

To demonstrate the effect of leaf age, the average numbers of lesions per leaf for three leaf positions (4, 6, 8) are presented for two isolates (NH and FR) (Fig. 4.5). Resistance to the Dutch isolate (NH) in the cultivars CA02, CA03 and BS05 was found in all three leaf levels, although some disease development took place at the oldest leaf level (leaf 4). On the contrary, inoculation with the French isolate (FR) resulted in disease development in all three leaf levels of the same cultivars, although disease severity levels decreased with decreasing leaf age. On the oldest leaf level (leaf 4) CA03-FR showed a lower disease severity than CA02-FR, whereas in the younger leaf levels CA03-FR tended to a higher disease severity than CA02-FR.

4.4 Discussion

In brassica plants resistance against the NH isolate of *M. brassicicola* can either be a result of a hypersensitive reaction in the mesophyll of leaves (resistance type I), or a consequence of a difference between the number of succesful penetrations of leaves of different cultivars (resistance type II) (Van den Ende, 1992b, 1993a). Results in the present study show that differences in resistance levels exists between cultivars of *Brassica* to other isolates of *M. brassicicola*.

Specificity in pathosystems is often indicated by significant isolate-cultivar interactions in the analysis of variance of an experiment where a number of pathogen isolates are tested on a set of host genotypes in all possible combinations (Kulkarni and Chopra, 1982; Vanderplank, 1982, 1984). Many authors have indicated that an interpretation of the analysis of variance can be misleading, when no information about the genetic background is available (Parlevliet and Zadoks, 1977; Winer, 1984; Jenns and Leonard, 1985; Carson, 1987). For instance, interaction effects can be due to higher order interactions such as the cultivar-isolate-environment (Kulkarni and Chopra, 1982; Zadoks and Van Leur, 1983). Therefore, the presence of an isolate-cultivar interaction in the analysis of variance is only an indication for the presence of physiological races of *M. brassicicola*. Hypersensitive responses on certain cultivars inoculated with the Dutch isolate (NH), and the absence of such responses on the same cultivars when inoculated with the French isolate (FR) provide additional evidence that differential adaptation within the pathosystem *M. brassicicola-Brassica* spp. can occur.

Tests of plants in the third leaf stage revealed resistance type II. Some cultivars showed a lower number of lesions without the presence of a hypersensitivity reaction (BS01 and

CA04, Fig. 4.2). It is remarkable that resistance type II against the NH isolate seems to be effective against the FR isolate too (BS01 and CA04), whereas resistance type I (CA02, CA03, BS05) does not seem to be effective against the FR isolate (Fig. 4.2).

The interaction effects age-cultivar-isolate and age-cultivar in the analyses of variance show that leaf age plays an important role in the expression of resistance in brassica cultivars, and consequently can influence conclusions about physiological specialization in *M. brassicicola*. Both types of resistance are influenced by leaf age. Discoloration of the mesophyll as a result of the hypersensitive reaction (resistance type I) seems to occur only in leaves and not in cotyledons. When leaves start yellowing with increasing age, the hypersensitive response is partially lost and lesions start to grow on leaves of previously resistant cultivars. Especially in Brussels sprouts and cauliflower cultivars which show early senesence, inoculation of resistant cultivars in the seventh leaf stage with the isolate NH resulted in a number of lesions on the oldest leaf. Cotyledons of Brussels sprouts and cauliflower cultivars started to wither much faster than cotyledons of cabbage cultivars which can explain the development of lesions on cotyledons of cultivars with high levels of resistance to the NH isolate (BS05 and CF07). The effect of leaf age on resistance type II seems to be present too. In some cases, resistance to some isolates increases with plant development though no hypersensitive response is present. As previous studies showed, in CA02 both types of resistance are present (Van den Ende, 1992b, 1993a). The French isolate (FR) showed a higher number of lesions on cotyledons of CA02 then on CA03 (Fig. 4.1). As plants developed disease severity on CA03 decreased, while that on CA02 remained at the level of the most susceptible cultivar (CA01) (Fig. 4.2 and 4.3). The same is true with increasing leaf age (Fig. 4.5).

Resistance type II can be related to compounds in the wax layer of brassica leaves which restrain fungi from leaf penetration (Rawlinson *et al.*, 1978; Hartill and Sutton, 1980; Conn and Tewari, 1989; Bansal *et al.*, 1990). It is possible that the production of these specific compounds depends on leaf age. Further research should give more information about the resistance mechanism involved.

Main effects of isolates in the analysis of variance are difficult to interpret. Zornbach (1990) tested many isolates of *M. brassicicola* from different geographical regions on a single cabbage cultivar and stated that there were differences in aggressiveness between isolates from different parts of the world. As in the present study, he treated his plants with inoculum consisting of mycelial fragments. Standardization of this kind of inoculum is hard to accomplish, which can lead to high variability in the results. Differences in disease severity of plants after inoculation can therefore be a result of differences in inoculum concentration. However, in the present study, the difference between the isolates FR and NH is consistent for all the plant stages tested, although cotyledons did not show hypersensitive reactions when tested with the NH isolate. Because the other isolates are only tested in one experiment reliable conlusions based on differences in disease severity

of the cultivars tested cannot be drawn. The four other isolates (OP, GE, UK and DK) all resulted in hypersensitive reactions on CA02, CA03 and BS05, and were therefore easy to distinguish from the FR isolate. A remarkeble difference exist between the numbers of lesions on the Brussels sprouts cultivar BS04 when tested with the FR-, OP- or NH isolate compaired to testing with the GE-, UK- or DK isolate. More insight in specialization of these isolates might have been revealed in some of these isolates, if additional cultivars had been added to the differential host set.

M. brassicicola isolated from certain host varieties in the field are also pathogenic on other host varieties. Adaptation of an isolate to specific host varieties does not seem to occur. The isolate OP from cauliflower showed a similar disease spectrum on the differential set of host genotypes as the isolate NH from cabbage. *M. brassicicola* isolated from *B. oleracea* was also infective on different species of *Brassica*, as can be concluded from the symptoms on *B. campestris* and *B. napus*. Results of the present study confirm the findings of Zornbach (1990) in Germany. He demonstrated that isolates of *M. brassicicola* from oilseed rape were also pathogenic on cabbage, which explained the severe epidemics of ring spot in areas were both crops are grown next to each other.

The present study shows that physiological specialization of *M. brassicicola* is present in the isolates under study. Consequently, rapid adaptation of the pathogen to a particular host cultivar and loss of effective resistance is possible. Stability of resistance is assumed to be highest when many resistance genes and so pathogenicity genes are involved, and when recombination in the pathogen is strongly restricted (Parlevliet en Zadoks, 1977). Inbreeding of brassica cultivars tends to eliminate much of the genetic variability, as only major genes for resistance are being selected. Due to the dependence on its sexual stage recombination in the pathogen can occur. It is therefore to be expected that the incorporation of only major genes for resistance in cultivars will result in a rapid loss of effective resistance, due to a strong selection pressure for virulent races of the fungus.

Chapter 5

Comparison of inoculation methods with *Mycosphaerella brassicicola* on *Brassica* spp.: ascospores versus mycelial fragments[1]

[1]J.E van den Ende and H.D. Frinking, 1993. Netherlands Journal of Plant Pathology 99, Supplement 3: 69-81.

Abstract

Ascospores can be collected from dried brassica leaves from the previous season, carrying lesions of the fungus. Discharge of ascospores is stimulated by light and takes place within a broad temperature range (5-20 °C). A method is described to isolate single ascospores of the fungus, or to collect sufficient ascospores for small inoculation experiments. To screen large numbers of plants under controlled conditions mycelial fragments can be used as the inoculum. Using mycelial fragments has the disadvantage of a long (4-5 days) leaf wetness duration necessary for infection. Ascospores need a much shorter leaf wetness duration to infect the host plant. The results of this study indicate that the minimum humidity requirement for infection in the field is lower (< 2 days) than generally accepted, and that the temperature range for infection by ascospores is wide (at least 10-20 °C).

5.1 Introduction

Mycosphaerella brassicicola is the causal agent of ring spot in brassica crops. The fungus was first described by Chevallier (1826) (in Zornbach, 1990) who found the spermatial stage of the fungus on cabbage leaves. The spermatia have long been considered as the imperfect stage of the fungus, but Dring (1961) and Zornbach (1990) showed that the spermatia were not infectious. Today, there is still no evidence about the existence of an imperfect stage of *M. brassicicola*. For infection of brassica plants and for dispersal the fungus depends on the production of ascospores within pseudothecia.

Production of ascospores of *M. brassicicola* in vitro is poor and unreliable. Therefore, inoculation studies have to be carried out with ascospores collected from diseased brassica leaves or with mycelial fragments. Using mycelial fragments as an inoculum has a disadvantage since a long period of high humidity is necessary for infection. Data on the minimum requirements of temperature and humidity for infection of plants after inoculation with mycelial fragments are available, but these data are contradictory (Nelson and Pound, 1959; Weimer, 1926; Zornbach, 1990). Inoculation experiments with ascospores of *M. brassicicola* are rare because a good selective method for the collection of large amounts of ascospores from diseased plant material has not been developed yet. Therefore, reliable data about the influence of environmental factors on infection of plants after inoculation with ascospores of *M. brassicicola* are hardly available. Optimalization of the use of the two types of inoculum is necessary, in order to screen large amounts of plants for breeding purposes, and to gain more insight in the epidemiology of ring spot.

The objective of this study was twofold: (1) to develop a selective method for the collection of ascospores of *M. brassicicola* from infected leaves of brassica and (2) to study the influence of temperature and humidity on the infection of brassica plants after inoculation by ascospores or by mycelial fragments of *M. brassicicola*.

5.2 Methods

Spore studies

Collection method

A selective collection method of ascospores is based on the active dispersal of the fungus. *M. brassicicola* shows typical characteristics of a drought-enduring xerophyte (Ingold, 1971). Drying does not destroy the fungus, but results in inactivity. The fungus quickly recovers and liberates the ascospores when wetted. When the correct conditions for discharge are present the ascus absorbs water and elongates until its tip is pushed out of the ostiole. The spores are then ejected in succession through a pore in the tip of the ascus (Dring, 1961). Many other common pathogens of brassica do not have this active dispersal of spores, so that the mechanism can be used for the selective collection of ascospores from diseased cabbage leaves.

Dried cabbage leaves with lesions of ring spot covered with pseudothecia were soaked in demineralized water for 30 minutes. After soaking the leaf material, the presence of ripe ascospores was checked by dissecting a random sample of fruiting bodies from the lesions under a microscope. To minimize contaminations by yeasts the leaves with lesions were washed in 70% ethanol for 3 minutes, and rinsed in sterile demineralized water. The ethanol treatment did not affect the sporulation of *M. brassicicola*. The leaves with lesions were placed on wet filter paper in the lid of a plastic petri dish. The bottom of the petri dish was covered by a thin layer of wateragar. After sealing the petri dish with parafilm it was placed upside down. Under optimal conditions for sporulation, ascospores will be shot upward into the wateragar within 1 or 2 days. Spore counts can be made under a dissection microscope at 50x.

Spore discharge

To study the influence of temperature and light on spore discharge in vitro, 40 lesions of ring spot were placed in petri dishes as described above. Per temperature (5, 10, 15, 18 and 20 °C) four petri dishes with one lesion each were placed in the light (8 klux) and four were wrapped in dark plastic. Spores were counted daily in each petri dish. Every day, lesions were moistened again and fresh water agar plates were placed above the lesions. After four days the average daily number of ascospores per treatment was determined.

Statistical analyses

Analysis of the raw data indicated an increase of error with increasing average number of lesions. Therefore, disease severity means were subjected to analysis of variance after logarithmic transformation of the data ($\log_{10}$ (number of lesions + 1)) (Gomez and Gomez, 1984). Data were analyzed using the Statgraphics computer software package (Statgraphics,

release 4.0). The Statgraphics procedure for a factorial design was used. Differences between treatments were tested with LSD at 95% probability level.

Inoculation studies

Inoculum preparation

For the production of mycelial inoculum ascospores from dried diseased plant material were collected according to the described method (at 15 °C and continuous light). With a needle single ascospores were transferred from the water agar to V8 agar (Miller, 1955). Colonies were grown under alternating light, 12 h UV (380 nm) - 12 h dark. Four weeks prior to inoculation, small pieces of mycelium (1-2 mm^2) were transferred to fresh V8 medium to start new colonies. After four weeks, eight of these colonies (1.0-1.5 cm in diameter) were suspended in 50 ml of distilled water by use of a microblender. The number of units in the suspension, which can possibly act as infection units, was estimated by counting individual mycelial fragments with a haemocytometer.

The same dried plant material was used to collect large amounts of ascospores as described above, at 15 °C under continuous light (8 klux), but without water agar in the lid of the petri dish. After discharge spores sticking to the plastic lid were removed by pouring a 0.03% Triton X-100 solution in the lid, brushing its surface softly with a brush.

Host preparation

Plants of *Brassica oleracea* var. *capitata* cv Bartolo were grown in a potting mixture consisting of decomposed sphagnum peat to which some clay and marl were added (TRIO 17: pH 5.4; organic matter 74%). Plants were grown in plastic pots (10 x 10 x 12 cm) in the greenhouse at 17-20 °C in daylight, supplemented with artificial light (Philips, HPIT, 400 W). Starting when the first two leaves of the plants were visible, fertilizer was added weekly (Kristalon blue: 19% N, 6% P, 20% K, 3% Mg). Plants were inoculated in the third leaf stage (Van den Ende, 1992b).

Inoculation technique

Inoculum was applied by spraying the suspensions of either mycelial fragments (7.6 x 10^5 infection units per ml) or ascospores (3 x 10^3 ascospores per ml) with a DeVilbiss atomizer under constant air pressure. Approximately 2 ml inoculum was used per plant. To ensure leaf wetness after inoculation, plants were covered individually with plastic bags that had been sprayed lightly on the inside with water. Bags were secured around the pot with an elastic band. The period during which the plants were kept covered with the plastic bags after spraying with inoculum is hereafter referred to as the wetness period (WP). It was only during the first 8 days that environmental factors were critically controlled, a period hereafter referred to as the inoculation period. Post-penetration temperature and humidity do not affect the

amount of disease (Van den Ende, 1992b). Therefore, after 8 days plants were moved to a greenhouse with a relatively low air humidity (60-70%) and a temperature fluctuating between 17 and 25 °C. Plants were randomized over trays. Water was applied to the trays, so that leaves remained dry. As mature pseudothecia only develop on lesions when they are exposed to a saturated or near-saturated atmosphere for at least 4 days (Nelson and Pound, 1959), conditions in the greenhouse did not favour ascospore production. Therefore, dispersal of the disease after inoculation could not occur.

Treatments

To study the influence of temperature and of wetness duration in relation to the type of inoculum, plants were subjected to various treatments during the inoculation period. Following inoculation with ascospores or mycelium at day t = 0, plants were placed at random in growth chambers with low light intensity (1 klux) and constant temperature of 10 or 20 °C. Wetness periods varying from 0-8 days were terminated by removal of the plastic bags. Per treatment four plants were inoculated with an ascospore inoculum and four with a mycelial inoculum. To check the influence of the plastic bags on plant vigour, four plants per temperature were sprayed with demineralized water. These plants were not covered with plastic bags. After the inoculation period, all plants were transferred to the greenhouse. To determine the average number of lesions per plant, the number of lesions on the inoculated leaves (1-4) were counted at 22 and 38 days after inoculation.

Statistical analyses

Disease severity means were subjected to an analysis of variance after logarithmic transformation of the data ($\log_{10}$ (number of lesions + 1)). The Statgraphics procedure for a factorial design was used. Multiple regression models were developed for the effect of wetness duration (WP) and temperature (T) on the transformed disease severity levels. Models were developed by using the stepwise regression procedure (Sokal and Rohlf, 1981). The choice of the models was based on their biological meaning, the significance of the estimated parameters, the normality of residuals and the coefficients of determination (R^2).

5.3 Results

Spore discharge

Fig. 5.1 shows the daily average number of ascospores per lesion discharged in relation to light and temperature. Table 5.1 shows the result of the analysis of variance on the logarithmically transformed data. No interaction effect between temperature and light was detected. The discharge of ascospores in the light is significantly higher than in the dark (LSD, $P \leq 0.01$). Relatively high numbers of ascospores were found at temperatures of 10 and 15 °C, but differences between temperatures were not significant (LSD, $P \leq 0.05$).

Table 5.1 *Analysis of variance for the log_{10} transformed average numbers of ascospores of M. brassicicola in relation to light (L) and five temperatures (T) treatments.*

Source of variation	df	MS[a]
Replication	3	1.26^{ns}
Light (L)	1	20.82^{**}
Temperature (T)	4	1.37^{ns}
L · T	4	1.19^{ns}
Residual	27	1.12
Error	39	

df degrees of freedom
MS Mean Square
[a] ns = *F*-value is not significant
* = *F*-value significant for $P \leq 0.05$
** = *F*-value significant for $P \leq 0.01$

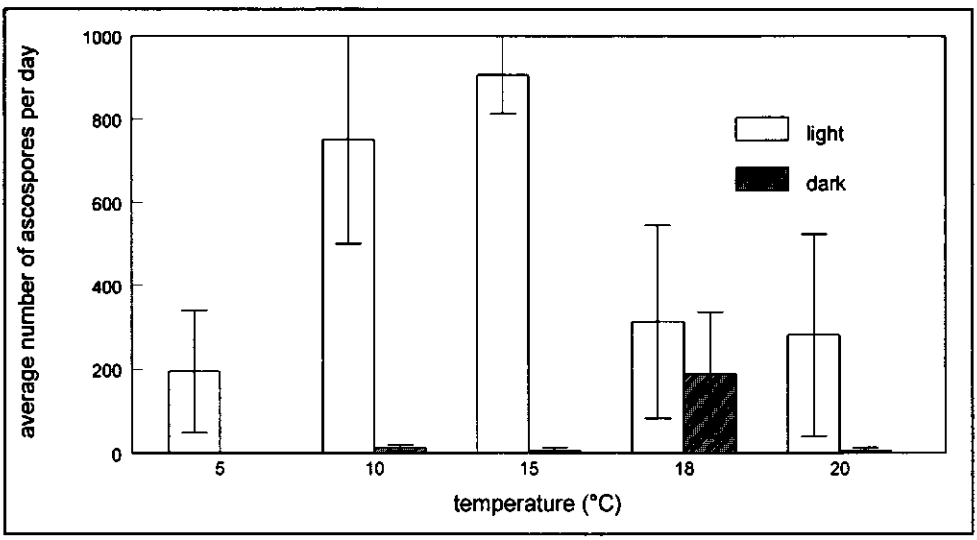


Figure 5.1 *Effects of temperature and light on spore discharge of M. brassicicola in vitro, expressed as the average number of ascospores per day per lesion. Error bars represent the SE of the mean.*

Table 5.2 *Analysis of variance for the log_{10} transformed average number of lesions per plant as a result of inoculation of Brassica plants with two inocula (I) of M. brassicicola at nine wetness periods (WP) and two temperatures (T). ANOVA's were carried out on data of 22 and 38 days after inoculation.*

Temperature	**22**		**38**	
Source of variation	df[a]	MS[b]	df[a]	MS[b]
Replication	3	0.01^{ns}	3	0.11^{ns}
Inoculum (I)	1	22.34^{**}	1	12.19^{**}
Wetness period (WP)	8	2.50^{**}	8	1.76^{**}
Temperature (T)	1	5.63^{**}	1	0.01^{ns}
I · WP	8	0.92^{**}	8	0.92^{**}
I · T	1	0.34^{**}	1	2.40^{**}
WP · T	8	0.40^{**}	8	0.186^{*}
Error	111(2)	0.04	108(5)	0.08
Total	141(2)		138(5)	

df degrees of freedom
[a] (..) = number of missing values
MS Mean Square
[b] ns = *F*-value is not significant
* = *F*-value significant for $P \leq 0.05$
** = *F*-value significant for $P \leq 0.01$

Inoculation studies

The results of the analyses of variance on the logarithmically transformed disease severity data of 22 days after inoculation showed the presence of significant interaction effects between the inoculation method (I), wetness period (WP) and temperature (T) (Table 5.2). Significant effects on the disease severity levels were present for inoculation method, wetness period and temperature. The same was true on 38 days after inoculation, except for the temperature effect.

Fig. 5.2 shows the average numbers of lesions per plant as a result of inoculation with mycelial fragments and ascospores, respectively. Control treatments showed no symptoms of ring spot. A temperature of 20 °C during a WP of at least 3-4 days resulted in low numbers of lesions 22 days after inoculation with mycelial fragments (Fig. 5.2-1A). Increase of the duration of the wetness period resulted in a slight increase of the number of lesions per plant. At 22 days, no lesions were found on plants which were kept at 10 °C during the wetness period. In contrast, the ascospore inoculum resulted in considerable numbers of lesions on plants 22 days after inoculation (Fig. 5.2-2A). At both temperatures lesions were found after a WP of 2 days. At both temperatures increase of the WP to 3 days resulted in

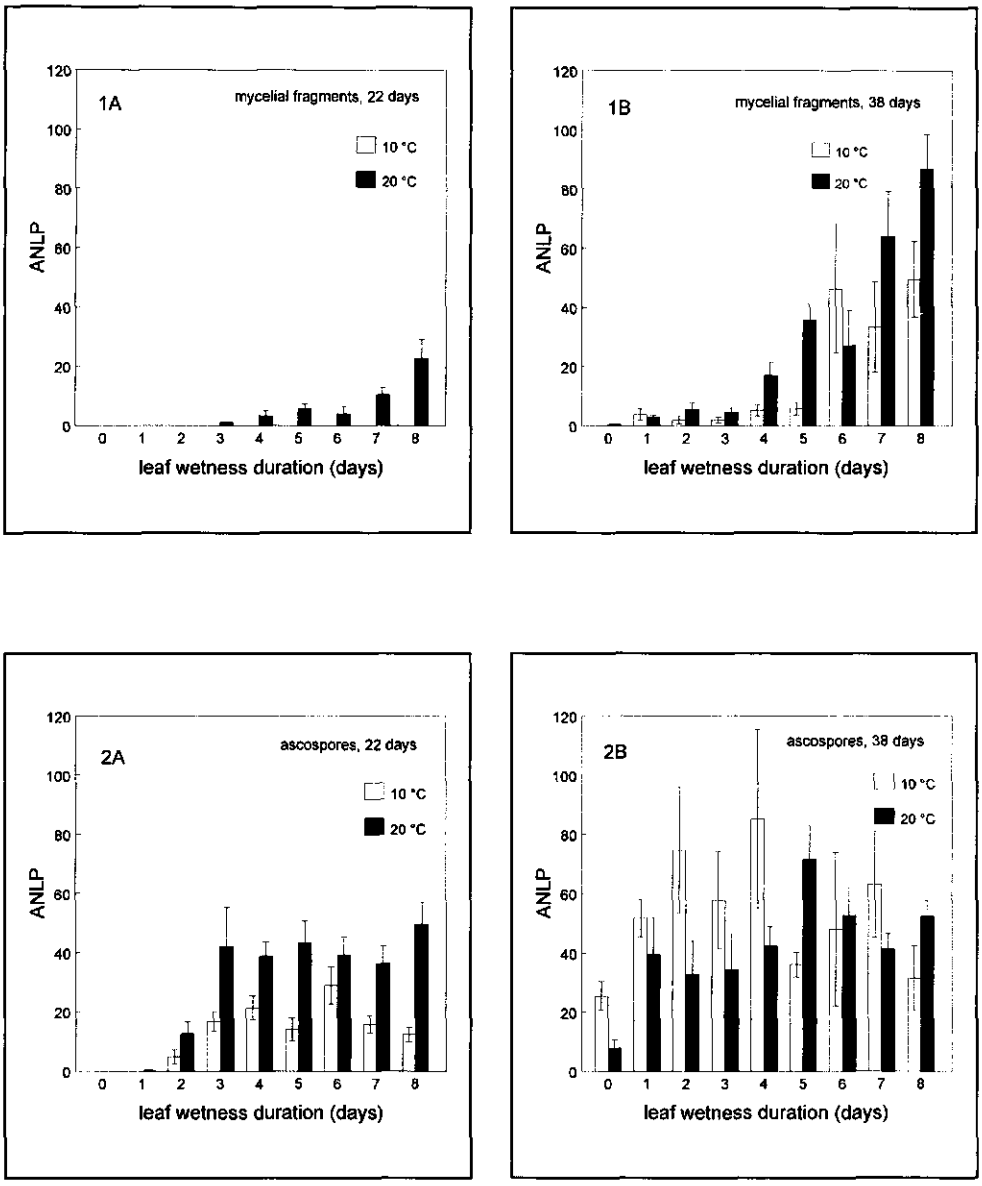


Figure 5.2 *Effects of temperature (10 and 20 °C) and wetness duration (0-8 days) on infection of Brassica plants by M. brassicicola.*
Average number of lesions per plant (ANLP) at 22 days (A) and 38 days (B) after inoculation of plants with mycelial fragments (1) and ascopores (2). Error bars represent the SE of the means.

an increase of the number of lesions per plant. Further increase of the wetness duration did not increase lesion numbers due to ascospores. A temperature of 20 °C during the wet period resulted in significantly more lesions per plant than that of 10 °C.

At 38 days after inoculation an increase in number of lesions was found for both methods of inoculation (Fig. 5.2-1B and -2B). Temperatures of 10 and 20 °C during a WP of 6 and 4 days, respectively, resulted in high numbers of lesions, compared to a WP of 0 days, 38 days after inoculation with mycelial fragments (Fig. 5.2-1B). Increase of the duration of the wetness period resulted in an increase of lesion numbers. On average, temperatures of 20 °C resulted in more lesions per plant than temperatures of 10 °C. At 38 days after inoculation by ascospores even plants with a WP of 0 days showed a high number of lesions for both temperature treatments (Fig. 5.2-2B). Wetness periods of 1 to 2 days seem to increased the disease severity, but longer wetness periods did not lead to a further increase in disease severity. A remarkable difference was found between the temperature treatments when plants were inoculated with ascospores. Hardly any increase was found between the number of lesions observed at 22 and 38 days after inoculation, when plants were held at 20 °C during a wet period of at least 3 days. However, the number of lesions on plants that were held at 10 °C during the wetness period increased between day 22 and day 38. At the end of the experiment, for relatively short wet periods (<5 days), plants held at 10 °C showed more lesions than plants at 20 °C.

Regression analyses

The combined effect of temperature (T) and leaf wetness duration (WP) on disease severity caused by mycelial infection was best described by the following equation:

$$y = b_0 + b_1 \cdot WP + b_2 \cdot T \qquad (1)$$

in which $y = \log_{10}(x + 1)$, x = average number of lesions per plant and b_0, b_1 and b_2 are the partial regression coefficients (Table 5.3). At 22 days after inoculation only the transformed data of the 20 °C treatment were fitted, resulting in a linear response to leaf wetness duration (Fig. 5.3-1A). At the final disease assessment, 38 days after inoculation, the transformed disease severity levels followed a linear response function to temperature and leaf wetness. Disease severity increased significantly at both temperatures with wetness duration. For each level of the wetness period an increase in temperature resulted in a significant increase in disease severity. No significant interaction effects were present (Fig. 5.3-1B). The residuals showed a random pattern and were normally distributed.

Table 5.3 *Estimated parameters of equations 1 and 2 for temperature (T) and wetness duration (WP) effects on infection of Brassica plants by M. brassicicola, together with the coefficients of determination (R^2) and standard deviations about the regression line (s)*

		Parameter estimates[a]						
Treatment[b]	**Day[c]**	b_0	b_1	b_2	b_3	b_4	R^2	s
mycelium	22	-0.167*	0.165**	-	-	-	0.71**	0.20
		(0.086)	(0.018)					
mycelium	38	-0.296*	0.201**	0.029**	-	-	0.73**	0.33
		(0.140)	(0.015)	(0.008)				
ascospores	22	-0.147*	0.481**	-	0.007**	-0.049**	0.86**	0.24
		(0.070)	(0.045)		(0.001)	(0.005)		
ascospores	38	1.267**	0.190**	-	-	-0.020**	0.24**	0.27
		(0.080)	(0.051)			(0.006)		

[a]Estimated parameters for Equation 2, 3 and 4 corresponding to intercepts, *WP*, *T*, *WP* · *T*, and WP^2, respectively. Numbers in parentheses under the parameters correspond to their standard deviations.
[b]Type of inoculum.
[c]Day of observation after inoculation.

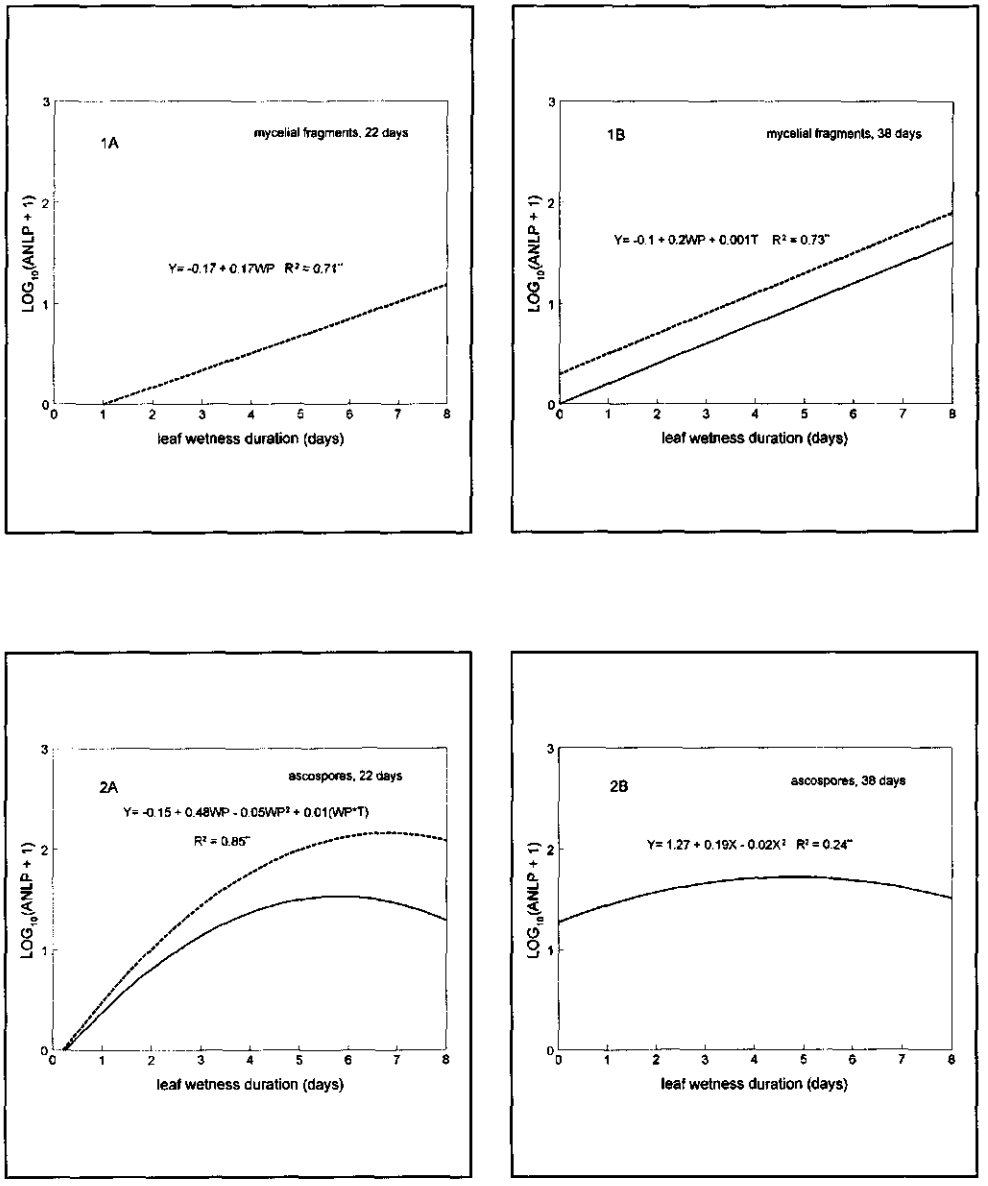


Figure 5.3 *Effect of temperature (T) on predicted infection of Brassica plants by M. brassicicola at wetness periods (WP) between 0 and 8 days, (A) 22 and (B) 38 days after inoculation with mycelial fragments (1) and ascospores (2). Curves were generated by using equation 1 and the parameters listed in Table 5.3. Solid and dotted lines represent the predicted logarithmically transformed average number of lesions per plant (log_{10} (ANLP + 1)) at 10 and 20 °C, respectively.*

The combined effect of temperature (T) and wetness duration (WP) on disease severity caused by ascospore infection was best described by the following equation:

$$y = b_0 + b_1 \cdot WP + b_3 \cdot WP^2 + b_4 \cdot (WP \cdot T) \qquad (2)$$

in which $y = \log_{10}(x + 1)$, x = average number of lesions per plant and b_0, b_1, b_3 and b_4 are the partial regression coefficients (Table 5.3). Transformed data from 22 days after inoculation showed a quadratic response function for wetness duration. A significant interaction between temperature and wetness duration was present resulting in maximum values for disease severity at a WP=6 at 10 °C and WP=7 at 20 °C (Fig. 5.3-2A). Transformed data from the final disease assessment at 38 days after inoculation showed also a quadratic response on wetness duration, though R^2 was low (Table 5.3). At 38 days, temperature did not affect disease severity levels significantly. The quadratic response was flat, since even at low levels of WP high disease severity levels were observed at both temperatures (Fig. 5.3-2B). The residuals had a random pattern and were normally distributed.

5.4 Discussion

The described method for the selective collection of ascospores of *M. brassicicola* from dried diseased leaves of brassica can be used to overcome the lack of ascospore production in vitro. As the collection of ascospores is labour intensive, the method is only suitable for small inoculation experiments. The method described is a modification of that by Hartill and Sutton (1980), which was not suitable for inoculation experiments due to lack of selectivity. A disadvantage of using dried leaf material is that dried lesions of *M. brassicicola* loose their ability to sporulate with time. The number of spores collected from lesions decreased after storage of approximately one year at room temperature. Hartill and Sutton (1980) also found that in case of prolonged storage leaves provided insufficient numbers of ascospores for large experiments.

The results of this study indicate that the fungus can sporulate in the light within a broad temperature range. Weimer (1926) and Quak (1957) showed that the fungus can even sporulate between 0 and 5 °C but that sporulation above 20 °C hardly occurs. Although no significant differences between temperatures were found, sporulation seems to proceed best at temperatures between 10-18 °C, conform Frinking and Geerds (1987). Results can be influenced by a slight increase of temperature within the petri dishes as a result of the heating capacity of the light source. Preliminary experiments showed that within the petri dishes an increase by 1-2 °C within the petri dishes could occur. Stimulation of the sporulation process by light was also found in field studies (Hartill, 1977; Humpherson-Jones and O'Brien, 1986). Hartill showed that ascospores were predominantly trapped during daytime when diseased cabbage leaves were wet. Few ascospores were trapped at night, and only when rain had

fallen earlier in the day.

It can be concluded that the collection of ascospores according to the described method will proceed best when petri dishes are placed at temperatures between 10 and 18 °C in light (8 klux). To ensure the collection of large amounts of spores it is advisable to use diseased leaves fresh from the field.

For many years it has been assumed that ring spot of cabbage only occurs under conditions of high humidity during a long period. Weimer (1926) concluded from his experiments that plants were infected by ring spot only under conditions of at least 3 days of high relative humidity (RH > 90 %) at temperatures between 10 and 18 °C. Nelson and Pound (1959) stated that infection would not occur unless inoculated plants were kept in a near-saturated atmosphere for about one week at temperatures between 12 and 20 °C. More recently Dixon (1981) and Frinking and Geerds (1987) assumed that the minumum period of high humidity for infection had to be 3 to 4 days. The results of inoculation experiments of Zornbach (1990) confirmed this assumption. All the research mentioned was based on inoculation experiments with mycelial fragments. Present results show that the minimum humidity requirements for infection are totally different when plants are inoculated with ascospores instead of mycelial fragments. Final disease severity data showed that inoculation with mycelial fragments is only succesful if a long period (4 to 5 days) of leaf wetness is present, conform the literature. The relatively long period of high humidity which Nelson and Pound (1959) found to be neccassary for infection at 12 to 20 °C is caused by the fact that final disease assessment in their experiments took place 22 days after inoculation. Present results show that due to a long incubation period after inoculation with mycelial fragments only a few lesions are found 22 days after inoculation, mainly at WP=7 and 8, conform Nelson and Pound. Disease assessment at 38 days after inoculation would have led them to a different conclusion.

A temperature of 20°C during the inoculation period resulted in higher disease severity levels and a shorter incubation period than a temperature of 10 °C in case of inoculation of plants with mycelial fragments. This result was also found by Nelson and Pound (1959), and is probably related to the temperature response of mycelial growth (Zornbach, 1990). The wetness duration also affects the duration of the incubation period and the disease severity level. With increasing wetness duration, the duration of the incubation period will decrease and disease severity will increase, possibly due to a positive effect on mycelial growth and hence infection of the plant (Nelson and Pound, 1959).

Final disease severity data of inoculation experiments with ascopores showed that infection of brassica plants could occur even at leaf wetness periods of less than one day. When disease assessment took place 22 days after inoculation with ascospores, the 20 °C treatment resulted in more lesions per plant than the 10 °C treatment. This is probably caused by a shorter incubation period at 20 °C, as was also shown in other experiments (Van den Ende *et al.*, 1998). The temperature effect on total disease severity after ascospore infection is not as clear as with mycelial infection, as disease assessment at 38 days after inoculation showed

no significant difference between temperatures. For short wetness periods, disease severity levels for the treatment at 10 °C seem to be even higher than for 20 °C. It is possible that when wetness periods are suboptimal for infection, conditions after the wetness period will affect the final disease severity. At a high temperature (20 °C) relatively more transpiration will occur, which could result in the death of germinated ascospores. At a lower temperature (10 °C) these germinated ascospores might still have a fair chance to penetrate the host, even though the wetness period had ended.

Regression analysis on the transformed disease severity data of the mycelial infection showed a linear response to leaf wetness duration. Regression analysis on the transformed disease severity data of the ascospore infection showed a quadratic response. The difference is apparently due to the characteristics of the inocula under study. Mycelial fragments form new epiphytically growing colonies on the leaf. Mycelial growth of *M. brassicicola* can be characterized by strong branching of the hyphae (Dring, 1961). The longer the wetness duration, the more branches are formed, and the more infection can occur, resulting in an exponential increase of disease severity. Ascospores form thin germination tubes, which branch rarely. As the wetness period increases, the growth of the germination tubes will decrease by exhaustion, and disease severity will approach a maximum value. The decrease of disease severity with long wetness periods is rather unexpected. It is possible that long wetness periods stimulate the growth of epiphytic organisms (e.g. bacteria and yeast) with a negative effect on the thin germination tubes. The colonies of *M. brassicicola* are less liable to suffer from negative effects of epiphytically growing organisms, as the mycelium consists of thick walled hyphae.

Results found in this study are important to understand the epidemiology of ring spot under field conditions. Using field data, Wicks and Vogelzang (1988) already suspected that infection by *M. brassicicola* on a susceptible cultivar could occur within 24 hours of high humidity. Hartill and Sutton (1980) proved that germination of ascospores occurred within 24 hours after inoculation, as we did. Staunton and Ryan (1977, 1983) mentioned results of experiments, which proved that the minimum period of high humidity for infection is much shorter than assumed by most researchers. Unfortunately, their experimental data are not available. Following Wicks and Vogelzang (1988), Hartill and Sutton (1980) and the results of the present study, it is suggested that the minimum humidity requirements for infection of ring spot on *Brassica* plants are much lower than is generally assumed. A warning systems which is presently used in the Netherlands is based on the assumption that at least 2 to 3 days of high humidity are necessary for infection by *M. brassicicola* in cabbage and Brussels sprouts. No inoculation studies with ascospores were undertaken to support this assumption. The present study clearly demonstrates that experiments should be undertaken to determine the exact requirements for infection by ascospores, to validate the use of the warning system.

Chapter 6

Effects of temperature, leaf wetness and humidity on infection of white cabbage by ascospores of *Mycosphaerella brassicicola*[1].

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Abstract

Data from infection studies on white cabbage with *M. brassicicola* ascospores from diseased leaf material showed great variability. Despite the variability of the data some general conclusions could be drawn. Ascospores are able to infect cabbage within 24 h of leaf wetness at temperatures from 5-20 °C. Lesion number and lesion size increases with increasing wetness period at all temperatures tested. Decreasing temperature during leaf wetness resulted in higher lesion numbers but reduced lesion size. Interruption of leaf wetness by dry periods did not necessarily lead to reduction of infection, it even could stimulate infection. Infection also occurred within 24 h of high relative humidity (90%) at 15 °C. Lesion number increases with increasing periods of high relative humidity. Interruption of periods of relative high humidity by dry periods (RH 60%) reduced infection. The complexity of the effects suggests that it will be difficult to find simple guidelines for infection conditions to apply in the field.

6.1 Introduction

Ring spot of brassica crops is caused by *Mycosphaerella brassicicola* (Duby) Lindau. Symptoms of the disease can appear on all aerial plant organs, but usually mature foliage is most heavily affected leading to early senescence and defoliation. In the Netherlands, severe damage is mostly found at the end of summer and during autumn when crops are fully grown and climatic conditions favour disease development (Frinking and Geerds, 1987; Van den Ende *et al.*, 1986). Severe infection of crops can cause serious economic losses as a result of both qualitative and quantative damages at harvest time (Everaarts and De Moel, 1991). Dutch growers use benzimidazoles (benomyl, carbendazim) and, more recently, pyrifenox to control the disease. As knowledge about the epidemiology of ring spot was lacking, very often pesticide applications were often ill timed and disease control poor. In 1991 general guidelines were introduced to optimize disease control, based on minimum humidity requirements for infection of Brussels sprouts and white cabbage by ring spot (De Moel *et al.*, 1991; Van Oeveren, 1991). Growers are advised to spray whenever an infection period is registered, but only if a previous spray has become ineffective.

Infection of white cabbage plants by *M. brassicicola* is said to occur only after two consecutive days with at least 18 h of high humidity (RH>90%) or leaf wetness. The assumption is based on literature about inoculation experiments carried out with mycelial fragments of *M. brassicicola*. However, the actual infection units, the ascospores, react differently. Van den Ende and Frinking (1993) showed that under controlled conditions ascospores are able to infect a host plant within 24 hours of leaf wetness. If the indoor result were also true in the field, the basic assumption of the guidelines would be incorrect.

Studies on ascospores of *M. brassicicola* are rare. Few data are available about the influence of environmental conditions on infection of brassica plants by ascospores. In preliminary experiments the effect of continuous leaf wetness on infection of white cabbage plants was studied (Van den Ende and Frinking, 1993), but only at two temperatures (10 and 20 °C). The effect of an interruption of the leaf wetness period on infection is not known. Reliable data on the influence of periods of high relative humidity on infection of cabbage plants by ascospores of *M. brassicicola* are not available. Götz *et al.* (1993) reported that *in vitro* produced ascospores needed at least a 7 days period of high humidity at 17 °C to penetrate white cabbage leaves, but she did not describe humidity conditions clearly.

We studied the effect of selected environmental factors on infection of white cabbage plants by ascospores of *M. brassicicola* under controlled conditions. Identification of optimum conditons for infection should help to understand the development of epidemics in the field, assist to formulate disease predicition models, and contribute to improve disease management.

6.2 Materials and methods

Host material

Plants of *Brassica oleracea* var. *capitata* cv Bartolo were grown in a potting mixture consisting of decomposed sphagnum peat to which some clay and marl were added (TRIO 17: pH 5.4; organic matter 74%). Plants were grown in polyethylene pots (10 x 10 x 12 cm) in a greenhouse at 17-20 °C in daylight, supplemented with artificial light (Philips, HPIT, 400 W). When the first leaf pair became visible, fertilizer was added weekly (Kristalon blue: 19% N, 6% P, 20% K, 3% Mg). Plants were inoculated in the fifth leaf stage (Van den Ende, 1992b).

Inoculum

Dried diseased cabbage leaves from a previous season were used to collect ascospores. Diseased leaves were soaked in water and placed in Petri dishes on wet filter paper. Under light (8 klux) and at temperatures of 10 to 18 °C the sporulation process begins within 1 or 2 days. Ejected ascospores stick to the lid of the Petri dish (Van den Ende and Frinking, 1993b). To obtain ascospores of the same age, the lids of dishes were replaced 12 h prior to inoculation of plants. Ascospores were collected by pouring a 0.03% Triton X-100 solution (demineralised water) in the lid, while brushing the surface lightly with a paint brush. The 0.03 % Triton X-100 did not affect viability or germination of the ascospores. Ascospore densities were determined by haemocytometer counts, and inoculations made with suspensions adjusted to ± 5.0 - 10^4 ascospores per ml.

Inoculation

Inoculum was applied by spraying the ascospore suspension with a DeVilbiss atomizer under constant air pressure. Approximately 2 ml inoculum was used per plant. In preliminary experiments spore viability and spore germination percentages had been assessed under a microscope, using collodion strips of inoculated leaves.

Treatments

In several experiments the effects of temperature, relative humidity and leaf wetness on infection of plants by ascospores were studied. It was only during the first 6 days following inoculation, unless otherwise indicated, that environmental factors were controlled, a period hereafter referred to as the inoculation period (IP).

Post-penetration temperature and humidity do not affect the amount of disease (Van den Ende, 1992b). Therefore, after termination of IP, plants were moved to a greenhouse with a relatively low air humidity (60-70%) and a temperature fluctuating from 17 to 25 °C. Plants were placed in trays at random. Water was applied to the trays, so that leaves remained dry. As mature pseudothecia only develop on lesions exposed to a (near)-saturated atmosphere for at least 4 days (Nelson and Pound, 1959), conditions in the greenhouse did not favour ascospore production. Thus, spread of the disease after inoculation was prevented.

Experiment 1 Effect of leaf wetness period and temperature. After inoculation plants were placed on a wet cloth in plastic cages (180 x 70 x 30 cm) and subjected to variable (0 to 6 days) leaf wetness periods (WP). Leaf wetness could be maintained by spraying the plants each day with demineralized water. Plastic cages were placed in four controlled environment chambers with constant temperatures (5, 10, 15 and 20 °C) and constant light (1 klux, Philips TL-F, 40 W). Per treatment 3 plants were inoculated. The experiment was replicated twice (I = $5.0 \cdot 10^4$ spores ml^{-1}, II = $4.9 \cdot 10^4$ spores per ml^{-1}) at each temperature, control plants were sprayed with demineralised water and subjected to a 6 days leaf wetness period. Lesions of leaves 3 to 5 were counted 30 days after inoculation and the average number of lesions per leaf was determined. On day 31 after inoculation, lesion diameters of approximately 50 lesions per replication were measured and averaged.

Experiment 2 Effect of interrupted leaf wetness period. After inoculation plants were placed in plastic cages (similar to experiment 1) and subjected to various dry periods: 0, 6, 12, 18, 21 and 24 h per day (DP) during each of 2, 4 or 6 days of leaf wetness (WP). The experiment was conducted at a temperature of 15 °C. During the dry periods, plants were exposed to ambient air (RH 60%) at 15 °C in the controlled environment chamber. No visible signs of leaf wetness could be detected after 20-30 min. Per treatment 3 plants were inoculated. The experiment was replicated three times (I = $5.1 \cdot 10^4$ spores ml^{-1}, II =

5.0 · 10^4 spores ml^{-1}, III = 4.7 · 10^4 spores ml^{-1}). Control plants were sprayed with demineralised water and subjected to 24 h leaf wetness during 6 days. Lesions of leaves 3-4 were counted 27 days after inoculation, and the average number of lesions per leaf was determined.

Experiment 3 Effect of interrupted relative humidity period. After inoculation, plants were placed in controlled environment cabinets (Weiss) and subjected to various dry periods (DP) : 0, 6, 12, 18, 21 and 24 h per day during each of 2, 4 or 6 days of 90% relative humidity (RHP). The experiment was conducted at 15 °C. During the dry periods (DP), plants were exposed to ambient air (RH 60%) at 15 °C in a controlled environment chamber. Per treatment two plants were inoculated, using 1 ml of ascospore suspension. The experiment was replicated twice (I and II = 10.0 · 10^4 ml^{-1}). Control plants were sprayed with demineralized water, and subjected to 24 h with 90% relative humidity during 6 days. Lesions of leaves 3 to 5 were counted 28 days after inoculation, and the average number of lesions per leaf was determined.

Experiment 4 Effect of interrupted leaf wetness period and relative humidity period. After inoculation plants were placed in plastic cages and subjected to a leaf wetness period of 1 day at a temperature of 17 °C. After that day plants were transferred to a controlled environment cabinet with a temperature of 17 °C and two relative humidities (70 and 90 %). Wetness on the leaves dried within 2 and 4 h at 70% RH and 90% RH, respectively. After 1 day plants were sprayed with demineralized water, and returned to the plastic cages in the climate chambers. After day 3 the IP ended and plants were transferred to the greenhouse. Per treatment 1 plant was inoculated. The experiment was replicated three times (I = 5.0 · 10^4 spores ml^{-1}, II = 4.2 · 10^4 spores ml^{-1}, III = 2.4 · 10^4 spores ml^{-1}). Control plants were inoculated with ascospores and subjected to a leaf wetness period of 3 days at a temperature of 17 °C. Lesions of leaves 3 to 5 were counted at 26 days after inoculation, and the average number of lesions per leaf was determined.

Statistical analyses

The average numbers of lesions per leaf were subjected to an analysis of variance after logarithmic transformation of the data. The Statgraphics procedure for a factorial design was used (Statgraphics, release 4.0). Differences among treatment means were detected using Duncan's multiple range test.

Regression models were developed by using the stepwise regression procedure (Sokal and Rohlf, 1981). The choice of the models was based on their biological meaning, the significance of the estimated parameters, the normality of residuals and the coefficients of determination (R^2).

6.3 Results

Experiment 1. Effect of leaf wetness period and temperature.

Control plants did not show symptoms of *M. brassicicola*. Temperature and wetness period had significant effects ($P \leq 0.01$) on the log transformed number of lesions per leaf (Table 6.1 and 6.2). No interaction effect between temperature and wetness period could be detected. In general, the number of lesions increased with increasing leaf wetness period. Even at relatively short wetness periods (< 2 days) a considerable number of lesions appeared on inoculated plants. When inoculation was followed by a wetness period at 5 °C more lesions appeared than at 20 °C. A regression model using the log transformed average lesion number was chosen to model the effects of temperature (T) and leaf wetness (WP):

$$y = b_0 + b_1 \cdot WP - b_2 \cdot WP^2 - b_3 \cdot T^2 \qquad (1)$$

in which $y = \log_{10}(x + 0.01)$, x = average number of lesions per leaf, and b_0, b_1, b_2 and b_3 are partial regression coefficients, all significant at $P \leq 0.01$ (Table 6.3). The estimated parameters of equation 1 were used to calculate y for the temperature/wetness period combinations. Fig. 6.1 shows the back-transformed average number of lesions per leaf in relation to temperature and leaf wetness period. An increase in lesion number was seen at all temperatures with increasing wetness duration. An increase in temperature during the wetness period resulted in a decrease of the average number of lesions per leaf.

Temperature and wetness period had a significant effect ($P \leq 0.01$) on the average lesion diameter (Table 6.1 and 6.2). No interaction between temperature and wetness duration was present. Short wetness periods (< 2 days) resulted in smaller lesions than longer wetness period. Low temperatures (5 °C) after inoculation resulted in smaller lesions than high temperatures (20 °C). The following regression model was chosen to model the effects of temperature (T) and leaf wetness (WP) on the average lesion diameter:

$$y = b_0 + b_1 \cdot WP - b_2 \cdot WP^2 + b_3 \cdot T \qquad (2)$$

in which y = average number of lesions per leaf, and b_0, b_1, b_2 and b_3 are partial regression coefficients, all significant at $P \leq 0.02$ (Table 6.3). Fig. 6.2 shows the average lesion diameter in relation to temperature and wetness as calculated by using the parameters estimated by equation 2. All curves show a clear increase in average lesion diameter with increasing wetness periods within the range of 0-3 days. Long wetness periods (> 3 days) have no strong effect on lesion size. Lesion size increases with increasing temperature during the wetness period.

Table 6.1 *The influence of leaf wetness (WP) and temperature (T) on the number of lesions per leaf and the lesion diameter of Mycosphaerella brassicicola on white cabbage.*

Lesion number	Temperature (°C)							
	5		10		15		20	
	AVG[a]	SE[b]	AVG[a]	SE[b]	AVG[a]	SE[b]	AVG[a]	SE[b]
WP (d)								
0	3.6	(0.57)	0.4	(0.28)	0.7	(-)	0.4	(0.17)
1	7.7	(0.35)	3.4	(1.06)	1.3	(0.25)	1.2	(0.06)
2	13.3	(5.53)	5.0	(2.44)	9.4	(2.23)	0.8	(0.25)
3	15.1	(6.78)	5.3	(2.00)	9.0	(0.67)	2.6	(2.22)
4	14.2	(7.83)	7.4	(0.75)	16.2	(3.81)	3.1	(1.61)
5	12.1	(2.32)	12.0	(5.96)	17.3	(8.66)	2.3	(0.77)
6	17.3	(0.19)	21.6	(9.15)	8.3	(1.08)	6.8	(5.29)
c	0.0		0.0		0.0		0.0	

[a] Average number of lesions per leaf of two replications (2 x 9 = 18 leaves).
[b] Standard error of the mean

Lesion diameter	Temperature (°C)							
	5		10		15		20	
	AVG[c]	SE[d]	AVG[c]	SE[d]	AVG[c]	SE[d]	AVG[c]	SE[d]
WP (d)								
0	5.4	(0.46)	6.8	(0.21)	5.9	(-)	8.9	(2.91)
1	5.6	(0.29)	5.5	(0.62)	6.7	(-)	6.6	(0.69)
2	6.8	(0.10)	8.5	(0.90)	10.9	(2.05)	11.6	(-)
3	7.3	(0.73)	9.7	(0.68)	13.3	(1.12)	13.8	(1.22)
4	6.4	(-)	9.1	(1.05)	11.1	(2.34)	11.6	(1.61)
5	7.4	(0.97)	9.5	(1.70)	11.3	(0.41)	10.8	(1.62)
6	6.7	(0.50)	8.7	(2.73)	11.9	(0.25)	13.8	(0.97)

Table 6.2. *Analysis of variance for the log_{10} transformed average number of lesions (ANLL) and the average lesion diameter (ALD) of Table 6.1.*

Source of variation	log_{10}(ANLL+0.01)		ALD	
	df[a]	MS[b]	df[a]	MS[b]
Replication	1	0.27^{ns}	1	2.20^{ns}
Wetness period (WP)	6	1.09^{*}	6	23.13^{*}
Temperature (T)	3	1.83^{*}	3	51.65^{*}
WP · T	18	0.11^{ns}	18	2.23^{ns}
Error	26(1)	0.09	24(3)	3.49
Total	54(1)		52(3)	

[a] df degrees of freedom, (..) = number of missing values
[b] MS Mean square, ns = *F*-value not significant, * = *F*-value significant at $P \leq 0.01$
ANLL average number of lesions per leaf
ALD average lesion diameter

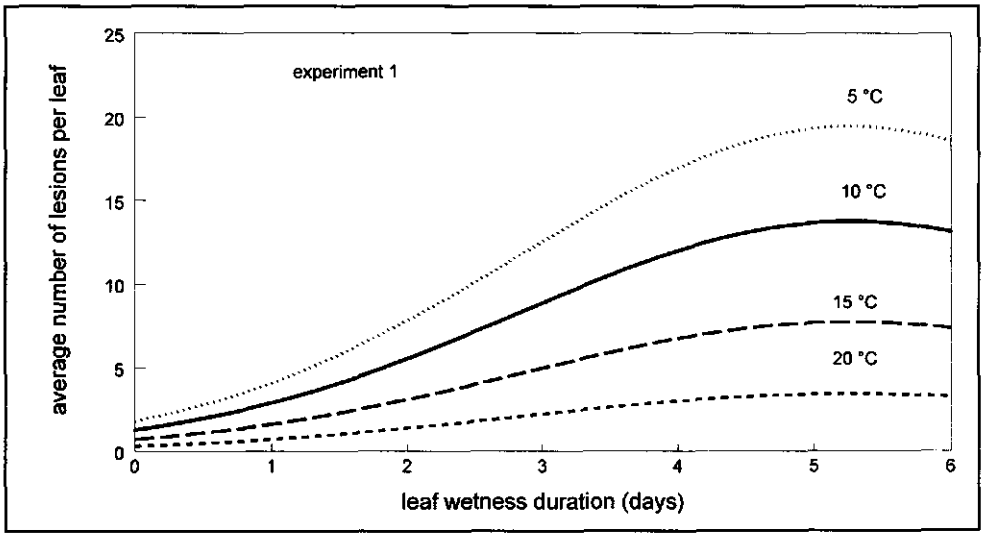

Figure 6.1 *Experiment 1. Effect of temperature on infection of white cabbage by Mycosphaerella brassicicola at wetness periods from 0 tc 6 days after inoculation with ascospores. Curves were generated by using equation 1 and the parameters listed in Table 6.3. Curves represent the back-transformed average numbers of lesions per leaf at 4 temperatures.*

Table 6.3 *Parameter estimates of equations 1, 2 and 3 for effects of environmental conditions on infection of white cabbage by ascospores of Mycosphaerella brassicicola, coeficients of determination (R^2) and standard error about the regression lines (SE). Standard errors of parameters are between brackets.*

Equation 1: $y = b_0 + b_1 \cdot WP - b_2 \cdot WP^2 - b_3 \cdot T^2$
Effect of leaf wetness (WP) and temperature (T) on the $\log_{10}$ transformed average number of lesions per plant.

Equation 2: $y = b_0 + b_1 \cdot WP - b_2 \cdot WP^2 + b_3 \cdot T$
Effect of leaf wetness (WP) and temperature (T) on the average lesion diameter.

Equation 3: $y = b_0 + b_1 \cdot DP - b_2 \cdot DP^2$
Effect of drying (DP) during wetness periods

Equation 4: $y = b_0 + b_1 \cdot DP + b_2 \cdot RHP^2$
Effect of drying (DP) during periods of 90% relative humidity

	Parameter estimates					
	b_0	b_1	b_2	b_3	R^2	SE
exp. 1 $\log_{10}$(ANLL+0.01)	0.312 (0.124)	0.392 (0.084)	0.037 (0.013)	0.002 (0.0003)	0.64	0.24
exp. 1 ALD	2.425 (0.834)	1.676 (0.473)	0.178 (0.075)	0.311 (0.046)	0.58	1.89
exp. 2 $\log_{10}$(ANLL)	0.432 (0.062)	0.066 (0.014)	0.002 (0.0006)		0.38	0.22
exp. 3 $\log_{10}$(ANLL)	0.145 (0.1000)	0.027 (0.005)	0.008 (0.003)		0.33	0.32

ANLL average number of lesions per leaf
ALD average lesion diameter

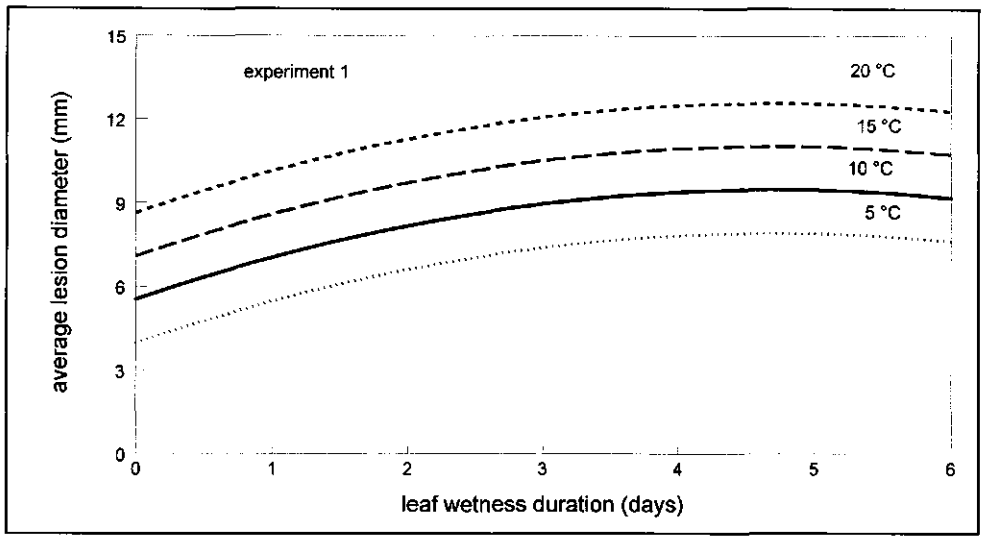

Figure 6.2 *Experiment 1. Effect of temperature on average lesion diameter (mm) of Mycosphaerella brassicicola on white cabbage at wetness periods from 0 to 6 days after inoculation with ascospores. Curves were generated by using equation 2 and the parameters listed in Table 6.3. Curves represent the average lesion diameter at 4 temperatures.*

Experiment 2 Effect of interrupted leaf wetness period.

Control plants did not show symptoms of *M. brassicicola*. Interruption of the wetness period had a significant effect ($P < 0.01$) on the log-transformed number of lesions per leaf (Tables 6.4 and 6.5). Duration of the wetness treatment in days (WP) had no significant effect on the transformed lesion number. No interaction could be detected between the duration of wetness in days (WP) and the duration of the daily dry periods (DP). A regression model based on the log-transformed average lesion numbers was chosen to model the influence of interruption of the leaf wetness period:

$$y = b_0 + b_1 \cdot DP - b_2 \cdot DP^2 \qquad (3)$$

in which $y = \log_{10}(x)$, x = average number of lesions per leaf, and b_0, b_1, and b_2 are the partial regression coefficients, all significant at $P \leq 0.01$ (Table 6.3). Fig. 6.3 shows the back-transformed average number of lesions per leaf in relation to the duration of dry periods. The response function shows a maximum number of lesions per leaf at a dry period of 6 hours per day (18 h of leaf wetness). Even on plants that were not wetted after inoculation (DP = 24 h) lesions appeared. Continuous wetness resulted in a lower number of lesions per leaf than a leaf wetness duration of 18 h per day.

Table 6.4 *The influence of the duration of dry periods (DP) during wetness on the average number of lesions per leaf after inoculation with ascospores of Mycosphaerella brassicicola on white cabbage.*

	WP (days)					
	2		4		6	
DP (h)	Mean[a]	SE[b]	Mean[a]	SE[b]	Mean[a]	SE[b]
24	3.1	(0.80)	2.0	(0.69)	2.3	(0.68)
21	4.3	(1.20)	8.3	(0.88)	3.3	(0.92)
18	7.1	(2.19)	8.7	(1.67)	6.2	(1.58)
12	5.1	(0.73)	11.1	(2.39)	9.3	(3.33)
6	5.2	(0.47)	9.9	(1.48)	8.3	(3.43)
0	7.8	(2.69)	6.9	(0.64)	8.6	(4.58)

[a] Mean number of lesions per leaf averaged over three replications (3 x 9 = 27 leaves)
[b] Standard error of mean
WP wetness period
DP dry period

Table 6.5 *Analysis of variance for the log_{10} transformed average numbers of Table 6.4.*

Source of variation	log_{10}(ANLL)	
	df[a]	MS[b]
Replication	2	0.10^{ns}
WP (days)	2	0.10^{ns}
DP (hours)	5	0.36^{*}
WP · DP	10	0.05^{ns}
Error	31(3)	0.04
Total	50(3)	

[a] df degrees of freedom, (..) = number of missing values
[b] MS Mean square, ns = *F*-value not significant, * = *F*-value significant at $P \leq 0.01$
ANLL average number of lesions per leaf
WP wetness period
DP dry period

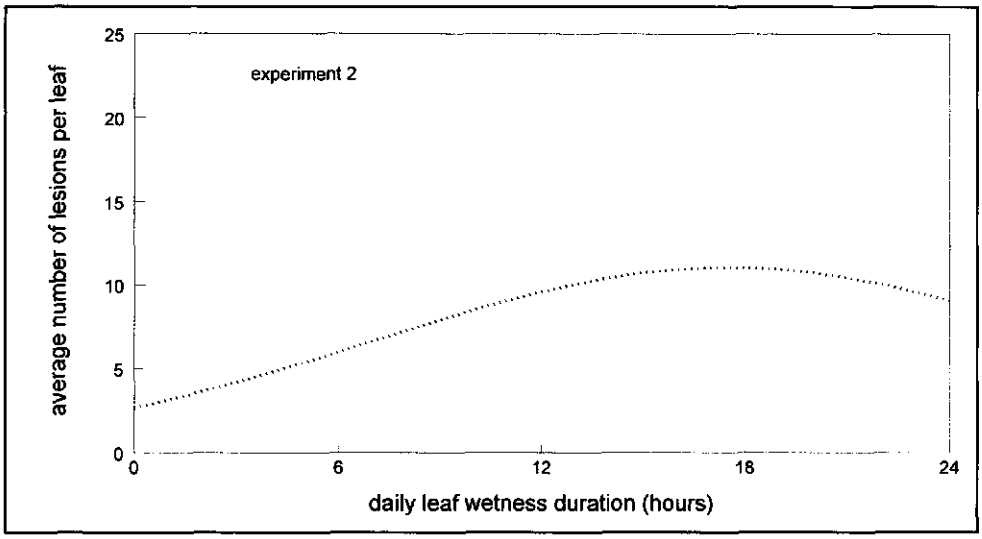

Figure 6.3 *Experiment 2. Effect of interruptions of leaf wetness periods on infection of white cabbage by Mycosphaerella brassicicola after inoculation with ascospores. The curve was generated by using equation 3 and the parameters listed in Table 6.3. It represents the back-transformed average numbers of lesions per leaf.*

Experiment 3 Effect of interrupted relative humidity period.

No symptoms of *M. brassicicola* appeared on the control plants. Interruption of periods with high relative humidity, and duration of the relative humidity period in days (RHP) had significant effects ($P < 0.01$) on the log-transformed number of lesions per leaf (Tables 6.6 and 6.7). No interaction effect could be detected between the duration of high relative humidity in days (RHP) and the duration of the daily dry periods (DP).

A regression model based on the log-transformed average lesion numbers was chosen to model the effect of interrupting relative humidity on infection. The equation for the model was:

$$y = b_0 + b_1 \cdot DP + b_2 \cdot RHP^2 \qquad (4)$$

in which $y = \log_{10}(x)$, x = average number of lesions per leaf, and b_0, b_1, and b_2 are partial regression coefficients, all significant at $P \leq 0.01$ (Table 6.3). Fig. 6.4 shows the back-transformed average number of lesions per leaf in relation to duration of dry periods and total length of the relative humidity treatment. The number of lesions after continuous periods of 2, 4 and 6 days of 90% relative humidity (DP = 24) seemed to be lower than under conditions of continous leaf wetness (Fig 6.2 and 6.3). A decreasing duration of daily dry periods (increase of the duration of 90% relative humidity) resulted in an increase of

lesion number. Furthermore, the longer the total duration of the relative humidity treatment in days, the higher the number of lesions.

Table 6.6 *The influence of the duration of dry periods (DP) during periods of 90% relative humidity on the average number of lesions per leaf after inoculation with ascospores of Mycosphaerella brassicicola on white cabbage.*

	RHP (days)						
		2		4		6	
DP (h)		Mean[a]	SE[b]	Mean[a]	SE[b]	Mean[a]	SE[b]
	21	2.2	(0.80)	3.3	(1.16)	5.5	(2.68)
	18	3.8	(1.61)	1.6	(0.58)	1.3	(0.46)
	12	5.4	(1.78)	4.1	(1.30)	9.5	(3.20)
	6	4.6	(0.90)	7.3	(3.00)	17.7	(3.80)
	0	7.1	(1.85)	4.8	(1.26)	15.2	(3.90)

[a] Mean number of lesions per leaf averaged over 2 replications (2 * 6 = 12 leaves).
[b] Standard error of mean
RHP relative humidity period
DP dry period

Table 6.7 *Analysis of variance for the log_{10} transformed average numbers of Table 6.6.*

	log_{10}(ANLL)	
Source of variation	df[a]	MS[b]
Replication	1	0.130^{ns}
RHP (days)	2	0.007^{**}
DP (hours)	4	0.000^{**}
RHP · DP	8	0.158^{ns}
Error	43(1)	0.085
Total	58(1)	

[a] df degrees of freedom, (..) = number of missing values
[b] MS Mean square, ns = *F*-value not significant, * = *F*-value significant for $P \leq 0.01$
ANLL average number of lesions per leaf
RHP relative humidity period
DP dry period

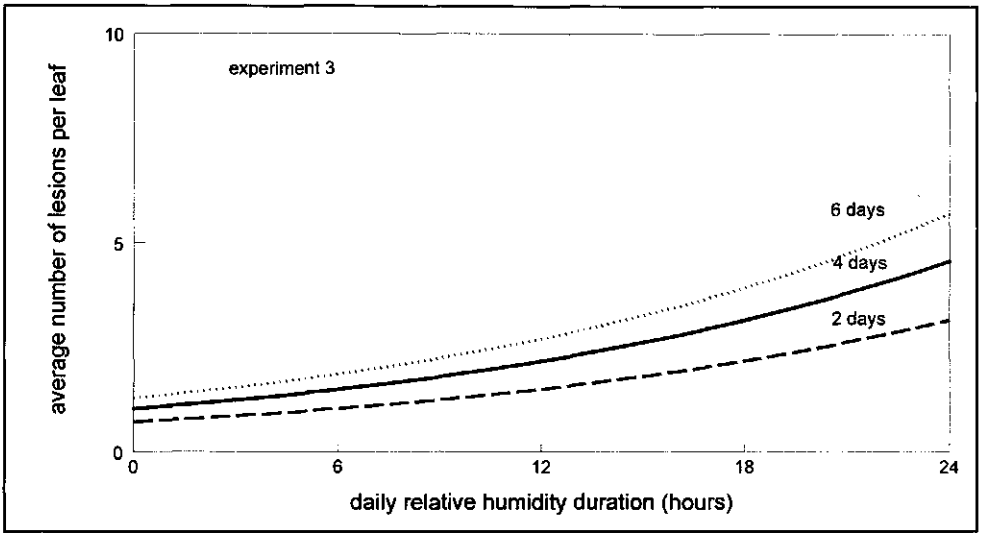

Figure 6.4 *Experiment 3. Effect of dry interruptions during periods of 90% relative humidity on infection of white cabbage by Mycosphaerella brassicicola after inoculation with ascospores. Curves were generated by using equation 4 and the parameters listed in Table 6.3. They represent the back-transformed average numbers of lesions per leaf at three inoculation periods of exposure to 90% relative humidity.*

Experiment 4 Effect of interrupted leaf wetness period and relative humidity

No symptoms of *M. brassicicola* appeared on control plants. Interruption of a period of leaf wetness of 3 days by a one day period of 90% relative humidity at the second day resulted in a significantly higher number of lesions (LSD, $P < 0.05$) than the other two treatments (Tables 6.8 and 6.9).

Table 6.8 *The influence of interrupted leaf wetness periods and relative humidity on the average number of lesions per leaf of Mycosphaerella brassicicola on white cabbage. WP = leaf wetness period, RHP = relative humidity period.*

treatment	ANLL[a]	SE[b]
WP - WP - WP	89 b	11.7
WP - 90% RHP - WP	132 a	10.7
WP - 70% RHP - WP	93 b	7.2

[a] mean number of lesions per leaf averaged over three replications (3 x 3 = 9 leaves)
[b] Standard error of the mean
ANLL average number of lesions
WP wetness period
RHP relative humidity period

Table 6.9 *Analysis of variance for the log_{10} transformed average numbers of Table 6.8.*

Source of variation	df[a]	log_{10}(ANLL) MS[b]
Replication	2	0.05^{ns}
Treatment	2	0.38^{*}
Replication · Treatment	4	0.11^{ns}
Error	75	0.06
Total	83	

[a] df degrees of freedom
[b] MS Mean square, ns = *F*-value not significant, * = *F*-value significant at $P \leq 0.01$
ANLL average number of lesions per leaf

6.4 Discussion

All experimental data showed great variability between and within treatments, resulting in poor coefficients of determination (R^2, Table 6.3) of the regression models. The great variation observed possibly relates to genetic variation among ascospores, as shown in a similar study with *M. fijiensis* var *difformis* (Jacome and Schuh, 1992), and the amount of inoculum deposited on a leaf. Results can also be influenced by the decline of viability of ascospores from stored leaf material. Storage of leaf material with perithecia of *M. musicola* at low humidity for long periods led to a decrease of viability of the ascospores (Stover, 1971). Despite the great variability of the experimental data some general conclusions can be drawn.

Ascospores of *M. brassicicola* are able to infect young white cabbage plants within 1 day of continuous leaf wetness at temperatures of 5-20 °C (experiment 1). Increase of the duration of continuous wetness up to 5 days led to an increase in lesion numbers. This agrees with previous findings (Van den Ende and Frinking, 1993), and confirms the observations of other scientists (Ryan and Staunton, 1983; Staunton and Ryan, 1977; Wicks and Vogelzang, 1988). The results contradict with data from infection studies with *in vitro* produced ascospores on white cabbage (Götz *et al.*, 1993). Although the latter studies were carried out under controlled conditions in growth chambers their results varied during the season. In summer, infection of white cabbage plants occurred only after a minimum humidity period of 7 days at 17 °C. This period was prolonged to more than 20 days in autumn. In winter no infection occurred. It seems that *in vitro* produced inoculum is probably less infectious than inoculum from diseased plant material. Seasonal effects are probably due to a loss of viability with time of the *in vitro* produced ascospores. Van den

Ende (1992b) showed that continuous subculturing of the fungus reduced the infectivity of mycelial inoculum.

Low temperatures (5 and 10 °C) during IP resulted in higher lesion numbers than high temperatures (15 and 20 °C). This temperature effect could be explained in several ways. (1) Relatively high temperatures (20 °C) reduce the viability of ascospores of *M. brassicicola* (Hartill and Sutton, 1980), which has an effect on the final lesion number. (2) Temperature may also affects the extramatricular hyphal growth of the infection hyphae on the leaf. Whiteside (1972; 1974) showed that ascospores of *M. citri* produced extramatricular hyphal growth on wet leaves of citrus, resulting in multiple penetrations of stomata. Once the fungus had penetrated the stomata, leaf wetness was not necessary for further colonization of the host. If low temperatures stimulate branching of the hyphae of *M. brassicicola* on the leaf, more penetrations would occur, and hence higher lesions numbers would be found.

Short wetness periods (<2 days) resulted in smaller lesions (longer incubation periods), possibly due to a direct effect on hyphal growth as shown with other *Mycosphaerella* species (McCoy and Dimock, 1972; Whiteside, 1972). Lesion diameter increases with increasing temperature during IP. Whereas relatively low temperatures seem to stimulate infection, relatively high temperatures seem to favour colonization of the host, resulting in bigger lesions and shorter incubation periods. The infection process of *M. zea-maydis* on corn showed similar effects (Castor, 1977).

Interruption of leaf wetness periods by short dry periods can lead to higher infection than continuous wetting (experiment 2). This result is not very common (Jones, 1987, Huber and Gillespie, 1992), although it has been observed for other diseases (Butler *et al.*, 1994; Carisse and Kushalappa, 1992; Moore, 1964; Shaw, 1991; Shuh, 1993; Sutton *et al.*, 1987; Trapero-Casas and Kaiser, 1992; Whiteside, 1974). The effect would be partially explained by the presence of large droplets of water on the leaf surface in case of continuous wetting, which may have reduced hyphal contact with the leaf surface (Carisse and Kushalappa, 1992).

Continuous periods of high relative humidity at different temperatures could not be tested due to lack of large controlled environment cabinets. Results at 15 °C (experiment 3) showed an increase of infection with increasing periods of continuous high relative humidity (>90 %). These observations are in agreement with the results of Ryan and Staunton (1983), who showed that increasing humidity periods up to 5 days resulted in an increase of infection. Although absolute lesions numbers after 6 days of continuous high relative humidity seem to be lower than after 6 days of continuous leaf wetness, no conclusions can be drawn as a consequence of the different inocula used. Interruption of the humidity period resulted in a decrease of lesion numbers, but even short periods of high humidity resulted in some infection. Nelson and Pound (1959) showed that after mycelial inoculation of cabbage plants with *M. brassicicola* RH need not be continuous.

Despite drying of 4, 8 or 16 hours daily, infection was obtained in all cases, although the rate and amount decreased as the dry period increased. Drying did not stimulate infection, probably due to the absence of negative effects of free water on the leaves.
Interruption of three days leaf wetness period with one day of 70% RH does not reduce lesion numbers. Interruption with one day with 90% RH even stimulates infection. The pathogen seems very well adapted to exploit leaf wetness interrupted by dry periods. Previously, Staunton and Ryan (1977) reported that a 5 day delay (at 18 °C and 80% RH) between inoculation of brassica plants with ascospores of *M. brassicicola* and moist incubation did not reduce infection. Ascospores of the fungus can withstand long periods of low humidity (Hartill and Sutton, 1980), as shown with other *Mycosphaerella* species (Brown *et al.*, 1978; Park, 1988; Van Steekelenburg, 1984).

All experiments are carried out with a susceptible white cabbage cultivar. Minimum humidity requirements for infection can be different on less susceptible cultivars, as Wicks (1988) showed with Brussels sprouts. Nevertheless, results of this study show that the pathogen is well adapted to survive dry periods during the infection process. Any attempt to define an infection period for *M. brassicicola* in terms of a continuous leaf wetness is unlikely to succeed. Therefore, the described guidelines for infection should be carefully evaluated under field circumstances, before they are used in warning systems.

Chapter 7

Incubation and latent periods of ring spot in brassica crops in relation to infection by ascospores: a field study[1]

[1] Van den Ende, J.E., A.P. Everaarts, C.P. de Moel and H.D. Frinking. To be submitted

Abstract

Severe infestations by ring spot of brassica crops, caused by *Mycosphaerella brassicicola*, result in qualitative and quantitative damage at harvest time. To improve the present control strategy, experiments have been carried out to gain insight in the length of the incubation and latent periods under field conditons. Artificial inoculations of Brussels sprouts plants with ascospores of *M. brassicicola* under field conditions indicated an incubation period of 14-21 days on fully grown leaves. The minimum length of the latent period varied between 31 and 41 days. Analysis of the development of an artificially induced epidemic of ring spot showed similar results. The incubation period varied from 13-33 days, dependent on the growth stage of the crop and the environmental condtions following critical periods for infection in the field. The warning system presently in use is based on the effects of temperature and humidity in relation to infection of brassica crops by ring spot. It is argued that such a warning system leads to superfluous fungicide use as the lengths of the incubation and latent periods are not taken into account.

7.1 Introduction

Ring spot of brassica plants is caused by the fungus *Mycosphaerella brassicicola* (Duby) Lind. As soon as expanded leaves are present, ascospores of the fungus can infect the host (Hartill and Sutton, 1980; Van den Ende, 1992b; Van den Ende and Frinking, 1993). In the Netherlands, disease symptoms are mostly found at the end of the summer and during autumn when climatical conditions and crop phenology usually favour disease development (Frinking and Geerds, 1987). Severe infestations lead to early leaf senescence and defoliation and result in qualitative and quantitative damage at harvest time (Everaarts and De Moel, 1991; Gladders *et al.*, 1992; Jouan *et al.*, 1972; Long, 1986; Rudnick, 1986; Wicks *et al.*, 1987). To minimize crop losses, fungicide applications against ring spot are an essential part of white cabbage and Brussels sprouts husbandry. Before 1990, sprayings with fungicides (benomyl or carbendazim) were calendar based. They began in August and were followed by 1-2 treatments until harvest, the number of treatments depending on the disease pressure. In 1991, general guidelines were introduced to improve the efficiency of ring spot control (De Moel *et al.*, 1991), using epidemiological data known from literature on the effects of temperature and wetness duration in relation to infection of brassica crops by *M. brassicicola*. A critical period for infection of white cabbage by *M. brassicicola* was defined as two consecutive days with at least 18 h of either a relative humidity above 90% or 18 h of leaf wetness per day. More recent research has shown that this definition of the critical period is unrealistic (Van den Ende and Frinking, 1993; Van den Ende *et al.*, 1998), which makes the use of the guidelines questionable. Moreover, because other components of the fungal life cycle (e.g.

incubation period and latent period) have not been taken into account, the guidelines should be reconsidered.

Humidity plays an essential role in the life cycle of *M. brassicicola*. Production of ascospores in pseudothecia will occur only after a 3-4 days period of high relative humidity (>90%; Nelson and Pound, 1959). Substantial dispersal of spores is stimulated by rainfall (Hartill, 1977; Humpherson-Jones and O'Brien, 1986; Staunton and Ryan, 1977). Small spore flights can occur after dew (Weimer, 1926; Zornbach, 1990). Germination of ascospores in free water takes place within 24 h (Snyder, 1946; Hartill and Sutton, 1980). Ascospores fail to germinate when free water is not available, even during long periods (3 days) of high humidity (RH > 90%) (Van den Ende, unpublished). Infection of cabbage leaves by ascospores is optimal after 1-2 days of leaf wetness, but even within 24 h of leaf wetness infection is possible within a broad temperature range (5-20 °C)(Van den Ende and Frinking, 1993; Van den Ende *et al.*, 1998). Data on the length of the incubation period are contradictory (Dring, 1961; Frinking and Geerds, 1987; Götz *et al.*, 1993; Nelson, 1958; Quak, 1957; Van den Ende, 1992b, 1993; Van den Ende and Frinking, 1993; Weimer, 1926; Wicks and Vogelzang, 1988; Zornbach, 1988) and vary from 1-4 weeks. Latent periods of *M. brassicicola* are long. Estimates based on greenhouse experiments and field data indicate a period of at least 35-40 days (Zornbach, 1988). Dring (1961) stated that fruiting bodies with ripe ascospores were only found in ring spot lesions on the older leaves which begin to yellow or have fallen from the plant.

In 1988 a field experiment was carried out in Wageningen to determine the incubation and latent periods of *M. brassicicola* in Brussels sprouts under field condition. In 1990 a field experiment was carried out by researchers of the PAV at Lelystad to illustrate the epidemiological development of ring spot on white cabbage in relation to host age and chemical control. In the present study results of these experiments are analyzed to validate existing knowledge on the life cycle of ring spot.

7.2 Materials and methods

Determination of incubation and latent periods under field circumstances.

In 1988, inoculations with ascospores of *M. brassicicola* were performed on fully expanded leaves under field conditions. The host plants, grown in the field, were Brussels sprouts of the susceptible cultivar Roger. Ascospore inoculum was obtained according to Van den Ende (1992b). Pieces of water agar (± 2 mm^2) with ascospores were placed upside-down on leaves, 5 on each side of the leaf. Inoculations were carried out 3 times during the growing season (15 July, 22 July and 1 Sept) on leaves of different ages of 10 randomly selected plants (Table 7.1). The diameters of resulting lesions were measured twice a week. Per date of inoculation and leaf number the appearance of the first fruiting bodies was recorded, and the average incubation period and the average lesion growth rate were

estimated by linear regression analyses of the data. Differences in incubation period and lesion growth rate of ring spots on the inoculated leaves were tested for significance at 95% probability with LSD.

Development of ring spot on white cabbage in relation to host age and fungicide application.

The 1990 field experiment was carried out in Lelystad. Transplants of white cabbage (*Brassica oleracea* var. *capitata*) cv. Bartolo (four - fifth leaf stage) were planted on six dates (P1 = 23.04, P2 = 10.05, P3 = 31.05, P4 = 12.06, P5 = 27.06, P6 = 04.07). Distance between plants within rows was 50 cm and between rows 60 cm. The experimental field (60 x 10 m) was divided into 6 plots (9.6 x 10). Distance between plots was 40 cm. Each plot contained a group of plants of the same age (Fig. 7.1). Plots were laid out in a fixed order of decreasing plant age. Within each plot, four subplots (3.5 x 3.6) were planted, each containing 35 plants. Subplots were randomized within plots and surrounded by two rows of cabbage plants. Inoculum consisted of dried cabbage leaves, collected during the previous season, full of lesions of *M. brassicicola*. The dry leaves were crushed and the resulting leaf fragments were homogeneously distributed between the rows shortly after planting. Per plant age, two subplots remained untreated, and two subplots were treated with fungicides, either penconazole or pyrifenox (a.i 0.20 and 0.25 kg/ha respectively) (Fig. 7.1).

Relative humidity was recorded by a thermohygrograph (Ties, Göttingen). Fungicide applications were carried out irrespective of critical periods for ring spot development. Dates of fungicide applications were 25 June (in P1), 16 July (in P1,P2), 7 September (in P2, P3, P4) and 28 September (in P3, P4).

From three randomly chosen plants per subplot the numbers of leaves (excluding head leaves) and the numbers of lesions per leaf were counted regulary. Lesions on the outer leaves of the cabbage heads were counted during the growing season. At harvest time the disease severity of all harvested cabbages was assessed by means of a disease scale. Disease severity was expressed as the percentage of the cabbage surface covered by lesions of *M. brassicicola* (0 = no lesions, 1 = 1-10%, 3 = 11-25% , 5 = 26-50% , 7 = 51-75% , 9 = >75%) and mean values per plot were determined.

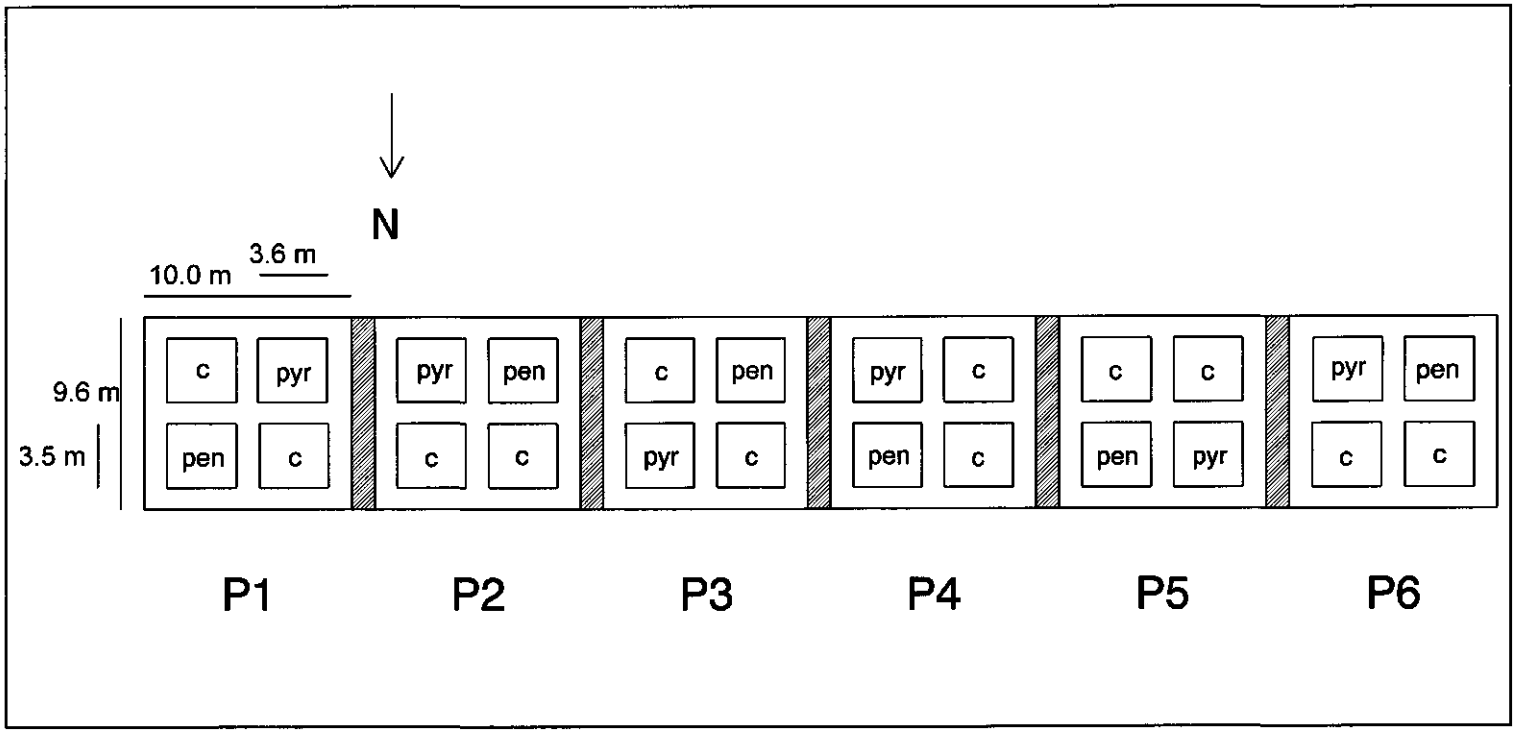

Figure 7.1 *Experimental lay-out of the field experiment, 1990, Lelystad. N = north, P1..6 = planting time, c = control, pyr = pyrifenox, pen = penconazole. Shaded parts are not planted.*

Table 7.1 *Average incubation period (IP) in days (d), average lesion growth (LG) in millimeter per day (mm/d) and appearance of the first fruiting bodies (FB) after inoculation of Brussels sprout leaves with ascospores of Mycosphaerella brassicicola on different dates (INOC) under field conditions, Wageningen, 1988. Inoculated leaves (n = number of leaves) are numbered (from bottom to top) according to their position (POS) on the plant; leaf age decreases with increasing POS number.*

INOC	POS	n	IP[1](d)	std[2]	LG[1](mm/d)	std[2]	FB
15 July	8	7	14.1 bc	(3.9)	0.45 a	(0.06)	38
	13	9	19.9 ab	(7.4)	0.34 bc	(0.07)	41
22 July	8	10	12.8 c	(2.0)	0.51 a	(0.17)	31
	13	9	14.7 bc	(1.3)	0.36 b	(0.04)	33
	18	10	19.3 ab	(7.8)	0.32 bc	(0.07)	41
1 Sept	43	5	21.4 a	(4.2)	0.23 c	(0.04)	-[3]

[1] Mean values with the same letter are not significantly different according to LSD (0.05).
[2] Standard deviation.
[3] No fruiting bodies present before leaf fall.

7.3 Results

Determination of incubation and latent periods under field circumstances

Results are shown in Table 7.1. Data from plants inoculated on 15 and 22 July show that the incubation period and lesion growth of *M. brassicicola* on Brussels sprouts were significantly affected by leaf age. Inoculations on relative young leaves (high leaf number) result in longer incubation periods and retarded lesion growth rate. Very young leaves, at position 43 and inoculated 1 September, show longest incubation periods and lowest lesion growth rates. Differences in lesion growth rate and incubation period of ring spot on leaves with the same leaf number but inoculated on different dates are not significant. The time of appearance of the first fruiting bodies (FB) follows the same tendency. Fruiting bodies appeared on average 37 days after inoculation.

Development of ring spot on white cabbage in relation to host age and fungicide application

Control plots. Lesion numbers of the control plots were averaged, log transformed and plotted against time. As hardly any lesions were found in P5 and P6, only the increases of lesion numbers in the first four plantings are presented. Following the guidelines metntioned earlier, several critical periods for infection of cabbage by *M. brassicicola* could be distinguished (CP 1-8) (Fig. 7.2) and 5 periods could be identified in which there was an obvious increase of lesion numbers in one or more plantings. Such an increase is here interpreted as an infection wave (W 1-5 in Fig. 7.3A). The number of days was determined between the end of the critical period and the date at which 50% of the total increase of lesion numbers during an infection wave was attained (Table 7.2). This number is used here as the length of the incubation period. Disease development will be discussed per planting.

Planting 1 (P1). At the first critical period (11-12 May) plants of P1 had on average 7 leaves (Fig. 7.4). Although the plants were at a very young stage of development, disease symptoms were found on the first 1-7 leaves after an incubation period of 31-32 days

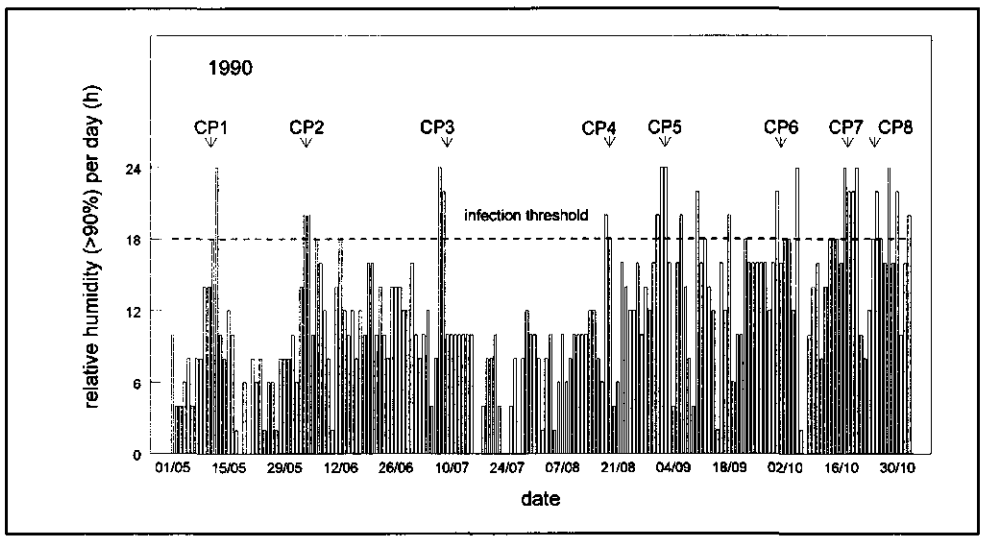

Figure 7.2 *Duration in hours of high relative humidity (>90%) periods per day. Critical periods for infection (CP 1-8) are indicated). The infection threshold is defined as two consecutive days with at least 18 h of a relative humidity above 90%*

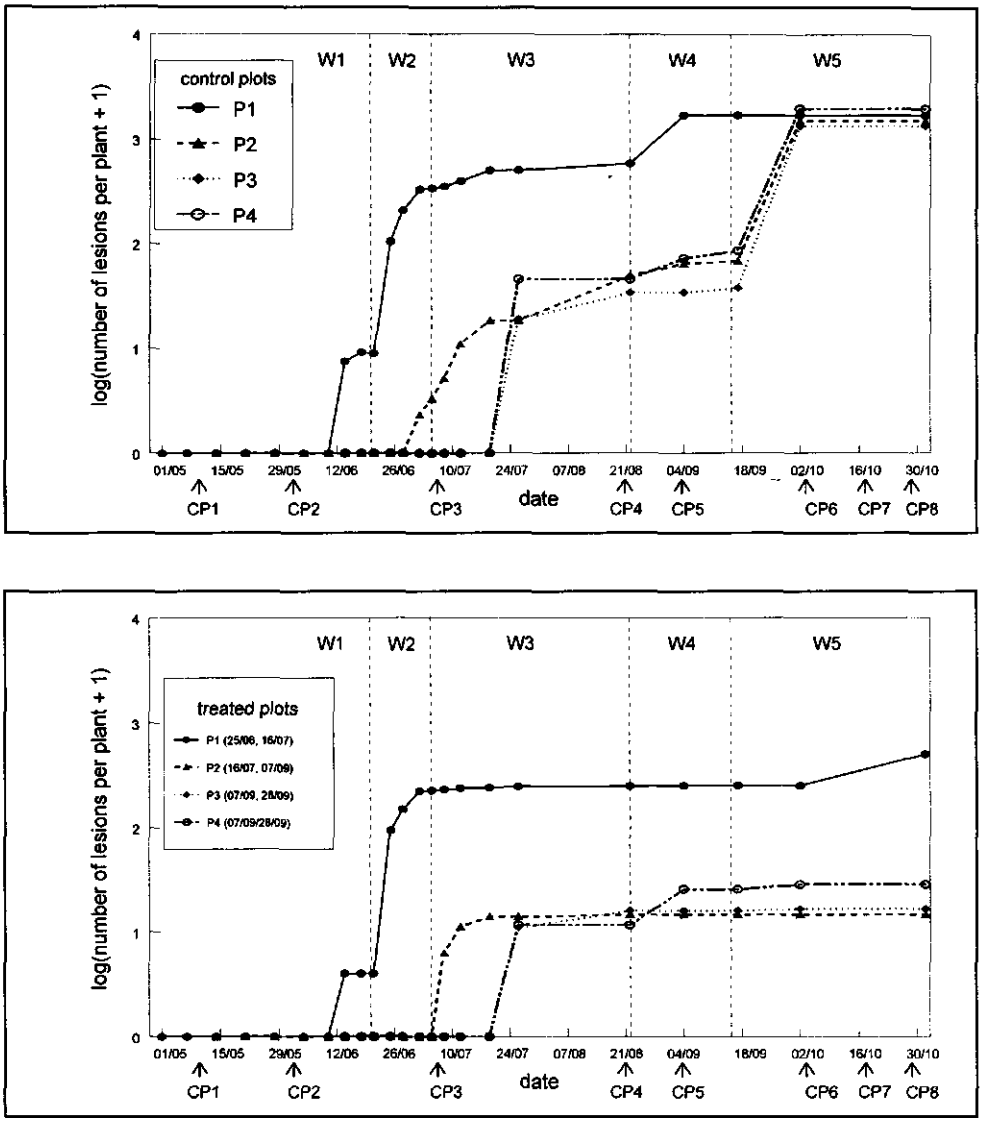

Figure 7.3 *Disease development of Mycosphaerella brassicicola in cabbage in control (A) and fungicide treated (B) plots in relation to planting time (P1 = 23/4, P2 = 10/5, P3 = 31/5, P4 = 12/6). Critical periods for infection (CP 1-8) and periods with a strong increase of lesion number (infection wave) in one or more plantings (W 1-5) are indicated. Spray data (B) are indicated between brackets.*

Table 7.2 *Estimation of the incubation period of Mycosphaerella brassicicola on cabbage under field conditions in relation to planting time (P1 = 23/4, P2 = 10/5, P3 = 31/5, P4 = 12/6) and supposed time of infection (CP = critical period). The incubation period (IP) is defined as the number of days from the critical period till the date at which 50% of the total increase of lesion numbers during an infection wave (W) is attained (50%W).*

	CP	50%W	W	IP (days)
P1	11 -12 May	13 June	W1	31-32
P1	3 - 4 June	25 June	W2	20-21
P1	7 - 8 July	22 July	W3	14-15
P1	18-19 August	1 September	W4	13-14
P2	3 - 4 June	30 June	W2	26-27
P2	7 - 8 July	22 July	W3	14-15
P3/P4	7 - 8 July	23 July	W3	15-16
P2/P3/P4	1 - 2 September	25 September	W5	23-24

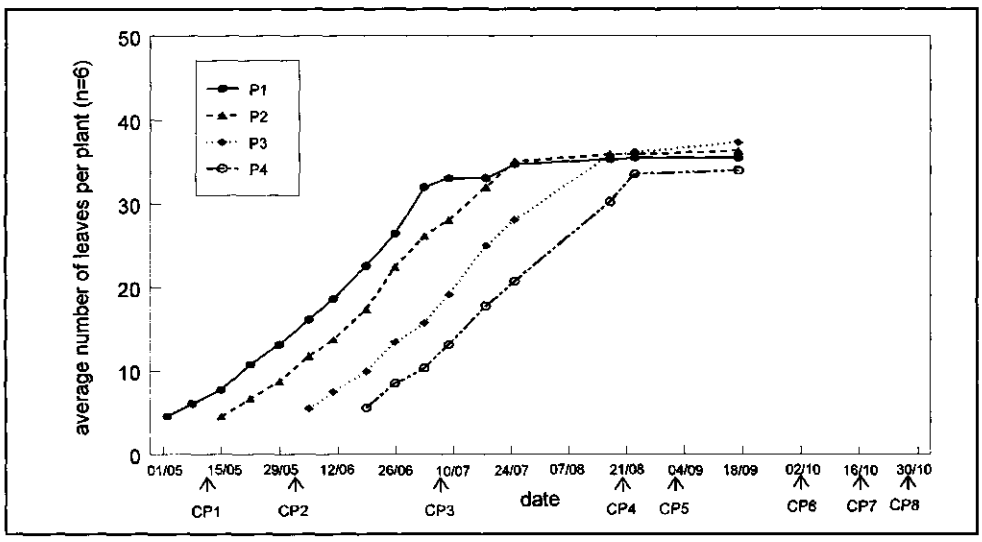

Figure 7.4 *Development of the number of leaves per cabbage plant (n=6) in relation to planting time (P1 = 23/4, P2 = 10/5, P3 = 31/5, P4 = 12/6). Critical periods for infection (CP 1-8) are indicated.*

(Table 7.2). The second infection wave (W2) during the first week of July on plants of P1 was found on leaves 1 to 16 (numbers from bottom to top), corresponding to the leaves present at the second critical period (Fig. 7.4). The incubation period was 20-21 days (Table 7.2). The third critical period, 7-8 July, resulted in a slight increase in lesion numbers by the end of July (W3), after an incubation period of 14-15 days. The critical period of 18-19 August was followed by the massive appearance of lesions on all the leaves of plants of P1 13-14 days later (W4).

Planting 2 (P2). Plants of P2, planted just before the first critical period (10 May), showed no symptoms until 3 July (W2). The increase in lesion numbers found after the third critical period in plants of P2 (W3) coincided partly with the increase in lesion numbers caused by infection during the second critical period which makes the estimation of the incubation period in both cases unreliable.

Plantings 3 and 4 (P3, P4). The first symptoms of ring spot on the plants of P3 (on leaves 1-17) and P4 (on leaves 1-12) appeared at the end of July (W3), 15-16 days after the critical period of 7-8 July.

At the fourth and fifth critical periods plants of all plantings were fully developed so that a strong increase in lesion numbers on all leaves of P2, P3 and P4 was possible by the end of September (W5). The incubation period of W5 was 23-24 days. Increases in P1 during W5 have not been taken into account as the highest lesion number per leaf (±100) that could be determined accurately had already been reached during W4.

The disease severity on the cabbage heads at harvest was significantly affected by planting time (ANOVA, $P = 0.0036$). Late planting resulted in low disease severity (Fig 7.5).

Treated plots

Since differences in disease severity between the plots treated with pecanozole or pyrifenox were small, treated plots were handled as replicates. The lesion numbers of the treated plots were averaged, log transformed and plotted against time. Spray data are indicated between brackets (Fig. 7.3B). In all plantings only two applications with fungicides were carried out. The first fungicide treatment per planting was applied after the appearance of the first generation of lesions in the respective planting. Although most treatments were poorly timed according to the guidelines, the fungicide applications significantly reduced the disease severity on leaves (Fig. 7.3A and B) and cabbage heads (LSD, $\alpha = 0.01$) (Fig 7.5).

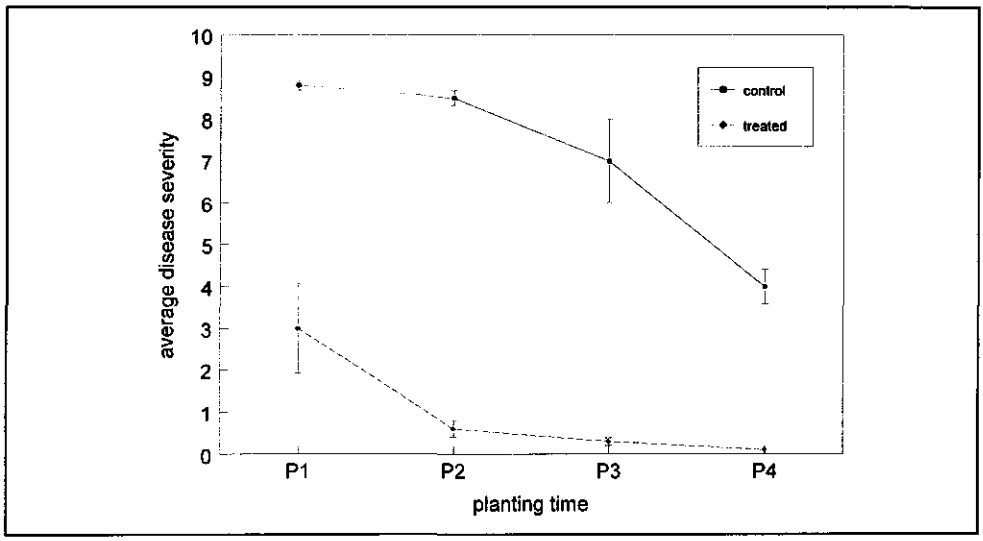

Figure 7.5 *Average disease severity of ring spot on cabbage heads in relation to planting time (P1 = 23/4, P2 = 10/5, P3 = 31/5, P4 = 12/6) and fungicide treatment. Disease severity is expressed as the mean percentage of the harvested cabbage surface covered by lesions of Mycosphaerella brassicicola (0 = no lesions, 1 = 1-10%, 3 = 11-25% , 5 = 26-50% , 7 = 51-75% , 9 = >75%). Standard errors are indicated per entry.*

7.4 Discussion

The results of the inoculation of Brussels sprouts plants with ascospores of *M. brassicicola* under field conditions showed that with increasing leaf age the length of the incubation period decreased and lesion growth rate increased. As temperature, leaf wetness duration and inoculation method (mycelial suspensions or ascospore inoculum) have an effect on the length of the incubation period too (Nelson, 1958; Ryan and Staunton, 1983; Staunton and Ryan, 1977; Van den Ende and Frinking, 1993; Van den Ende *et al.*, 1998; Wicks and Vogelzang, 1988; Zornbach, 1988) it is not surprising that the reports on the length of the incubation period are contradictory. Field data in this study indicate an incubation period of 14-21 days on fully grown leaves of a susceptible Brussels sprouts cultivar after inoculation with ascospores. The minimum length of the latent period in our experiment varied between 31 and 41 days. Although these observations on the length of the latent period are few in number, they are in agreement with the suggestion by Zornbach (1988) that the latent period of ring spot on susceptible white cabbage plants varied from 5-6 weeks under field conditions.

Analysis of the development of an epidemic of ring spot in cabbage showed results similar to those from the artificial inoculation of plants under field conditions. The incubation period varied from 13-32 days. The length of the incubation period may have been influenced by the growth stage of the cabbage crop and the environmental conditions following each critical period, as stated earlier. Many other *Mycosphaerella* species are known to have similar, relatively long incubation periods under field conditions (Mobambo *et al.*, 1996; Moulion-Pefoura *et al.*, 1996; Shaw, 1990) or even longer ones (Park, 1988; Sutton *et al.*, 1987; Whiteside, 1972).

If the latent period of ring spot is at least 5 weeks, the increase in lesion numbers found in P1 during the infection waves W1 and W2 must have been caused by ascospores from the inoculum still present on the soil, since the lesions of the first generation (W1) were not capable of producing ripe ascospores within 24 days after the first critical period. Leaf fragments containing fruiting bodies of ring spot are indeed capable to produce ascospores for a long period as found by Frinking and Geerds (1987), who showed in *in vitro* studies that single lesions of ring spot produced ascospores for at least 4 weeks. The increases of lesion numbers in P1 during the infection waves W3 and W4 were probably caused by ripe ascospores from lesions formed during W1 and W2, respectively. The increases in lesion numbers found in plantings P2, P3 and P4 during W5 are thought to result from ascospores produced by lesions during W3 and W4.

The growth of cabbage usually takes 22-25 weeks, which means that with a latent period of 5-6 weeks the fungus will have 4-5 generations per year at most (Zornbach, 1988). In the 1990 field experiment, *M. brassicicola* could have had 3-4 generations in the early planting, according to our estimates of the critical, incubation and latent periods. Hardly any lesions developed on cabbage that was planted late (P5 and P6). The third critical period (CP3) did not cause a significant increase of lesion numbers in P5 and P6, probably due to the young stage of the cabbage plants. This shows that when the first generation lesions appear late in the growing season (August/September), epidemic increase of the disease will not occur.

The good control achieved with fungicides cannot be explained by proper timing as most applications were carried out irrespective of the critical periods for ring spot development in cabbage. In all plantings the first treatment was carried out well after the appearance of the first lesions, but still prevented increases in lesion numbers due to the curative effect of both fungicides on ring spot lesions that were already present. The good curative control of pencanozole has been reported in other fungicide trials (Wicks and Vogelzang, 1988). The results show that a delay in the production of ripe ascospores by the first generation lesions after application of curative fungicides may also prevent the epidemic development of ring spot in cabbage. The treatment on 7 September was well timed according to the guidelines. Since the increase of lesion numbers in plots of P2 treated on 7 September was slight indeed the fungicide application in P3 and P4 on 28 September was

superfluous. As the increase in lesion numbers in the untreated plots of P2, P3 and P4 early in the season (up to the beginning of September) was slight, the fungicide treatment on 16 July might have been superfluous. We conclude that, in 1990, one fungicide application would have been sufficient for disease control in cabbage planted after 10 May.

The increases of lesion numbers in all plantings were found on almost all the leaves which had been present at the time of the corresponding critical periods. It seems that *M. brassicicola* not only infects fully grown cabbage leaves (Hartill and Sutton, 1980), but that the fungus is also able to penetrate young leaves of cabbage if the circumstances are favourable to infection. The susceptibility of young plants was already shown in studies under controlled conditions (Van den Ende, 1992b), but only few data are available on susceptibility of young plants under field conditions (Van den Ende, 1993; Wicks *et al.*, 1987; Wicks and Vogelzang, 1986). Infection of young plants at an early stage may results in severe damage to the crop at harvest time, as has been shown in previous studies (Van den Ende, 1992). Earlier and present results stress the importance of avoiding early infections caused by inoculum produced on infected debris from previous crops or infected oilseed rape and winter cauliflower (Gladders, 1993; Humpherson-Jones, 1989; Zornbach, 1991).

The design of the 1990 field experiment could have been better, with complete randomization of planting dates, but its results are clear and their interpretation leads to a better understanding of ring spot epidemiology under field conditions. The field experiment shows that proper control of the first generation lesions can prevent the epidemic development of ring spot, even when a high inoculum potential is present early in the season. Under natural conditions without a high inoculum potential at the onset of the season, at least 4-5 infection periods are needed until the inoculum potential is large enough to produce an epidemic (Zornbach,1991). Apparently, growers can wait until symptom development begins before they decide whether to spray or not, because symptoms provide the most reliable warning they can get.

The guidelines for infection of ring spot in cabbage have been incorporated in an computerized warning system (Stallen, 1995). Using the more preventive fungicides such as benomyl and carbendazim in combination with this warning system is risky because the present guidelines for fungicide input have not been confirmed by field experiments and thus remain subject to discussion. Although the present criteria for critical periods of ring spot seem to explain sudden increases in lesion numbers, the ensuing recommendation may lead to superfluous fungicide applications because the long incubation and latent periods of ring spot have not been taken into account. An evaluation of the warning system as it is used by the cabbage growers shows indeed that they apply fungicides more often than in the past (Anonymous, 1991; Kooistra, personal communication). We are faced with the curious situation that a warning system, designed to minimize fungicide input, in fact increases it and thus is counterproductive. The warning system can be improved by

utilizing the new data on the effects of temperature and humidity on infection of *Brassica* spp. by *M. brassicicola* (Van den Ende and Frinking, 1993; Van den Ende *et al.*, 1998) and incorporating the data on incubation and latent periods of ring spot presented in this study.

Chapter 8

General discussion

Ring spot disease of brassica crops, caused by *Mycosphaerella brassicicola*, has long been considered to be of minor importance. This view changed drastically when in the early eighties severe epidemics of ring spot occurred in the Netherlands, Germany and the UK (Frinking and Geerds, 1987; Humpherson-Jones and O'Brien, 1986; Zornbach, 1990). The outbreaks of ring spot were a result of the introduction of new, more susceptible, brassica cultivars and the increased intensity of brassica growing yearround in certain coastal regions of these countries (Zornbach, 1990). The economic damage caused by the fungus emphasized the need for resistance breeding and improvement of the ring spot control strategies.
The aim of the research presented in this thesis was twofold: to develop a routine screening method for resistance against *M. brassicicola* in *Brassica oleracea* and to determine effects of environmental factors on the life cycle of ring spot in order to optimize disease control in the field.

8.1 Development of a routine screening method

Although sources of resistance were available (Dixon, 1981; Mulder, 1985; Zornbach, 1990), breeding of resistant cultivars was hampered by the time-consuming procedure of the available screening method. Plants were screened under field conditions without artificial inoculation with spores of *M. brassicicola*, which in many cases resulted in expensive field trials without proper results. Screening tests for most diseases in brassica are well described, and many of these tests are carried out on seedlings (Williams, 1985). The use of seedlings has an advantage in that large populations of plants can be screened in a short period of time, with minimum use of growth chamber and greenhouse space.
At the start of this project the reason for not using a seedling test in screening for resistance against ring spot was twofold. It was assumed that young plants of brassica crops were not susceptible to ring spot (Osmun and Anderson, 1915; Butler and Jones, 1949) and that growth and sporulation of the fungus *in vitro* was poor which frustrated infection studies under controlled conditions.

Susceptibility of seedlings

Results of a field study showed that infection by *M. brassicicola* was not restricted to the fully grown plants, but that the cotyledons and the first two leaves of cabbage seedlings are also susceptible to ring spot (chapter 2). This result does not contradict the conclusions by Hartill (1977, 1978) and Hartill and Sutton (1980) that symptoms rarely develop until leaf expansion is complete, and never develop on rapidly expanding leaves. Two or three weeks after sowing, cotyledons and the first two leaves are likely to be fully expanded.

Inoculum production

Due to the difficulty of producing high numbers of ascospores of *M. brassicicola in vitro*, tests with plants under controlled conditions were hard to accomplish at a large scale. Nelson (1958) inoculated plants with mycelial inoculum to overcome the lack of spores. Improvement of his inoculation method was achieved by adding a 3% sucrose solution to a mycelial suspension which ensured high disease levels on a susceptible cultivar (chapter 3). The mycelial inoculum was easy to produce and could be standardized.

Greenhouse screening method

The resistance expressed in the cabbage seedlings under field conditions was correlated with adult plant resistance (chapter 2). This observation and the development of reliable inoculation method led to the development of a greenhouse screening method for ring spot resistance on cotyledons and young plants (third leaf stage). Data from the indoor tests on several cabbage and Brussels sprouts cultivars, obtained with mycelial inoculum, corresponded to field data resulting from natural infection by ascospores (chapters 3 and 4). Testing plants in the third leaf stage gave the best results since the resistance reactions were more distinct than on cotyledons and because older seedlings could better withstand the long wetness periods necessary for infection than young ones.

Environmental effects on the results of the screening method

The screening method was tested on several cultivars of Brussels sprouts, cabbage and cauliflower. Resistance was expressed as a lower number of lesions per leaf and/or hypersensitive reactions (chapters 3 and 4). Hypersensitive responses in *B. oleracea* after penetration of pathogens are a common phenomenon (Conn *et al.*, 1988; Greenhalgh and Dickinson, 1975; Wang, 1949). The lower number of lesions can be caused by the presence of the epicuticular wax layer. In many *Brassica* species the wax layer provides a mechanical barrier to infection and its presence has been found to play a significant role in resistance against several pathogens (Bansal *et al.*, 1990, Conn *et al.*, 1984; Rawlinson *et al.*, 1978, Skoropad and Tewari, 1977). Hartill reported on the presence of compounds in the wax layer of *B. oleracea* leaves that resulted in inhibition of ascospore germination of *M. brassicicola*.

Resistance associated with the wax layer of *Brassica* species may well be overlooked in the developed screening method. Wax layer production will not only be influenced by the developmental stage of the plants, but the environment will affect (Baker, 1974) differences in wax production on greenhouse and field grown plants which will lower the desired correlation.

Therefore our screening method was tested on different locations by the potential users. In this way, more insight was provided in the practical use of the test. Although much variability existed in disease levels at the different locations, resistant cultivars and highly

susceptible cultivars could be easily distinguished (chapter 3). The resistance based on the hypersensitive response correlated well between young and old plants as shown with other brassica-pathogen combinations (Mithen and Lewis, 1988). Partial resistance against ring spot is difficult to assess in the greenhouse screening method, maybe because it is more related to the wax layer of the brassica plants.

Differential interaction

Physiological specialization of *M. brassicicola* is present in the isolates used in this research (chapter 4). The result underlines the importance of using isolates of different origin in routine screening. As *M. brassicicola* depends on its sexual stage recombination in the pathogen can occur. We expect that the incorporation of only major genes for resistance in cultivars will result in a rapid loss of effective resistance, due to a strong selection pressure for virulent races of the fungus (Parlevliet, 1993).

Recommendations

Screening *B. oleracea* in the greenhouse for resistance against *M. brassicicola* should be considered as a preliminary step to field screening. The ability to screen *B. oleracea* for resistance to *M. brassicicola* under controlled conditions offers the potential for a rapid estimation of the suitability of *B. oleracea* selections for inclusion in a breeding and selection program. With such a test, resistant and highly susceptible cultivars can be distinguished but partial resistance is still difficult to assess. Using multi-spore isolates or isolates from different regions in the screening test would prevent the risk of selecting a resistance factor which is isolate-specific.

Improvement of the screening method described in this thesis could be achieved by optimizing the methods of *in vitro* production of ascospores. The use of ascospores as inoculum will probably not affect differences in susceptibility between cultivars, but the screening method will be simplified. Infection of young plants by ascospores is less dependent on the humidity conditions during incubation (chapter 5 and 6). Methods have been described to obtain ascospores *in vitro* (Götz *et al.*, 1993) but the production of the spores remains low, unreliable and hence not suitable for routine screening.

Other ways of screening may be found in using the production of active secondary metabolites produced by *M. brassicicola* as described by Götz and Boyle (1993). Germination of seeds of several white cabbage cultivar was inhibited by these metabolites *in vitro*. However, their results show a low correlation with field data and results with similar cultivars as described in this thesis, which makes the practical value of the metabolites approach questionable.

8.2 Effects of environmental factors on the life cycle of ring spot

Improvement of the ring spot control strategies could be achieved by studying the effects of environmental factors on the life cycle of the fungus. The slow growth and inability of the fungus to produce large amounts of spores *in vitro* frustrated studies under controlled conditions. Some studies were carried out by inoculating plants with suspensions of mycelial fragments (Nelson and Pound, 1959; Weimer, 1926; Zornbach, 1990), but results from these studies were contradictory and hardly representative for the occurrence of ring spot under field conditions.

Mycelial inoculum contra ascospore inoculum

Using a new method for the collection of ascospores from diseased leaf material (Chapter 5) it was possible to study effects of temperature and humidity on plants under controlled condition inoculated with ascospore suspension. The results showed that the minimum humidity requirements for infection were different for plants were inoculated with ascospores instead and with mycelial inoculum. Inoculation with mycelial fragments is only successful if a long period (4-5 days) of leaf wetness is maintained. Inoculation experiments with ascospores showed that infection of brassica plants could occur at much shorter leaf wetness period, less than one day, within a broad temperature range (5-20 °C).

Effects of temperature and wetness duration on infection

Despite the great variability of the experimental data some general conclusions can be drawn (chapter 6). Ascospores of *M. brassicicola* are able to infect cabbage plants within 1 day of continuous leaf wetness at temperatures of 5-20 °C. Increase of the duration of continuous wetness up to 5 days leads to an increase in lesion numbers. This result confirms field observations by other scientists (Ryan and Staunton, 1983; Staunton and Ryan, 1977; Wicks and Vogelzang, 1988) but contradicts the findings of Götz *et al.* (1993). Their Infection experiments carried out with *in vitro* produced ascospores were only successful when the minimum high humidity period was at least 7 days. It seems that *in vitro* produced inoculum is probably less infectious than inoculum from diseased plant material. Effects of continuous high relative humidity (90%) on the infection of cabbage by *M. brassicicola* was only tested at one temperature (15 °C). If free water is available for the germination of the ascospores, infection could very easily occur at 90% RH and increased number of lesions as found with increased periods of high RH (0-5 days).

The pathogen seems very well adapted to exploit leaf wetness interrupted by dry periods. Short interruptions (6 h) of leaf wetness periods at low RH or interruptions by one day at high RH (90%) even stimulate infection. The effect might be partially explained by the presence of large droplets of water on the leaf surface in case of continuous wetting, which may have reduced hyphal contact with the leaf surface (Carisse and Kushalappa, 1992).

Relatively low temperatures (5, 10 °C) seem to stimulate infection, possibly because at suboptimal wetness periods for infection conditions after the wetness periods affect the final disease severity. High temperatures (15, 20 °C) after short wetness periods may result in the death of germinated ascospores. At low temperatures these germinated ascospores might still have a fair chance to penetrate the host even though the wetness period had ended. A second reason may be a stimulating effect on branching of the germination hyphae by relatively low temperatures (Whiteside, 1972; 1974). In this way more penetrations would occur and hence higher lesion numbers will be found. Relatively high temperatures (15, 20 °C) favour colonization of the host, resulting in shorter incubation periods and larger lesions. This effect is not uncommon and has been shown with other *Mycosphaerella* species (Castor *et al.*, 1977).

Long wetness periods and ring spot

The data presented in chapters 5 and 6 show that the temperature and humidity requirements for infection of cabbage by *M. brassicicola* are complex. Any attempt to define an infection period for *M. brassicicola* in terms of a continuous leaf wetness or a continuous period of high humidity is therefore unlikely to succeed. We have shown that leaf wetness is not as limiting to infection as suggested by other scientists (Götz *et al.*, 1993; Nelson and Pound, 1959; Meier, 1985; Zornbach, 1990). Leaf wetness (rain or dew) plays a dominant role in spore formation and dispersal. Long periods (4 days) of high relative humidity (>98%) are necessary for the formation of ripe ascospores (Dring, 1961; Nelson and Pound, 1959). Spore dispersal is stimulated by wetness, either rainfall or dew (Hartill, 1977; Hartill and Sutton, 1980; Humpherson-Jones and O'Brien, 1986; Staunton and Ryan, 1977; Zornbach, 1991).

Thus, the fact that long periods of leaf wetness stimulate the fungus does not only depend on wetness requirements for infection, but is also strongly influenced by the wetness requirements for spore formation and dispersal. Therefore, long periods of wetness (> 2 days), considered as critical periods, might explain the massive appearance of lesions in the field (chapter 7). Environmental conditions in the autumn when temperatures are moderate and humidity inside the crop is relatively high ensure the presence of ripe pseudothecia, and probably result in infection waves after shorter wetness periods (< 2 days).

Incubation and latency

The *in vitro* studies indicated already a long incubation period for ring spot (chapter 5). These findings were confirmed by experiments in the field, which showed that incubation periods of *M. brassicicola* varied between 14-21 days on fully developed Brussels sprouts leaves. The developmental stage of the host and variable weather conditions during the growing season may even result in incubation periods of more than 30 days. Latent

periods have not been well studied. Field observations indicate a latent period of at least 30 days.

Due to the long incubation and latent periods of the fungus the number of generations of *M. brassicicola* in the field is limited. In isolated brassica fields at least 4-5 generations are needed to reach an epidemic level (Zornbach, 1991). So, in most years ring spot will not reach an epidemic level in isolated brassica fields, simply because the number of generations is not enough to reach an epidemic level. Two conditions would change the situation drastically, the presence of initial inoculum such as infested plant material (chapter 1) and yearround cultivation in regions with intensive brassica growing (Gladders, 1993; Van den Ende, 1993; Zornbach 1990) would change the situation drastically. Early infections in brassica crops may result in a rapid build up of inoculum in the crop, resulting in severe infestations at the end of the growing season (chapter 1).

8.3 How to optimize the present control strategy?

In the past, control treatments against ring spot were calender based. It was the common feeling that improved disease control could be achieved by well timed fungicide sprayings. Wetness requirements for infection were available from the literature and seemed so predominant that a prediction system could be operated reasonably well on the basis of meteorological data alone. Similar prediction system have been used in other diseases such as *Botrytis* blight (Bastiaansen *et al.*, 1997; Van den Ende *et al.*, 1998). This approach suffers from the obvious objection that if conditions permit infection but spores are not available unnecessary sprays may be applied. Hence, for most diseases biological observations are not just a desirable but an essential supplement to weather information (Campbell and Madden, 1990) and this is certainly the case with ring spot.

The present warning system recommends to use preventive fungicides (benomyl or carbendazim) after two days of at least 18 hours of 90% RH or leaf wetness. Both fungicides prevent germination of ascospores of ring spot, but will hardly prevent the colonization of tissue after penetration. When conditions in autumn are optimal for spore formation and dispersal, it can be expected that infection of brassica tissue will occur even within 24 hours. Thus, the input of the above mentioned fungicides becomes questionable, in relation to the warning system. If the present warning system can be improved by incorporating the data on infection and incubation of ring spot in brassica crops presented in this thesis, the preventive fungicides can be used more efficiently.

The curative fungicide pyrifenox is also reecommended as it has the strong advantage that lesion present in the leaves can be destroyed (chapter 7). Thus, it is not necessary to use the warning system, as growers can await the appearance of the first lesions before they decide to spray or not.

Some growers do not want to use the relatively expensive pyrifenox for ring spot control. However, one should be aware of the fact that repeated fungicide applications with

carbendazim and benomyl may result in the development of resistance in the fungus, as has been shown in other studies (Gladders *et al.*, 1992; Wicks *et al.*, 1987). Resistance of ring spot against pyrifenox has not yet been reported.

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Summary

Ring spot disease of brassica crops, caused by the fungus *Mycosphaerella brassicicola* is a major disease in cabbage, Brussels sprouts and cauliflower in the Netherlands. Severe infestations lead to early leaf senescence and defoliation and result in quantitative and qualitative damage at harvest time. The aim of the research presented in this thesis was twofold: to develop a routine screening method for resistance against *M. brassicicola* in *Brassica oleracea* (chapters 2,3,4) and to determine effects of environmental factors on the life cycle of ring spot in order to optimize disease control in the field (chapters 5, 6, 7).

The general introduction (**Chapter 1**) presents a detailed account of symptomatology, host range, life cycle and control strategy of the fungus. *M. brassicicola* is able to produce typical circular lesions on a wide range of brassica crops and some cruciferous weeds. Symptoms of the disease appear on all aerial plant organs, but usually mature foliage is most heavily affected. For dispersal and infection *M. brassicicola* depends on ascospores. No imperfect stage of the fungus is known. Although it is general accepted that wetness plays a dominant role in the life cycle of the fungus, reported quantitative effects on sub-processes of the life cycle are not well studied or contradictory. Fungicides are used to control ring spot, often in calender based spray schedules. Improvement of this control strategy with a reduced input of chemicals can be found in resistance breeding and timing of fungicide applications in relation to the development of the fungus.

At the start of the project breeding of resistant cultivars was hampered by the time-consuming procedure of the available screening method in fully grown crops under field conditions. Seedling tests were not used in screening as it was assumed that young plants of brassica crops were not susceptible. **Chapter 2** presents results on the artificial inoculation of seedlings of cabbage under field condition. Seedlings of three cultivars of cabbage with known and different levels of resistance were grown in a seedbed, to which infected plant debris was added as an inoculum. Differences in disease severity on the cotyledons and the first two leaves of the seedlings reflected the adult plant resistance.

This result opened the way for the development of a greenhouse screening method for resistance to ring spot in *B. oleracea* as described in **chapter 3.** To overcome the difficulties with the *in vitro* production of spores of *M. brassicicola* a new inoculation method was developed. High infection levels were achieved by spraying young plants with mycelial inoculum enriched with 3% sucrose. The screening method was tested on 9 cultivars, 3 Brussels sprouts, 3 cabbage and 3 cauliflower, respectively, with known reactions to ring spot in the field. Resistance was expressed both in cotyledons and true leaves by a lower number of lesions than the susceptible control and/or by hypersensitive reactions. Results of the tests reflected differences in resistance in the field. Testing plants in the third leaf stage gave the best results, as the resistance reactions were more clear

and plants could better withstand the long wetness periods necessary for infection than the cotyledons. The developed screening method was tested on different locations by potential users. Although much variability existed in disease levels at the different locations, resistant and highly susceptible cultivars could be easily distinguished. Under controlled conditions the greenhouse screening test can be applied year-round to young plants thus accelerating selection procedures.

The greenhouse screening method was used to test isolates of *Mycosphaerella brassicicola*, originating from various locations in Europe on a differential set of brassica cultivars (**chapter 4**). The isolates differed in their virulence, as measured by the number of lesions per leaf on the different cultivars. Hypersensitivity and significant cultivar-isolate effects were observed, indicating a differential host-pathogen interaction. Although expression of resistance depends on plant development, the differential host-pathogen interaction was found in all plant stages tested. The existence of physiological specialization of *M. brassicicola* was not known yet, and should be taken into consideration when breeding for resistance in brassica crops.

Using a new method for the collection of ascospores from diseased leaf material (**chapter 5**) it was possible to study effects of temperature and humidity on plants under controlled conditions after inoculation with an ascospore suspension. The results showed that the minimum humidity requirements for infection were different when plants were inoculated with ascospores instead of mycelial inoculum. Inoculation with mycelial fragments is only successful if a long period (4-5 days) of leaf wetness is present. Inoculation experiments with ascospores showed that infection of brassica plants could occur at shorter leaf wetness periods.

Quantitative data on the effects of wetness duration and temperature on infection of cabbage by ascospores of *M. brassicicola* are presented in **chapter 6**. Despite the great variability in the data general conclusions can be drawn. Ascospores are able to infect cabbage within 24 h of leaf wetness at temperatures from 5-20 °C. Lesion number and lesion size increase with increasing wetness period at all temperatures tested. Decreasing temperature during leaf wetness resulted in higher lesion numbers but reduced lesion size. Interruption of leaf wetness by dry periods did not necessarily lead to reduction of infection, it even could stimulate infection. Infection also occurred within 24 h of high relative humidity (90%) at 15 °C. Lesion number increases with increasing periods of high relative humidity. Interruption of periods of relative high humidity by dry periods (RH 60%) reduced infection. The complexity of the effects made clear that definition of simple guidelines for infection based on continuous wetness or high humidity is questionable.

Experiments have been carried out to gain insight in the length of the incubation and latent period under field conditions (**chapter 7**). Artificial inoculations of Brussels sprouts plants with ascospores of *M. brassicicola* under field conditions indicated an incubation period of 14-21 days on fully grown leaves. The minimum length of the latent period varied between 31 and 41 days. Analysis of the development of an epidemic of ring spot showed similar results. The incubation period varied from 13-33 days, dependent on the growth stage of the crop and the environmental conditions following critical periods for infection in the field. In practice, a warning system is used based on the effects of temperature and humidity in relation to infection of brassica crops by ring spot. It is argued that such a warning system leads to superfluous fungicide use as the length of the incubation and latent period are not taken into account.

A general discussion of the results presented in this thesis is given in **chapter 8**. It is concluded that the developed greenhouse screening test offers the potential for rapid determination of suitability of *B. oleracea* selections for inclusion in a breeding and selection program. Assessment of partial resistance in young plants of brassica cultivars remains difficult. In breeding for resistance one should be aware of the fact that physiological specialization occurs in *M. brassicicola*.
Further, it is concluded that reported quantitative data in literature on the effects of wetness duration and temperature on infection of brassica crops by *M. brassicicola* are questionable. Results from studies with ascospore inoculum differ from those with mycelial inoculum, as carried out in the past. Data presented in this study provide a better insight in temperature and wetness requirements for infection of brassica crops by *M. brassicicola*. Field studies indicate that the fungus has long incubation and latent periods, which should be taken into consideration when applying fungicides to control the disease.

Samenvatting

De ringvlekkenziekte van brassica gewassen, veroorzaakt door de schimmel *Mycosphaerella brassicicola*, is één van de belangrijkste ziekten in sluitkool, spruitkool en bloemkool. Zware aantasting leidt tot vroegtijdig bladverwelking en bladafsterving, en geeft kwalitatieve en kwantitatieve opbrengstverliezen. Het doel van het onderzoek gepresenteerd in dit proefschrift was tweeledig: het ontwikkelen van een praktische resistentietoets voor *M. brassicicola* in *Brassica oleracea* en het bepalen van effecten van omgevingsfactoren op de levenscyclus van *M. brassicicola*, zodat de bestrijding onder veldomstandigheden geoptimaliseerd kan worden.

De algemene inleiding (**hoofdstuk 1**) geeft een gedetailleerde beschrijving van de symptomologie, de waardplantenreeks, de levenscyclus en de bestrijdingswijze van de schimmel. *M. brassicicola* produceert karakteristieke ronde lesies op vele brassica soorten en een aantal crucifere onkruiden. Symptomen komen voor op de gehele plant, maar meestal zijn de oudere bladeren het zwaarst aangetast. De schimmel heeft geen imperfect stadium en is voor de verspreiding en vermenigvuldiging afhankelijk van ascosporen. Hoewel algemeen wordt aangenomen dat vocht een belangrijke rol speelt in de levenscyclus van de schimmel, zijn de gerapporteerde kwantitatieve effecten op de levenscyclus slecht bestudeerd of tegenstrijdig. De ringvlekkenziekte wordt bestreden met fungiciden, vaak in kalenderbespuitingen. Verbetering van de bestrijding gericht op een verminderd middelengebruik kan bereikt worden door resistentieveredeling en door het tijdstip van bespuiten afhankelijk te maken van de ontwikkeling van de schimmel.

Bij de start van het project werd veredeling van resistente cultivars bemoeilijkt door de tijdrovende methode om in volgroeide gewassen onder veldomstandigheden resistentie te toetsen. Toetsen met jonge planten werden niet gebruikt omdat werd aangenomen dat jonge brassica planten ongevoelig waren voor *M. brassicicola*. In **hoofdstuk 2** staan resultaten van kunstmatige infectie van jonge koolplanten onder veldomstandigheden. Jonge planten van drie koolcultivars die verschilden in gevoeligheid voor de ringvlekkenziekte, werden gekweekt in een kiembed waaraan geïnfecteerde gewasresten van kool was toegevoegd als inoculum. Aantastingsverschillen op cotylen en de twee eerste bladeren van de jonge koolplanten kwamen overeen met de gevoeligheidsverschillen zoals die in volwassen planten was bepaald.

Dit resultaat maakte de ontwikkeling van een resistentietoets mogelijk voor de bepaling van gevoeligheidsverschillen in *B. oleracea* voor de ringvlekkenziekte zoals beschreven in **hoofdstuk 3**. Een nieuwe inoculatiemethode werd ontwikkeld om de problemen met *in vitro* productie van ascosporen van *M. brassicicola* te ondervangen. Wanneer jonge planten besproeid werden met een mycelium suspensie verrijkt met 3% sucrose konden hoge aantastingniveau's worden bereikt. De resistentietoets werd getest op 9 rassen: 3 spruitkool-, 3 sluitkool- en 3 bloemkoolrassen met een bekende veldresistentie voor de

ringvlekkenziekte. Resistentie kwam tot uiting op de cotylen en bladeren door een lager aantal lesies dan de gevoelige controle en/of door de aanwezigheid van overgevoeligheidsreacties. De resultaten van de resistentietoets kwamen overeen met verschillen in resistentie gevonden onder veldomstandigheden. Het toetsen van planten in het "drie-blad" stadium leverde de beste resultaten. De resistentiereacties waren duidelijker en de jonge planten waren in vergelijking tot de cotylen beter bestand tegen de langdurige vochtperiode die noodzakelijk was om infectie te verkrijgen. De resistentietoets werd getest op verschillende locaties door potentiële gebruikers. Hoewel er sprake was van veel variatie in aantastingsniveaus op de verschillende locaties, konden zeer gevoelige en resistente cultivars duidelijk worden onderscheiden. Onder gecontroleerde omstandigheden kan de resistentietoets op jonge planten het gehele jaar worden gebruikt, waarmee de selectie en veredeling van resistente rassen versneld kan worden

De resistentietoets werd gebruikt om verschillende isolaten van *M. brassicicola* afkomstig uit diverse gebieden in Europa te testen op een differentiërende reeks van brassica rassen (**hoofdstuk 4**). De isolaten verschilden in virulentie, gemeten in aantal lesies per blad op de verschillende cultivars. Overgevoeligheidsreacties en significante ras-isolaat-interacties werden waargenomen, hetgeen duidde op een differentiërende waard-pathogeen-interactie. Hoewel resistentie afhankelijk was van plantontwikkeling werd de differentiërende waard-pathogeen-interactie aangetroffen in allle getoetste plantstadia. Het bestaan van fysiologische specialisatie bij *M. brassicicola* was niet bekend. Men zal hier bij de resistentieveredeling in brassica gewassen rekening mee moeten houden.

Door gebruik te maken van een nieuwe methode voor de verzameling van ascosporen uit aangetast bladmateriaal (**hoofdstuk 5**) was het mogelijk om de effecten te bestuderen van temperatuur en vocht na inoculatie van planten met ascoporen. De resultaten toonden aan dat de voor infectie noodzakelijke lengte van de bladnatperiode afhankelijk was van het type inoculum. Aantasting na bespuitingen met myceliumsuspensies werd alleen verkregen na langdurige bladnatperioden (4-5 dagen). Experimenten met ascosporeninoculum toonden aan dat de benodigde bladnatperiode voor infectie van koolplanten korter was.

Kwantitatieve gegevens over de effecten van bladnatduur en temperatuur op infectie van sluitkool door ascosporen van *M. brassicicola* worden gepresenteerd in **hoofdstuk 6**. Ondanks de grote variatie in de gegevens konden algemene conclusies getrokken worden. Ascosporen zijn in staat om koolplanten binnen 24 uur bladnat te infecteren bij temperaturen van 5-20 °C. Het aantal lesies en de lesiegrootte nam toe met toenemende duur van de bladnatperiode bij alle getoetste temperaturen. Verlaging van de temperatuur resulteerde in een hoger aantal lesies maar een verminderde lesiegroei. Onderbreking van de bladnatperiode leidde niet direct tot verminderde infectie; de infectie kon zelfs gestimuleerd worden. Infectie kon ook ontstaan bij 24 uur hoge relatieve luchtvochtigheid

(90%) bij 15 °C. Het aantal lesies nam toe bij een toenemende relatieve luchtvochtigheid. Onderbreking van perioden van hoge relatieve luchtvochtigheid door perioden met een lage relatieve luchtvochtigheid (60%) verminderde de aantasting. De complexiteit van de waargenomen effecten maakt duidelijk dat het definiëren van simpele richtlijnen voor infectie gebaseerd op continue perioden bladnat of hoge luchtvochtigheid dubieus is.

Om de lengte van de incubatie- en latentieperiode te bepalen onder veldomstandigheden zijn experimenten uitgevoerd (**hoofdstuk 7**). Kunstmatige infectie van spruitkoolplanten met ascoporen van *M. brassicicola* leidde onder veldomstandigheden tot een incubatieperiode van 14-21 dagen op volgroeide bladeren. De minimumlengte van de latentieperiode varieerde tussen de 31 en 41 dagen. De analyse van de ontwikkeling van een epidemie van de ringvlekkenziekte leidde tot vergelijkbare resultaten. De incubatieperiode varieerde van 13-33 dagen, afhankelijk van het groeistadium van het gewas en de klimatologische omstandigheden.

In de praktijk wordt een waarschuwingssysteem gebruikt op basis van de effecten van temperatuur en vocht in relatie tot infectie van brassica gewassen door de ringvlekkenziekte. Er wordt beargumenteerd dat een dergelijk waarschuwingssysteem leidt tot overbodige fungicidebespuitingen, aangezien de lengte van de incubatieperiode en de latentieperiode niet in beschouwing worden genomen.

Een algemene discussie van de resultaten van dit proefschrift staat in **hoofdstuk 8**. Er wordt geconcludeerd dat de ontwikkelde resistentietoets de mogelijkheid biedt om snel geschikte *B. oleracea* selecties te identificeren om te gebruiken in een veredelingsprogramma. Het bepalen van partiële resistentie in jonge planten van brassica rassen blijft moeilijk. In de resistentieveredeling dient men rekening te houden met het voorkomen van fysiologische specialisatie in *M. brassicicola*.

Vervolgens wordt geconcludeerd dat de kwantitatieve literatuurgegevens over de effecten van bladnatduur en temperatuur op infectie van brassica gewassen door *M. brassicicola* discutabel zijn. Resultaten van onderzoek met ascosporeninoculum waren duidelijk verschillend van myceliuminoculum, zoals in het verleden werd uitgevoerd. De gegevens die in dit onderzoek worden gepresenteerd geven een beter inzicht in de temperatuur- en bladnat-benodigheden voor infectie van brassica gewassen door *M. brassicicola*. Uit veldonderzoek bleek dat de schimmel een lange incubatie- en latentieperiode heeft, hetgeen in acht genomen moet worden bij fungicidebespuitingen.

Curriculum vitae

Johannes Ernst van den Ende werd geboren op 7 december 1962 te Hattem. Na het VWO-diploma begon hij in 1982 een studie Plantenziektenkunde aan de Landbouwuniversiteit in Wageningen. De doctoraalfase bevatte afstudeervakken bij de vakgroepen Fytopathologie en Entomologie. In 1986 deed hij gedurende 7 maanden onderzoek bij het Malang Research Insititute for Food Crops (Indonesië) naar populatiedynamica en natuurlijke vijanden van diverse insektenplagen in de soja teelt. Na het behalen van het doctoraaldiploma in 1988 werkte hij gedurende vier jaar als assistent in opleiding (AIO) bij de vakgroep Fytopathologie aan het in dit proefschrift beschreven onderzoek. Eind 1992 startte hij als wetenschappelijk onderzoeker Entomologie bij het Proefstation voor de Fruitteelt te Wilhelminadorp. Zijn onderzoek richtte zich op verbetering en implementatie van GABY, een computer adviessysteem voor de geïntegreerde bestrijding van insektenplagen in de fruitteelt. In februari 1995 werd hij aangesteld als wetenschappelijk onderzoeker Fytopathologie bij het Laboratorium voor Bloembollenonderzoek in Lisse. Naast bestudering van epidemiologische aspecten van *Botrytis* sp. in bloembolgewassen is een deel van zijn taak gericht op ontwikkeling en implementatie van een waarschuwingssysteem voor de bestrijding van *Botrytis* sp. in lelie, tulp en gladiool.

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..... en nu moet papa slapen, en Jikke achter de petjoeter

Jikke van den Ende, juli 1998