Clubroot in the cole crops: the interaction between *Plasmodiophora brassicae* and *Brassica oleracea*

Knolvoet in de koolgewassen: de interactie tussen *Plasmodiophora brassicae* en *Brassica oleracea*

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Abstract

The clubroot disease of the cole crops (*Brassica oleracea*) and other crucifers is caused by the fungus *Plasmodiophora brassicae*. It is an important disease, affecting an estimated 10 % of the total cultured area world-wide. The potential of cultural practices to reduce crop losses due to clubroot are limited, and chemical treatments to control the fungus are either banned due to environmental regulations or are not cost effective. Breeding of resistant cultivars therefore is an interesting alternative. This thesis addresses some aspects of the *P. brassicae* - *B. oleracea* interaction associated with resistance breeding.

A seedling test for clubroot resistance was developed. Symptom development in this test was shown to correlate well with symptom development in the field situation. The seedling test was used to identify *B. oleracea* accessions resistant to a Dutch field isolate of clubroot.

Resistance to the earliest stages of development of *P. brassicae* occurring in root hairs was shown not to be correlated with resistance to the development of clubroot symptoms. Presumably, only an absolute resistance to root hair stages of *P. brassicae* would prevent further symptom development, but no such absolute resistance was found.

The probability of infection of a plant by individual spores of *P. brassicae* was studied in a series of experiments with varying inoculum densities. The probability could be well described by a one-hit model, involving no between-spore interactions. A large between-test variation in the infection probability was shown to occur, even between tests performed in the same climate room.

Two single-spore isolates (SSIs) obtained from one field isolate of *P. brassicae* proved to be indistinguishable from each other by testing their interactions with a series of 21 differential accessions, but to differ from the field isolate in their interaction with four of those hosts. The pathotype of those two SSIs was shown to occur at a low frequency in the field isolate. The fact that both SSIs contained the same pathotype indicated that pathotypes may differ in their likelihood to be isolated as SSIs. Results of inoculation experiments with mixtures of field isolate and one of the SSIs suggested that in some hosts resistance to clubroot may be induced by certain pathotypes present in the field isolate.

From four clubroot-resistant accessions doubled haploid lines (DH-lines) were obtained through microspore culture and resistant DH-lines were selected. Progenies (F_1, F_2, backcrosses and a DH-population derived from one F_1) of crosses of each of these four resistant DH-lines with a susceptible DH-line were obtained. Segregation for resistance in these progenies was studied and several genetic models were fitted to the observations. Classical genetic studies indicated evidence for one or two major resistance genes in three of the four resistant parental DH-lines. For the fourth resistant x susceptible cross, a genomic map was constructed based on RFLP and AFLP markers scored in a population of DH-lines obtained from the F_1. Two major resistance genes were mapped in this

population. Evidence for the presence of at least one more resistance gene in the resistant parent was also found.

This study indicates that markers are a very useful tool to optimize breeding programmes for resistance to clubroot. The value of each gene has to be evaluated for specific growing regions as populations of *P. brassicae* may differ in their pathogenicity.

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Outline of the thesis

This thesis is the result of research intended to support the breeding of cultivars of the cole crops resistant to clubroot in The Netherlands. Several topics in plant pathology and plant resistance concerning the interaction between the host and the pathogen are addressed. The main issue of the thesis is the genetic control of various forms of resistance to clubroot found in *Brassica oleracea*. While developing the necessary populations and methods several aspects of the *P. brassicae* - *B. oleracea* interaction were investigated, and reported in various chapters.

In Chapter 1 an overview is presented of the recent literature concerning the clubroot disease in *B. oleracea* and its causal agent, the fungus *Plasmodiophora brassicae*.

Chapter 2 addresses the development of a clubroot resistance test method which can be applied under controlled conditions, in a greenhouse or phytotron chamber. The correspondence of the test results with those obtained under field conditions is considered. Further, the application of the test to screen various accessions for resistance is described.

In Chapter 3, the development of a test for resistance to a particular developmental stage (the root-hair stage) of the pathogen is described. For 13 *Brassica* accessions the results of this test are compared with those of the seedling test described in the previous chapter.

In Chapter 4, models for the probability of plant infection by fungal spores are presented, and their success in explaining the results of inoculation tests with varying amounts of fungal spores is discussed.

In Chapter 5, the production and characterization of single-spore isolates by microscopical isolation of spores is described. The results of mixed inoculations with field and single-spore isolates are also discussed.

Chapters 6 and 7 deal with the observed segregation of clubroot resistance in various progenies of crosses between resistant and susceptible parents. For four different sources of clubroot resistance conclusions about the genetic control of the resistance are presented.

Chapter 8 again addresses the genetic control of clubroot resistance in one of the parents studied in the previous two chapters. In this case molecular markers are used to locate genes for clubroot resistance on a map of the genome and to assess their individual effects.

Finally, the General Discussion aims to integrate results obtained during this project and to indicate opportunities for research and breeding.

Chapter 1

Plasmodiophora brassicae: aspects of pathogenesis and resistance in *Brassica oleracea*

Abstract

Clubroot is one of the most damaging diseases in *Brassica oleracea* crops world-wide. The pathogenicity of *Plasmodiophora brassicae* is highly variable between as well as within field populations. Several sources of resistance to clubroot have been identified in *B. oleracea*. Generally, resistance tends to inherit partly as a recessive, partly as an additive trait, and appears to be controlled by few major genes. Progress in the understanding of the inheritance of resistance is being made through the use of single-spore isolates of the pathogen, and the use of molecular markers for resistance genes.

Abbreviations: cv: cultivar; DH: doubled haploid; ECD: European Clubroot Differential set; RFLP: Restriction Fragment Length Polymorphism

Introduction

Clubroot, caused by the fungus *Plasmodiophora brassicae* Wor., is probably the most damaging disease of cole crops (*Brassica oleracea* L.) and other cruciferous crops worldwide. The pathogen causes swelling of parts of the roots and sometimes of the stem base into characteristic clubs. These clubs inhibit nutrient and water transport, stunt the growth of the plant and increase the susceptibility to wilting. After some weeks the clubbed roots decay, weakening the support of the plant.

The incidence of clubroot was discussed by Crête (1981). He estimated that in Northwestern Europe, Japan, North America and Australia, about 10% of a total of 660.000 ha of *B. oleracea* crops was infested with clubroot. For all cruciferous crops, the infested area in those regions amounted to 196.000 ha. The spores can remain infectious for at least 15 years (Mattusch, 1977). Cultural practices, especially the application of calcium and boron, and liming to decrease the pH of the soil, may reduce disease pressure, but are often not sufficient to keep the crop healthy. The effects of chemical control are limited, since most treatments are either banned due to environmental regulations, or are too expensive.

The introduction of cultivars with resistance or tolerance to clubroot would be desirable. Nevertheless, only a very small number of resistant cultivars have been released. Breeding programmes aimed at the introduction of resistance to clubroot have been few,

and the results of these programmes were often disappointing. This general lack of success is due to the relatively small number of sources of resistance, the recessive and often apparently complex inheritance of resistance, and the genetic variability of the pathogen.

In this paper the literature on resistance to clubroot in *Brassica oleracea* is reviewed. In addition, relevant studies of the biology of *P. brassicae* and results obtained in host species other than *B. oleracea* are discussed. Earlier reviews of Colhoun (1958), Karling (1968) and Crute et al. (1980) have been complemented with more recent publications.

Terminology

Some terms have been employed ambiguously in clubroot literature. To prevent misinterpretation, they are briefly introduced here.

A *field isolate* is a population of the pathogen obtained either directly from the soil of an infested field, or from clubs of susceptible plants grown in the field, and maintained in isolation on plants not carrying any known resistance. A *single-spore isolate* is a population derived from clubs of a plant inoculated with one single resting spore and maintained in isolation.

Pathogenicity is the ability of a pathogen population to cause clubroot symptoms on host plants. *Differential pathogenicity* (*sensu* Crute et al., 1980) indicates different ranking of pathogenicity of populations in dependence of the host genotype. Conversely, the relative ranking of populations exhibiting *non-differential pathogenicity* does not depend on the host genotype.

Resistance to clubroot indicates the ability of a plant genotype to limit the development of clubs on the roots, when infected by *P. brassicae*. *Partial resistance* and *complete resistance* refer to the level of reduction of club formation in comparison with a completely susceptible genotype. *Differential* and *non-differential resistance* are defined analogous to differential and non-differential pathogenicity.

Pathogenesis

The pathogenesis of clubroot is well described by Ingram & Tommerup (1972). The disease cycle can be roughly divided into two stages, the first occurring in the root hairs and the second in the root cortex.

The first stage starts with the germination of a primary zoospore from a haploid resting spore in the soil. The zoospore attaches to a root hair and injects its cell contents into the host cell (Aist and Williams, 1971). In the infected root hair, repeated nuclear divisions of the pathogen lead to a multinucleate plasmodium, which later develops into

tens or hundreds of uninucleate zoosporangia. In contrast to the second stage, the development of *P. brassicae* in root hairs into zoosporangia has also been observed in non-crucifers, including some monocotyledons (Webb, 1949; MacFarlane, 1952; Kole and Philipsen, 1956). From the zoosporangia, haploid secondary zoospores are released. Naiki et al. (1984) showed that secondary zoospores again can infect root hairs, which results in a rapid, asexual propagation of the pathogen.

The second stage in the pathogenesis starts with the secondary zoospores. Two zoospores can fuse, resulting in a dikaryotic zoospore, as reviewed by Ingram and Tommerup (1972). It is not known whether fusion is necessary for infection of the root cortex to occur, nor whether different mating types of *P. brassicae* exist. However, genetically uniform single-spore isolates can complete the disease cycle, implying that either fusion of zoospores is not necessary, or that homothallic genotypes of *P. brassicae* exist.

After infection of the root cortex, the pathogen exists as intracellular, multinucleate plasmodia. Dekhuijzen (1975, 1981) observed that, at least in infected callus tissue, isolated plasmodia did not penetrate the host cell wall. He postulated that the spread of the pathogen occurs mainly by the stimulated division of infected cells, a process which results in the formation of the clubs. The enhanced cell division is thought to be stimulated by the elevated concentrations of cytokinins (Dekhuijzen & Overeem, 1971; Dekhuijzen, 1980) and auxins. The auxins are presumably derived from indole glucosinolates, normally present in roots of crucifers, due to the presence of intracellular *P. brassicae* plasmodia (Butcher et al., 1974, 1976).

Later in development, the haploid nuclei in multinucleate plasmodia fuse in pairs (Tommerup and Ingram, 1971). After meiosis the newly formed diploid nuclei develop into haploid resting spores which are released into the soil when the clubbed roots decay. During this second stage in the pathogenesis as many as 10^{11} resting spores per plant can be produced (Voorrips, unpublished results).

Mechanisms and components of resistance

At any stage in the life cycle, host resistance could conceivably block or hamper pathogen development. Investigations have focussed on two stages: the infection of root hairs by primary zoospores, and the production of auxins in the root cortex.

Voorrips (1992) demonstrated large differences in the levels of root hair infection among 13 host genotypes with varying levels of resistance, but observed no correlation between resistance to root hair infection and resistance to clubroot development. Conceivably, even a very low survival of *P. brassicae* during the root hair stage allowed infection of the root cortex. Only complete resistance to root hair infection, which was not observed in this study, would confer resistance to clubroot.

If the enhanced auxin levels present in infected roots are indeed processed from glucobrassicin and other indole glucosinolates (Butcher et al., 1974, 1976), then host genotypes producing no indole glucosinolates might be (non-differentially) resistant to clubroot. However, the evidence for a correlation between indole glucosinolate content and clubroot susceptibility is conflicting. Butcher et al. (1976) and Ockendon and Buczacki (1979) found clear correlations between resistance and low indole glucosinolate content among cruciferous species. Chong et al. (1981, 1984) obtained similar results with cabbage breeding lines. In contrast, Mullin et al. (1980) found no such correlation in 43 rutabaga and turnip cultivars. Ludwig-Müller et al. (1993) found no difference in indole glucosinolate content between control plants of resistant and susceptible Chinese cabbage, but found an enhanced level in infected plants of the susceptible genotype. Rausch et al. (1983) proposed that only very small amounts of auxins, and therefore of indole glucosinolates are needed for club formation. So, although presumably the auxin production from indole glucosinolates is an important factor in the development of clubroot, no general relation between indole glucosinolate content and clubroot resistance has been found.

Thus, although processes occurring during pathogenesis are known in some detail, the mechanisms responsible for resistance have not yet been elucidated.

Pathogenicity of *P. brassicae*

Field populations of *P. brassicae* exhibit clear differences for pathogenicity. Several test series of host genotypes carrying resistance from different sources have been employed to classify populations with respect to differential pathogenicity (reviewed by Crute et al., 1980). Currently the differential series of Williams (1966) and the European Clubroot Differential set (ECD, Buczacki et al., 1975) are commonly used.

It was first shown by Haji Tinggal (1980, cited in Haji Tinggal & Webster, 1981) and Jones et al. (1982a) that field isolates were not genetically uniform, as they found differences between inocula obtained from different slices of the same club or from clubs of different plants from the same field. The variation within field isolates was even more clearly demonstrated by using single-spore isolates (Buczacki, 1977; Haji Tinggal & Webster, 1981; Jones et al., 1982b; Scott, 1985; Schoeller & Grunewaldt, 1986). Since resting spores are haploid (Tommerup & Ingram, 1971), single-spore isolates are genetically uniform except for mutations. Frequently single-spore isolates were obtained which were less pathogenic than the original field isolate. This could be indicative of segregating pathogenicity genes in the pathogen population. However, Jones et al. (1982b) also obtained single-spore isolates pathogenic on host genotypes that were resistant to the original field isolate. Possibly genotypes with rare pathogenicity genes were extracted from the population, perhaps even selected for by the isolation procedure.

Jones et al. (1982b) showed that a pathogenic isolate was inhibited by a non-pathogenic isolate, suggesting competition for infection sites or host resources. Another explanation may be a rapidly induced resistance, as described in other host-pathogen interactions (*e.g.* Ward, 1983).

The existence of genetic variation for pathogenicity within field populations is also demonstrated by the response of field isolates to selection pressure. Crute & Pink (1989) showed that five successive passages of a field isolate through a partially resistant line of Böhmerwaldkohl yielded an isolate with a higher pathogenicity on the resistant line as well as on a susceptible control. Also, the erosion of the resistance of cabbage cv Badger Shipper within a few years after its release was probably due to selection of pathogenic genotypes (Seaman et al., 1963). In absence of the resistant host, the pathogenicity of the population in the field towards Badger Shipper diminished again (Seaman et al., 1963), supporting the hypothesis of reduced fitness of genotypes carrying pathogenicity genes.

This genetic variation implies that the characterization of a field isolate using a differential test series is in fact only valid for the inoculum used in the test. Other clubs, and especially clubs obtained after further propagation of the isolate may contain a population with different pathogenicity. It is therefore essential that relevant control host genotypes are included in every clubroot test. The concept of races is not very effective in the case of field isolates of clubroot, since neither the pathogen populations nor the differential hosts fulfill the necessary conditions of genetic uniformity and stability (Parlevliet, 1985). Rather, a system is required based on identified pathogenicity and resistance genes.

Progress in this area may become possible by the use of host plants carrying well characterized resistance genes, and by studying genetically uniform single-spore isolates. The advantages of single-spore isolates are the stability of the genotype of the isolate over propagation cycles, and the absence of interaction between different pathogen genotypes. Meanwhile, it must be recognized that single-spore isolates may represent atypical pathogen genotypes. It will therefore be necessary to compare the results obtained with single-spore isolates, with those obtained with the original field isolates and mixtures of single-spore isolates.

Sources of resistance to clubroot in *B. oleracea*.

Contrary to the situation in *B. rapa* and *B. napus*, completely resistant accessions have only rarely been described in *B. oleracea*. This has stimulated much screening work in the cole crops, uncovering several accessions partially resistant to field isolates (Table 1). Most of these accessions were cabbage, kale or curly kale types. In other cole crops, only low levels of resistance were found (e.g. Dixon & Robinson, 1986; Crisp et al., 1989; Voorrips

Table 1. Original sources of resistance to clubroot and derived lines and cultivars in *Brassica oleracea*.

Original source	Lines and Cultivars	References
Böhmerwaldkohl and Bindsachsener	Respla, Resista	Gante, 1951; Weisaeth, 1977
Irish fodder cabbage		Crisp et al., 1989; Crute & Pink, 1989
Shetland fodder cabbage		Dennis & Gray, 1954
Russian cabbage	cabbage cultivars	Giessmann & Bauch, 1974
unknown cabbage[a]	cabbage lines Oregon-100, -123, -140, -142	Baggett, 1983
unknown kale[b]	cvs Badger Shipper, Richelain, cabbage line 8-41[e]	Walker & Larson, 1960; Chiang & Crête, 1989
curly kale	curly kale cultivars	Nieuwhof & Wiering, 1962; Voorrips & Visser, 1993
B. napus cv Wilhelmsburger	cv Richelain, cabbage line 8-41[e]	Karling, 1968; Chiang & Crête, 1989
cauliflower[c]		Catovic-Catani & Rich, 1964
unknown	MSU 134 broccoli	Vriesenga & Honma, 1971
unknown[d]	broccoli lines OSU CR-1 to -8	Baggett, 1976; Baggett & Kean, 1985

[a] Baggett (1983) used an accession obtained from the Institute for Horticultural Plant Breeding, the Netherlands (now CPRO-DLO) as source of resistance. This accession was most likely derived from Bindsachsener, Böhmerwaldkohl or from the breeding programme leading to cv Badger Shipper, since those were the only clubroot-resistant cabbage accessions available at the institute at that time.

[b] Cabbage cv Badger Shipper was derived from a chance cabbage x kale hybrid

[c] The results of Catovic-Catani & Rich (1964) were based on only five plants per accession and were therefore questionable.

[d] The clubroot resistant parent used by Baggett (1976) was a leafy annual with small, loose flower heads of unknown origin.

[e] Cabbage line 8-41 and the derived cv Richelain were descended from both *B. napus* cv Wilhelmsburger and, via cv Badger Shipper, from an unknown kale accession

& Visser, 1993). Several highly resistant lines were bred from some of the resistant accessions. However, the cultivars carrying clubroot resistance have found only limited application, because of an insufficient level of resistance, a quick erosion of resistance, or because of insufficient quality.

Voorrips & Visser (1990) produced doubled haploid lines (DH-lines) from partially resistant cabbage and kale accessions. Some of these DH-lines were almost completely resistant. This suggested that some accessions described as partially resistant did in fact segregate for resistance. As *B. oleracea* is a cross-fertilizing species many accessions are likely to be heterogeneous. Since the level of resistance of an accession is expressed as the average disease severity of several plants, a partially resistant accession may still harbour genes for complete resistance. It is perhaps unfortunate that research and breeding for clubroot resistance have concentrated on the few sources with high levels

of resistance, and ignored the potential of accessions with low partial resistance. Other sources of resistance could possibly have been identified in this way.

Also of interest is the range of pathogen isolates to which a host genotype is resistant. Toxopeus et al. (1986) reported a summary of ECD tests (Buczacki et al., 1975) involving 299 field isolates, carried out by 18 researchers in different countries. None of the 15 ECD host genotypes was resistant to all field populations tested. The five *B. oleracea* ECD hosts were susceptible to the majority of the isolates. Crute et al. (1983) and Crute (1986) demonstrated that cvs Böhmerwaldkohl, Bindsachsener and Verheul interacted non-differentially with a range of isolates, whereas cv Badger Shipper interacted differentially. Tests of Voorrips and Thomas (unpublished) also indicated that cvs Böhmerwaldkohl and, to a lesser extent, Bindsachsener were partially resistant to different clubroot isolates in Europe. Badger Shipper and related accessions, as well as the OSU broccoli lines (Baggett, 1976; Baggett & Kean, 1985) were highly resistant or highly susceptible, depending on the clubroot isolate. Nevertheless, some field isolates were highly pathogenic to Böhmerwaldkohl and Bindsachsener (Voorrips and Thomas, unpublished). The distinction between differential and non-differential resistance is therefore gradual rather than absolute, and is dependent on the isolates used. The suggestion of Crisp et al. (1989) that genes for differential resistance should be excluded in order to breed for non-differential resistance is therefore likely to be counter-productive. What is really needed is information on the interaction of resistance genes with pathogenicity genes, and information regarding the distribution of pathogenicity genes in pathogen populations. Studies in this field would greatly benefit from the use of well-defined single-spore isolates of *P. brassicae*.

Classical genetic studies of resistance and tolerance

Several classical genetic studies of clubroot resistance in *B. oleracea* have been published, using either diallel crossing schemes, or segregating progenies from crosses between two lines (Table 2). Generally, symptoms were assessed visually and rated on a symptom scale. For genetic analysis, either a qualitative approach was used by classifying plants with low symptom grades as resistant and the others as susceptible, or a quantitative approach based on the symptom grades. Hansen (1989) studied tolerance to clubroot, defined in this case as the yield on infested relative to uninfested test plots in the field. This measure of tolerance has a practical foundation, but it is seriously confounded with resistance.

Most of these studies pointed in the same general direction: the various types of resistance and tolerance inherit as partly or completely recessive traits and are under control of a limited number of major genes. Laurens & Thomas (1993) obtained different results: they found evidence for many dominant alleles for resistance in kale accessions. These kales were selected for resistance to field isolates from Brittany, which are rather

Table 2. Summary of classical genetic studies of clubroot resistance and tolerance in *Brassica oleracea*.

Resistant parents	Populations	Test	Analysis	Conclusions	Reference
cabbage line 8-41	F_2, BC	field	qualitative	2 recessive genes	Chiang & Crête, 1970
cabbage line 8-41, cv Badger Shipper	diallel	glasshouse	quantitative	mainly additive, one recessive gene group	Chiang & Crête, 1976
broccoli MSU 134	F_2	glasshouse	qualitative	1[a] or 2 recessive genes	Vriesenga & Honma, 1971
Böhmerwaldkohl	F_2, BC	glasshouse	quantitative	4 genes[b]	Yoshikawa, 1983
Böhmerwaldkohl	F_2, BC	glasshouse	quantitative	Additive and recessive	Crute & Pink, 1989
kale K269	F_2, BC	glasshouse	qualitative	1 recessive gene	Yoshikawa, 1983
several cabbage accessions	diallel	field	quantitative	additive and recessive	Hansen, 1989
several kale accessions	diallel	field, glasshouse	quantitative	many genes, mostly dominant	Laurens & Thomas, 1993
several kale and cabbage accessions	F_1	glasshouse	qualitative	all recessive	Voorrips & Visser, 1993

[a] Crute et al. (1980) re-interpreted the data of Vriesenga and Honma (1971) and concluded to 1 rather than 2 genes.
[b] Yoshikawa (1983) apparently neglected the fact that his accession of Böhmerwaldkohl was not a pure line, which invalidates his analysis.

different from most European and American isolates (Voorrips and Thomas, unpublished results).

Quantitative genetic theory as applied in the studies mentioned in Table 2 is only applicable to continuous, normally distributed data. With the exception of Hansen (1989) however, all the quantitative genetic studies were based on ordinal data, the symptom grades. It would be interesting to verify the conclusions by analyzing the original data using more appropriate statistical methods (e.g. Jansen, 1991).

Molecular markers for resistance genes

Landry et al. (1990, 1992) were the first to report linkage studies of clubroot resistance genes with genetic markers. They studied an F_2-population from a cross between a cabbage breeding line resistant to race 2 (Williams, 1966) and a susceptible rapid-cycling *B. oleracea* line. The parentage of the resistant line included *B. napus* cv Wilhelmsburger, resistant to races 2, 3, 6 and 7 as well as cv Badger Shipper, resistant to races 1, 3 and 6. The resistance tests were performed with a field isolate designated as race 2, using

the test series of Williams (1966). For the QTL analysis, the symptom ratings were transformed to obtain normally distributed data. Two QTL's for clubroot resistance were mapped, located in two separate linkage groups. Together they explained 61% of the variance in the F_2. Presumably these QTL's originated from *B. napus*, but this was not verified.

Figdore et al. (1993) studied the segregation of RFLP markers and clubroot resistance to race 7 (Williams, 1966) in an F_2 from a cross between a susceptible cauliflower cv and broccoli line OSU CR-7 (Baggett & Kean, 1985), which was resistant to race 7. Symptoms were rated in three grades. Linkage of resistance with RFLP markers was determined using contingency Chi-square tests, appropriate for ordinal data. They found strong evidence for one resistance gene on chromosome 1C, and possibly spurious indications for two other resistance genes. The resistance gene on chromosome 1C was expressed in a semi-dominant way. Treating the symptom ratings as quantitative data, they estimated that this gene accounted for 12.6% of the variance in the F_2.

Breeding perspectives

Selection for clubroot resistance might in some cases be expected to be reasonably efficient in view of the relatively large heritabilities and the small number of major genes involved (Chiang & Crête, 1976; Table 2). However, for practical breeding the partially recessive nature of resistance implies that both parental lines of a resistant F_1-hybrid cultivar need to carry the same resistance genes. Backcrossing recessive alleles into lines involves alternating generations of selfing or of doubled haploids in order to reselect for resistance. The need for those generations could be eliminated by the use of molecular markers for resistance genes. Introgression could be accelerated even further by selecting for several markers from the recurrent parent.

The main problems in breeding resistant cultivars are the large variation for pathogenicity in *P. brassicae*, which may require the incorporation of several resistances in cultivars intended for use in a large area, and the lack of information on the interaction of resistance genes with pathogen populations. These problems may in the near future be addressed more effectively, using techniques such as production of single-spore isolates, microspore culture and molecular mapping of resistance genes. Pathogen isolates can be more precisely characterized by their interaction with plants carrying known resistance genes. For genetic studies, populations of DH-lines derived from resistant x resistant or resistant x susceptible crosses will be more useful than F_2 or backcross populations, as such lines can be tested repeatedly to reduce environmental variation. In addition, resistance to several pathogen isolates, preferably of monospore origin, can be mapped in the same population of DH-lines. In this way the level and specificity of resistance conferred by any resistance gene can be determined. In the

future, it will be worthwhile to combine efforts to characterize genes for clubroot resistance obtained from various sources and to find molecular markers to trace those genes in successive generations, in order that they can be rationally employed in breeding for resistance.

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Chapter 2

Examination of resistance to clubroot in accessions of *Brassica oleracea* using a glasshouse seedling test

Abstract

A glasshouse test was elaborated for assessing large numbers of seedlings of *Brassica oleracea* for resistance to clubroot, a disease caused by the fungus *Plasmodiophora brassicae*. The method offers good control of inoculum density per plant, and requires 6-7 weeks from sowing. The results from the glasshouse test correlated well with field test results. With this method, 71 accessions of *B. oleracea* reported to carry resistance to clubroot, and one susceptible control cultivar were tested with a Dutch clubroot isolate. High levels of resistance were found in several accessions of cabbage, broccoli and curly kale. F_1-populations of resistant cabbage or curly kale x susceptible cabbage were fully susceptible, indicating recessive inheritance of resistance in all cases.

Abbreviations: cv: cultivar; ECD: European Clubroot Differential Set

Introduction

Plasmodiophora brassicae Wor., the causal agent of the clubroot disease of cruciferous crops is a widespread and harmful pathogen. It induces the infected roots to develop large galls, so-called clubs. The structure of vascular elements in the clubbed roots is disrupted (Ikegami and Yamashita, 1983), resulting in a reduced uptake and transport of water and nutrients. The diseased plant wilts and usually has a slightly darker, blue-grey leaf colour. Decaying clubs release large numbers of newly formed resting spores of *P. brassicae* in the soil, which can remain infectious for periods up to 15 years (Mattusch, 1977).

The clubroot disease has been the subject of numerous investigations (Colhoun, 1958; Crute et al., 1980). The occurrence of club formation has been studied in plants grown in naturally and artificially infested fields. More recently, seedling tests were devised for use under glasshouse conditions, using artificial inoculation methods. Several of these seedling test methods were compared by Dixon (1976). Buczacki et al. (1975) proposed a standardized test method and symptom grading scale, and the use of a set of differential hosts known as the European Clubroot Differential Set (ECD).

The research reported here is part of a project aimed at the characterization of genes regulating resistance to Dutch populations of *P. brassicae* and their introduction into modern cultivars. An effort was made to collect and test most of the known sources

of resistance (reviewed by Crute et al., 1980), as well as other genotypes described as potential sources of resistance. The present paper reports experiments to evaluate seedling test methods, to determine the effectiveness of the resistance in collected accessions to a Dutch field population of *P. brassicae*, and to investigate its mode of inheritance.

Materials and methods

Pathogen

A field isolate of *P. brassicae* was obtained from a heavily infested field of the Experimental Station Brabant at Breda, the Netherlands. Large clubs of an unknown, highly susceptible cauliflower cultivar were collected, washed, and stored at -20 °C. This isolate is hereafter designated as the Breda isolate.

Spore suspensions were prepared by macerating frozen clubs in 4-5 volumes demineralized water using an electric blender, and filtering the crude suspensions through four layers of cheese-cloth. Suspensions generally contained $1\text{-}3\cdot10^8$ spores·ml^{-1} and were stored for up to 8 weeks at 4°C before use. Prior to inoculation, they were diluted to 10^7 spores·ml^{-1}.

Plant material

The European Clubroot Differential Set (ECD; Buczacki et al., 1975) was obtained from H. Toxopeus (CPRO-DLO). Cabbage cv. Septa (Bejo Seed b.v., Warmenhuizen, the Netherlands), which is equivalent to ECD host 14 was used as a susceptible control in all tests. In some tests, susceptible Brussels sprouts cv. Leander (Asmer Seeds Ltd, Leicester, UK) was also used.

The 71 accessions screened can be subdivided in 11 groups (Table 2). Bindsachsener and Böhmerwaldkohl were German cabbage landraces of which several accessions were obtained. A number of Russian cabbage cultivars was described as carrying field resistance (Giessmann and Bauch, 1974; Crute and Pink, 1989; N.V. Krasheninnick, VNIISSOK Institute, Moscow, Russia, pers. comm.). The material labeled as 'Wisconsin' was derived from a breeding programme of Walker and Larson at the Wisconsin Agricultural Experimental Station, Madison, USA; it consisted of cv. Badger Shipper (ECD host 11), two lines derived respectively from "Larson 8353 T" and from a cross involving cv. Resistant Detroit (Nieuwhof and Wiering, 1963), and one line derived from a cross involving line 8-41 (Chiang and Crête, 1970). The Oregon cabbage and OSU CR broccoli material were developed by Baggett (1976, 1983) and Baggett and Kean (1985). The Brussels sprouts lines were derived by Nieuwhof at the Institute for Horticultural Plant Breeding (now CPRO-DLO), Wageningen, the Netherlands from crosses with cv.

Bindsachsener. The curly kale material included both open-pollinated and F_1-hybrid cvs. The Portuguese Tronchuda cvs were obtained from local suppliers.

F_1-seeds were obtained by bud-pollination of individual plants grown in insect-free glasshouse compartments.

Test methods

The standard seedling test method, hereafter referred to as "pipette method" was as described by Lamers and Toxopeus (1977), with the following modifications. Seven sets of six 4.5 x 4.5 cm^2 square, 110 ml pots were filled with potting compost (pH 6.0, sterilized by gamma irradiation) and placed on a tray in 1 cm deep water, refilled daily. Up to 30 trays were placed on glasshouse benches warmed to 23 °C, with a minimum air temperature of 18 °C. During the winter period (October-March), daylight was supplemented by 45 $\mu E \cdot m^{-2} \cdot s^{-1}$ radiation supplied by 400 W SON-T lamps for 14 $h \cdot day^{-1}$. No tests were performed in December and January.

One seed was sown per pot at a depth of 2.5 cm. Two ml of a suspension of 10^7 spores$\cdot ml^{-1}$ was pipetted to each pot. The pots were covered for 3 days with black plastic until the seedlings emerged. After 6 to 7 weeks, plants were washed and disease symptoms assessed on a scale of 0-3, according to Buczacki et al. (1975): 0, no swelling visible; 1, very slight swelling, usually confined to lateral roots; 2, moderate swelling on lateral and/or tap roots; 3, severe swelling on lateral and/or tap roots.

In all glasshouse experiments, each treatment or accession was tested in two replicates of 18 and 24 pots respectively. A disease index, ranging from 0 (no symptoms) to 1 (severely affected) was calculated by dividing the mean disease rating of each accession by three. Where necessary, plants were recovered after the test by cutting off the root system, rinsing the plants in running tap water, allowing the cut ends to dry overnight, and planting the cuttings in sterile potting compost at high air humidity.

A preliminary experiment was performed with cvs. Septa and Leander to test the effect of growing the seedlings for 10 days before inoculation. In one half of the experiment seeds were sown 10 days earlier than in the other half. All pots were inoculated on the same day using the pipette method.

In another experiment the pipette method was compared with the root-dip method (Johnston, 1968), using cvs. Septa and Leander. The same spore suspension was diluted to 10^8 spores$\cdot ml^{-1}$ for the root-dip method and to 10^7 spores$\cdot ml^{-1}$ for the pipette method.

Resistance under field conditions was determined by growing seedlings in trays and transplanting them into an infested field. Six accessions were planted in four replicates of 16 plants each. After 4 months the symptoms on the root system were rated in four grades equivalent to those used for the classification of seedling symptoms, and a field disease index was calculated as above.

Data analysis

Most accessions in the collection were tested once, using two replicates per accession. A small number of accessions was studied in two or more tests. The evaluation data of the accessions were collected from eight separate tests, with cv. Septa and an accession of Böhmerwaldkohl used as standards.

For statistical analysis, plants were classified in two groups: "healthy" (symptom grades 0 and 1) and "diseased" (grades 2 and 3). To overcome problems caused by observations equal to 0 % and 100 %, data were transformed to (y+½) and (n+1), where y represents the number of healthy plants and n represents the total number of plants tested (Cox, 1970). These data were analyzed according to a generalized linear model for binomial data (McCullagh and Nelder, 1989).

Results

Development of the seedling test method

A preliminary experiment was performed to investigate the effect of a growing period of 10 days prior to inoculation, as described by Lamers and Toxopeus (1977), on the results of the pipette method. The disease indices of cvs. Septa and Leander were 1.00 and 0.94 respectively when inoculated directly after sowing, and both 0.97 when inoculated 10 days later. Since elimination of the 10-day growing period resulted in a shorter test duration without affecting symptom development, in all further experiments inoculation was performed directly after sowing.

In contrast to the results obtained with the pipette method, the results of the root-dip method (Johnston, 1968) were erratic. About half of the plants treated with the root-dip method remained healthy, while all plants developed severe symptoms when inoculated using the pipette method. Although the root-dip method could possibly have been improved, the pipette method was chosen for further experiments because of its superior control of inoculum load per plant and shorter test duration.

Six accessions with seedling disease indices ranging from 0.03 to 0.98 as established using the pipette method were also tested in the heavily infested field from which the Breda isolate was obtained. Results obtained under glasshouse and field conditions were highly correlated. Spearman's rank correlation coefficient between seedling and field disease indices was 0.83 ($P \leq 0.05$).

Characterization of the P. brassicae field isolate

The Breda isolate used for the resistance tests was characterized using the ECD (Table 1). Most differential hosts showed a reasonably uniform reaction, allowing classification as resistant or susceptible. Intermediate values were however obtained for the disease indices of ECD hosts 08 and 15. Host 08 was not genetically uniform, as was indicated by the

Table 1. Assessment of differential reactions of *Brassica* hosts to the Breda isolate of *Plasmodiophora brassicae*.

ECD host[1]	Symptom scale[2]				n[3]	D.I.[4]
	0	1	2	3		
01	35	0	0	0	35	0.00
02	34	0	0	0	34	20.00
03	33	0	0	0	33	0.00
04	38	0	0	0	38	0.00
05	0	0	0	37	37	1.00
06	0	0	0	40	40	1.00
07	0	1	0	32	33	0.98
08	23	1	5	6	35	0.28
09	36	0	0	0	36	0.00
10	35	0	0	0	35	0.00
11	17	5	2	0	24	0.13
12	0	0	4	32	36	0.96
13	0	0	0	36	36	1.00
14	0	0	0	21	21	1.00
15	10	3	16	7	36	0.52

[1] ECD: European Clubroot Differential set (Buczacki et al., 1975).

[2] Symptom scale: the scale ranges from 0 (no symptoms) to 3 (severely affected). The number of plants in each grade is indicated.

[3] n: the total number of plants tested.

[4] D.I.: Disease Index. The index is calculated by dividing the mean symptom rating by 3.

fact that some inbreds were produced which were highly resistant to the Breda isolate. This host was therefore considered to be resistant. For ECD host 15 such indications of genetic variability for resistance were not obtained. Using a cut-off value of 0.50 or lower resulted in a susceptible classification. The Breda field isolate could therefore be characterized as ECD 16/3/30 (Buczacki et al., 1975).

Resistance tests of collected accessions

Seventy-one *B. oleracea* accessions with reported or putative resistance to clubroot were screened in eight tests (Table 2). Two replicates (42 pots) each of cv. Septa and of one accession of Böhmerwaldkohl were included as controls to compare the levels of infection between tests. The disease indices of these two accessions were 0.95-1.00 and 0.60-0.80 respectively.

In 9 out of 11 groups of accessions studied, resistance to the Breda isolate was found. The Wisconsin cabbage and OSU CR broccoli groups were consistently resistant. In the Böhmerwaldkohl, Brussels sprouts and curly kale groups large differences in level of resistance were observed between accessions. All four accessions of the Oregon cabbage group, and some of the Russian, Bindsachsener and "other cabbage" groups showed intermediate levels of resistance, while most accessions in the latter three groups were fairly susceptible. No resistance was found in the Irish "Flat Dutch" cabbage landraces, nor in the Portuguese Tronchuda cvs.

Table 2. Proportions of plants not or slightly affected by clubroot (class 0 and 1) in groups of accessions and in the most resistant accession of each group.

Accession group	Number of accessions	Proportion in grade 0 or 1[1]	
		mean	most resistant
Cabbage			
Septa (control)	8[2]	0.03[2]	
Bindsachsener	5	0.18***	0.68***
Böhmerwaldkohl	8	0.44***	0.87***
Irish "Flat Dutch"	9	0.03	0.15
Oregon	4	0.48***	0.59***
Russian	17	0.17***	0.56***
Wisconsin	4	0.77***	0.97***
other cabbage	2	0.13*	0.40**
Broccoli			
OSU CR	8	0.85***	0.94***
Brussels sprouts			
breeding lines	5	0.28***	0.79***
Curly kale			
cultivars	6	0.33***	0.93***
Tronchuda			
cultivars	3	0.06	0.08

[1] Proportions near 0 or 1 are over- or underestimated by approx. 0.025.

[2] Results of eight tests of cv. Septa.

*, **, and *** indicate significant differences with cv. Septa at the 5%, 1% and 0.1% confidence level, respectively.

Very high levels of resistance were observed in Wisconsin cabbage breeding line Dr Larson, in a line of Böhmerwaldkohl selected by Dr Crute (HRI, Wellesbourne, UK), in broccoli OSU CR-7 and in curly kale cv. Petibor. The seedling disease indices of these accessions were below 0.15, and more than 85 % of the plants were scored in grades 0 and 1.

Resistance of F_1 progenies

Randomly chosen plants of 11 partially resistant accessions (three Böhmerwaldkohl, four Wisconsin and four curly kale) were self-pollinated and crossed with susceptible cv. Septa to determine the inheritance of resistance factors (Table 3). All F_1-populations were highly susceptible, while the inbreds showed moderate to high levels of resistance. These preliminary results indicated that in these accessions the resistance was expressed as a recessive character.

Three resistant cabbage accessions of the Wisconsin group, cv. Badger Shipper and breeding lines Resistant Detroit and Dr. Larson were crossed pairwise for an allelic test. As shown in Table 4, the resistance of the F_1-populations was similar to that of the inbred lines. In all cases a small number of plants was rated as grade 2 or 3, while the majority was rated as grade 0 or 1. In combination with the recessive inheritance of resistance

Table 3. Disease indices of progenies of plants from three groups of accessions, obtained by self-pollination (I_1) and by crossing with cv. Septa (F_1).

Accession group	Number of accessions	Disease index	
		I_1	F_1
Böhmerwaldkohl	3	0.25 - 0.63	0.99 - 1.00
Wisconsin	4	0.22 - 0.40	0.96 - 1.00
Curly kale	4	0.58 - 0.69	0.94 - 1.00

Table 4. Disease indices of inbreds and F1's of three cabbage accessions in the Wisconsin group. Number of plants tested is indicated in parentheses.

Accession	Resistant Detroit	Dr. Larson	Badger Shipper
Resistant Detroit (breeding line)	0.22 (27)		
Dr. Larson (breeding line)	0.29 (14)	0.31 (79)	
cv. Badger Shipper (ECD11)	0.30 (85)	0.21 (42)	0.28 (57)

observed in the crosses with cv. Septa, this indicated that the resistance genes in these accessions were allelic.

Discussion

Reliability of the seedling test method

The seedling test method used in this study proved to be suitable for the screening of resistance of relatively large numbers of plants. It has the advantage over other methods that the number of spores applied to each plant is well controlled. In the widely used root-dip (Johnston, 1968) and slurry (Toxopeus and Jansen, 1975) methods, the number of spores per plant can vary significantly due to unknown and varying amounts of spores adhering to root systems of different sizes, or to the difficulty of obtaining a uniform distribution of spores in batches of slurry. Another advantage of the pipette method is that inoculation can be made directly after sowing, thus reducing the test duration by 10-14 days. A slight drawback of this method is that the number of plants tested depends on the germination potential of the seed lot, which is not always known in advance. The results obtained with this seedling test were shown to correlate well with the symptom development found under normal field conditions.

Identification of resistant accessions

The aim of the present research was to identify accessions with resistance to Dutch populations of *P. brassicae*, for further genetic studies. Useful resistance was identified in nine groups of accessions, although a common source of resistance was involved in several cases. From these experiments it is not possible to conclude whether the occurrence of both healthy and affected plants in a specific accession is caused by genetic

variation within the accession or by partial resistance which is genetically uniform. This will be investigated by studying the resistance of doubled haploid lines derived from these accessions.

The resistance found by Crisp et al. (1989) and Crute and Pink (1989) in Irish landraces was shown to be completely ineffective to the Breda isolate used in this study. The same was true for the resistance in many Russian cultivars described by Krasheninnick (pers. comm.). These accessions therefore must carry differential resistance, or the Breda isolate used in this study is generally more aggressive than the isolates to which these accessions were found to be resistant. The Portuguese Tronchuda cvs were also susceptible in our tests, although Crisp et al. (1989) found some resistance in related material. The resistance in two accessions received from the Wisconsin Agricultural Experimental Station in the 1950's was shown to be related to that in cv. Badger Shipper.

The complete susceptibility of all resistant x susceptible crosses agrees with earlier findings in *B. oleracea* (Crute et al., 1980), where resistance appeared to be generally recessive. This has now also been shown to be true for the resistance present in curly kale.

Further work is needed to determine whether the resistance found in different groups of accessions is caused by different genes. If so, these could possibly be accumulated in commercial cultivars to achieve higher and more stable resistance. Work in progress concentrates on genetic analysis of resistance, using doubled haploids derived from selected accessions.

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Chapter 3

Root hair infection by *Plasmodiophora brassicae* in clubroot-resistant and susceptible genotypes of *Brassica oleracea, B. rapa* and *B. napus*

Abstract

The pathogenesis of clubroot, a disease of cruciferous crops caused by the fungus *Plasmodiophora brassicae*, starts with infection of the root hairs. This process was studied in 13 accessions of *Brassica oleracea, B. napus and B. rapa* with varying levels of plant resistance to *P. brassicae*. Seedlings were grown in a mineral solution, inoculated with resting spores of *P. brassicae*, and the number of plasmodia developing in root hairs was recorded. When compared with the standard susceptible cultivar Septa, both higher and lower resistance to root hair infection was found in the accessions of the different *Brassica* species. No complete resistance to root hair infection was found. Over the accessions studied, there was no correlation between the plant resistance estimated from greenhouse tests and the resistance to root hair infection of seedlings. The resistance of all accessions must at least partly be caused by other mechanisms which operate after the root hair plasmodia are formed.

Introduction

Clubroot, an important root disease of cruciferous crops, is caused by the fungus *Plasmodiophora brassicae*. Over several decades, plant breeders have tried to incorporate resistance to clubroot in cultivars of *Brassica oleracea*. Several sources of resistance have been described in this crop (reviewed by Crute et al., 1980 and Crisp et al., 1989), but little is known about the genetics of resistance. Most resistance tests suffer from a considerable environmental variation, which complicates the assessment of resistance of individual plants. Consequently, no commercial resistant cultivars of *B. oleracea* are yet available, and a more fundamental approach to the mechanisms and genetics of clubroot resistance in this species appears to be necessary.

The life cycle of the pathogen starts with infection of root hairs by primary zoospores, which have germinated from resting spores. Subsequently plasmodia and zoosporangia are formed in the root hairs, followed by the production of secondary zoospores. At later stages, occurring in the root cortex, galls are formed and resting spores are produced (Ingram and Tommerup, 1972).

The occurrence of root hair infection has been reported in several non-cruciferous species which are non-hosts of *P. brassicae* (Kole and Philipsen, 1956; Webb, 1949). Root

hair infection has also been reported in some host species with complete resistance (Butcher et al., 1976; Dekhuijzen, 1979). In a resistant *B. rapa* genotype, the resistance was shown to be caused by a hypersensitive reaction in the root cortex (Dekhuijzen, 1979). However, there are no reports of the quantification of root hair infection in these accessions, nor of root hair infection in resistant *B. oleracea* genotypes. The resistance present in *B. oleracea* is often incomplete and generally recessive (Crute et al., 1980). In these respects, the resistance in this species differs from that present in *B. rapa*, *B. napus* and many other cruciferous species (Crute et al., 1980). Therefore, it is conceivable that also the mechanisms of resistance in *B. oleracea* are different. In the present study the occurrence and development of plasmodia in the root hairs was investigated in several *B. oleracea* accessions with varying levels of plant resistance to clubroot. For comparison, two completely resistant accessions of *B. rapa* and *B. napus* were included. Resistance to root hair infection is defined as the ability of the host plant to restrict the formation of plasmodia in root hairs. The term plant resistance is used to indicate the resistance of the plant to gall formation when grown in infested soil.

Materials and methods

Plant material

Cabbage cv. Septa, host 14 of the European Clubroot Differential set (ECD, Buczacki et al., 1975), was used as a susceptible control in all experiments. In the resistance tests, *Brassica* accessions were used with varying levels of resistance. The source of resistance of these accessions is indicated in Table 1. All accessions were maintained at the CPRO-DLO *Brassica* collection.

Pathogen

A field isolate of *P. brassicae* was obtained from the Experimental station "Proeftuin Brabant" in Breda, in the south of The Netherlands. This isolate was characterized as ECD 16/3/30. Clubs from highly susceptible broccoli plants were harvested, washed and stored at -20 °C for up to 14 months. Spore suspensions were prepared by grinding one part of frozen clubs with four parts of deionized water for 1 min in a high-speed blender. The spore suspensions were filtered through four layers of cheese-cloth, and diluted with deionized water to a density of 10^7 spores·ml^{-1}.

Test for plant resistance

Clubroot resistance was assayed in a greenhouse. Seeds were sown in 4.5 cm square pots in potting compost (pH 6.0) at a depth of 2.5 cm. Two ml of a spore suspension was applied to each pot. The pots were covered with plastic for 3 days until emergence of the seedlings. Forty-two pots were placed in a tray, which was watered with 1 cm water each

day and kept at 23 °C. After 6 weeks, plants were washed and symptoms graded on a scale of 0-3, according to Buczacki et al. (1975): 0, no swelling visible; 1, very slight swelling, usually confined to lateral roots; 2, moderate swelling on lateral and/or tap roots; 3, severe swelling on lateral and/or tap roots. A disease index, ranging from 0 (no symptoms) to 1 (severely affected) was calculated by dividing the mean disease grading of each accession by three.

Test for root hair infection

Root hair infection was determined in seedlings growing in a mineral solution in 12 ml polystyrene centrifuge tubes. The mineral solution was adapted after Crute et al. (1981) by replacing the spore elements by those of the Murashige and Skoog (1962) medium, and had the following composition: 6.00 mM KNO_3, 3.05 mM $Ca(NO_3)_2$, 1.50 mM $MgSO_4$, 1.33 mM NaH_2PO_4, 109 μM NaFeEDTA, 100 μM H_3BO_3, 100 μM $MnSO_4$, 30 μM $ZnSO_4$, 5.00 μM KI, 1.03 μM Na_2MoO_4, 0.10 μM $CuSO_4$ and 0.10 μM $CoCl_2$. After autoclaving the mineral solution for 30 min at 121 °C the pH was adjusted to 6.3, unless stated otherwise. The centrifuge tubes were filled with 11 ml of this mineral solution and 1 ml of water or a resting spore suspension (10^7 spores/ml H_2O). The spores were allowed to settle for 2 hours before seedlings were placed on the tubes.

Seeds were sown on moist filter paper in 9 cm Petri dishes, and incubated for 3 days at 18 °C in the dark. Germinated seeds with an emerging root of about 1 cm were then placed on the caps of the centrifuge tubes, with the root protruding through a 2 mm hole in the caps. The tubes were placed in 19 mm holes drilled in the lid of a box, leaving the tubes with the roots in the dark while exposing the emerging shoots to the light. The box was placed in a conditioned climate room at a temperature of 23 °C, with a photoperiod of 16 h at 82 $\mu E \cdot m^{-2} \cdot s^{-1}$ (Philips TLD 33 fluorescent light).

Six tests were performed to evaluate root hair infection in the different accessions. In each test, 22 infested tubes of each of two resistant accessions, 22 infested tubes of cv. Septa and 11 non-infested tubes of cv. Septa were used. Seven days after inoculation, all poorly developed seedlings were discarded. Nine or ten of the remaining plants of each accession were randomly chosen for assessment of root hair infection, as well as five non-inoculated control plants of cv. Septa. Each plant was placed with its roots in 1 ml of a 125 ppm aniline-blue solution in 50% (v/v) acetic acid for 1 min at room temperature and then rinsed with tap water. The main root was cut off just below the root neck, and mounted in 50% (v/v) glycerin. Lateral roots were discarded. The length of the main root was measured and the number of plasmodia in root hairs on the main root was determined at 200x magnification (Fig. 1).

Data analysis

Fungal development in root hairs was expressed as the number of plasmodia per cm root length. An analysis of deviance for a generalized linear model was performed using the

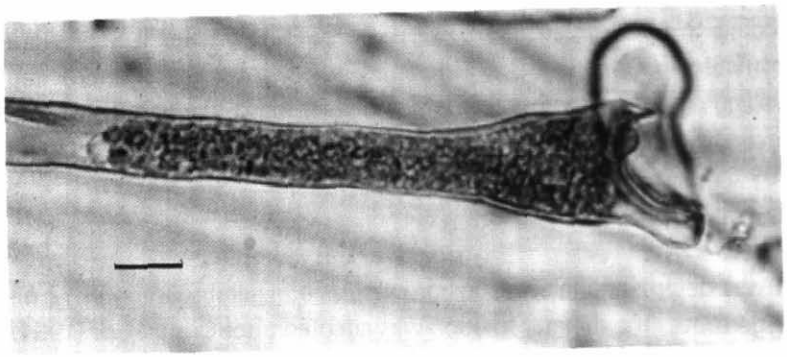

Figure 1. Root hair with multinucleate plasmodium stained with aniline-blue, 7 days after inoculation with resting spores of *P. brassicae* in a nutrient solution. The bar represents 10 μm.

logarithmic link function; the variance function was assumed to be proportional to the mean (McCullagh and Nelder, 1989). In this model, $\mu_{ij} = c \cdot t_i \cdot a_j$, where μ_{ij} is the expected response of accession j in test i, c is a constant, t_i is the effect of test i and a_j is the effect of accession j. Mean values and 95% confidence intervals are given for the severity of infection relative to that of cv. Septa, calculated as a_j/a_{Septa}.

Spearman's rank correlation coefficient was used to test for correlation between the severity of root hair infection and the percentage of healthy plants or the disease index as determined by tests for plant resistance.

Results

Test for plant resistance

All accessions were tested for clubroot resistance at the plant level using the seedling test described above. Three accessions of *B. oleracea*, *B. rapa* and *B. napus* respectively showed complete resistance to the clubroot isolate used in these experiments. Apart from the susceptible control cv. Septa, the other *B. oleracea* accessions showed medium to high levels of partial resistance (Table 1).

Development of the root hair infection test method

Preliminary experiments with cv. Septa were conducted to optimize the experimental conditions. The mineral solution S/5 of Macfarlane (1958, 1970) was found to be much less conducive to plant development than the solution described in this paper, although with both solutions good root hair infection was obtained. To determine the optimal pH, the initial pH of the solution was varied from 3.0 to 7.0. High and comparable numbers of plasmodia were obtained at pH 5.0, 6.0 and 7.0. At pH 4.0 two-fold shorter roots

Table 1. Plant resistance (percentage of plants in each grade of the symptom scale, and disease index), and severity of root hair infection for 13 *Brassica* accessions.

	Plant resistance					Severity of root hair infection	
	Scale				Disease	Mean[2]	Confidence interval
Accession[1]	0	1	2	3	index		(p = 0.95)
cv. Septa (ECD14)	0	0	4	96	0.99	1.00	
cv. Bindsachsener	9	20	63	9	0.57	0.87	0.30 - 2.55
cv. Verheul (ECD15)	28	8	44	19	0.52	0.16*	0.05 - 0.46
cv. Losinoovstrovskaja 8	33	26	5	36	0.48	1.44	0.81 - 2.57
cv. Badger Shipper (ECD11)	6	53	39	3	0.46[3]	1.56	0.61 - 4.00
cv. Petibor	10	52	31	7	0.45	1.05	0.57 - 1.92
cv. Böhmerwaldkohl F	29	16	48	6	0.44	0.92	0.52 - 1.63
cv. Iras	53	13	27	7	0.29	0.34*	0.14 - 0.83
cv. Resistant Detroit	57	43	0	0	0.14	0.91	0.41 - 2.02
line 8-41	74	26	0	0	0.09	0.66	0.40 - 1.10
Dr. Larson	100	0	0	0	0.00	3.05*	1.62 - 5.75
B. rapa (ECD04)	100	0	0	0	0.00	0.32*	0.17 - 0.61
B. napus (ECD10)	100	0	0	0	0.00	2.65*	1.56 - 4.50

[1] Accessions are identified by the source of the resistance they carry. The *B. oleracea* accessions consist of 3 curly kales (cv. Verheul, cv. Iras and an F_2-population from cv. Petibor), susceptible cabbage cv. Septa, and six cabbage accessions carrying varying resistances. "Dr. Larson" is a line derived from breeding material obtained in 1955 from Dr. R.H. Larson; "Resistant Detroit" is derived from crosses of cv. Resistant Detroit with susceptible cabbages; "line 8-41" is derived from line 8-41 of Chiang and Crête (1970).

[2] Means marked * are significantly different from control cv. Septa ($p < 0.05$).

[3] It should be noted that this accession, a mass propagation of ECD11 (cv. Badger Shipper) has a much higher disease index (0.46) than ECD11 itself. ECD11 has been tested repeatedly with the same field isolate as used in the present work, always yielding disease indices between 0.12 and 0.25. This indicates that ECD11 is not uniformly homozygous for its clubroot resistance genes.

were produced with a ten-fold reduction in plasmodia, while at pH 3.0 all plants died. Since the pH in all treatments except for initial pH 3.0 slowly converged to pH 6.3 after 1 week due to the low buffering capacity of the solution, in further experiments the initial pH was set at pH 6.3.

No evidence for root hair infection by secondary zoospores

In general, primary zoospores infect root hairs, while secondary zoospores infect the root cortex cells (Ingram and Tommerup, 1972). However, it is not known whether secondary zoospores can also infect root hairs. Two experiments were performed to check whether the root hair infections observed could be attributed to secondary, as well as to primary zoospores.

In the first experiment, tubes containing solutions with resting spores were incubated in the presence or absence of a seedling. After a preculture period varying from 0 to 16 days, a non-inoculated seedling was placed on the same tubes, and the number of plasmodia was examined 7 days later. In all cases the infectivity of the spore suspensions

declined to almost zero within 5 days. Since there was no difference in the number of root hair infections with or without seedlings present during the preculture period, it was concluded that the number of infections caused by secondary zoospores was insignificant compared with the infection by primary zoospores.

More evidence of the absence of root hair infection by secondary zoospores was obtained from a re-infection experiment. Seedlings were grown in an infested solution and removed at 3, 7 or 14 days after inoculation. Subsequently they were rinsed in deionized water to eliminate most spores from the outside of the roots. They were then placed in a fresh solution containing no spores, together with a non-inoculated 3-day-old seedling. The younger seedling might thus be infected by secondary zoospores released from the root hairs of the older seedling. In order to check for the occurrence of infections caused by primary spores remaining on the roots after rinsing, rinsed roots of other infested seedlings were homogenized in a mortar and added to an otherwise uninfested solution on which a young seedling was placed. Plasmodia were only found occasionally, as well in the control as in the re-infection treatments. Presumably these originated from spores still present in the rinsed roots of the older seedling, and not from secondary zoospores produced by the older seedlings.

From these experiments the conclusion was drawn that the plasmodia observed in root hairs on seedling roots grown in nutrient solution were always derived from infections by primary zoospores, emerged directly from the resting spores. Infection by these primary zoospores ceased about 5 days after inoculation.

Primary root hair infection in resistant and susceptible accessions

Using the test method described above, the differences in root hair infection in a range of *Brassica* accessions differing in plant resistance were studied. Six separate tests were performed, in each of which the numbers of plasmodia were scored in nine or ten plants of each of two resistant accessions and the susceptible cv. Septa.

The number of plasmodia per mm root length varied from 0.5 for cv. Verheul to 6.0 for *B. napus* (ECD10). After correction for test effects, accession effects for severity of root hair infection were obtained (Table 1). Large differences were found, with the accession most susceptible to root hair infection ("Dr. Larson") having a 20-fold higher effect than the least susceptible accession (cv. Verheul). No complete resistance to root hair infection was present in any of the 13 accessions tested.

Control cv. Septa, which is highly susceptible at the plant level, showed an intermediate susceptibility to root hair infection. A significantly different root hair infection was found in five accessions tested. Of these five, accessions "Dr. Larson" and *B. napus* (ECD10) were significantly more susceptible, while cv. Verheul, cv. Iras and *B. rapa* (ECD04) were significantly more resistant to root hair infection than cv. Septa. It is interesting to note that the two accessions most susceptible to root hair infection, "Dr. Larson" and *B. napus* (ECD10), were completely resistant to clubroot at the plant level.

No correlation was found between the plant resistance estimated from the greenhouse test and the severity of root hair infection. Spearman's rank correlation coefficients between root hair infection and percentage healthy plants or disease index were -0.02 and -0.13 respectively; if only the *B. oleracea* accessions were considered, these values were 0.08 and -0.09. None of these values was significantly different from zero.

The analysis of deviance is presented in Table 2. Apart from the clear accession effect there was also a strong test effect. This could not have been caused by environmental effects, since all tests were incubated in the same climate room. Presumably, the test effect was mainly due to the fact that a fresh spore suspension was prepared for each test. Since only small amounts of frozen clubs were used for each inoculum preparation, any between-club variation in spore viability or maturity could have resulted in inocula of varying infectivity.

Table 2. Analysis of deviance for number of plasmodia per mm root length.

Source of variation	Degrees of freedom	Deviance	Mean deviance	Deviance ratio
Tests	5	64.39	12.88	10.88*
Accessions	12	95.75	7.98	6.74*
Residual	157	185.89	1.18	
Total	174	346.03	1.99	

* $p < 0.005$

Discussion

Resistance to root hair infection has been interpreted in this work as the ability of the host plant to limit the formation of plasmodia in root hairs, when exposed to a very high number of resting spores. A high inoculum pressure was used to ensure that ample zoospores would be present to attack all root hairs. Differences in infection therefore reflect the ability of the plant to defend itself against the pathogen, rather than differences in the ratio of zoospores to root hairs. In order to avoid the necessity to count all root hairs, which often formed a tangled mass, the infection was expressed as the number of plasmodia in root hairs per unit length of the main root.

Resistance to root hair infection is only one component of the complex trait, clubroot resistance. This resistance is usually considered as the ability of the plant to limit club formation, which is a rough indication of the number of resting spores produced.

During the early stages of infection occurring in the root hairs, one primary zoospore, germinated from one resting spore, causes the production of secondary

zoospores, which can infect the root cortex. However, the multiplication of the pathogen in the root hairs appears to be limited, compared to the multiplication during club formation. Since club formation and resting spore production are limited by the resources of the host plant rather than by the number of infecting secondary zoospores, resistance to root hair infection would only be an important component of resistance if it were almost complete. In that case almost no secondary zoospores would be formed, preventing infection of the root cortex. A quantitative resistance however, which would reduce but not completely prevent secondary zoospore formation, would not be enough to prevent root cortex infection. This would agree with the work of Naiki et al. (1978), who found evidence that highly susceptible Chinese cabbage plants with only a few infected root hairs could nevertheless become severely clubbed, as well as with the observations of several authors that inoculation with a single resting spore can lead to club formation.

This hypothesis was confirmed by the results of the present study. Large differences in root hair infection were found. However, no complete resistance to root hair infection was observed. The susceptible control cv. Septa had a resistance to root hair infection approximately equal to the average of the resistant accessions, indicating that a certain measure of resistance to root hair infection is not enough to prevent severe club formation. In the other accessions partial to complete plant resistance was demonstrated, while no complete resistance to root hair infection was present. In these accessions, other mechanisms of resistance must exist which operate after the root hair plasmodia are formed. From the absence of correlation between the root hair infection and club formation it is concluded that the resistance to plasmodia formation in root hairs, and the resistance to club formation are governed by different genes.

Further study would be useful in order to find genotypes with a near-complete resistance to root hair infection. The wide range of severity of root hair infection found in this study is an indication that such high levels of root hair resistance may still be found. The level of resistance to root hair infection found in this study however is only of limited use in an attempt to unravel the genetics of clubroot resistance and in breeding for clubroot-resistant cultivars.

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Chapter 4

A one-hit model for the infection of clubroot-susceptible cabbage (*Brassica oleracea* var *capitata*) by *Plasmodiophora brassicae* at various inoculum densities

Abstract

In two glasshouse and three phytotron experiments, clubroot-susceptible cabbage (*Brassica oleracea* var *capitata*) cv Septa was inoculated with clubroot resting spores at inoculum densities ranging from 0 to $2 \cdot 10^7$ spores·plant^{-1}. At densities of 10^5 spores·plant^{-1} and higher all plants developed clubroot symptoms, except in one glasshouse experiment conducted in winter. The proportion of plants developing symptoms plotted against inoculum density showed a sigmoid curve. Although the shape of the curve was similar in all experiments, the inoculum densities required to induce 50% disease incidence varied from 10^3 to 10^5 resting spores·plant^{-1}. The data of all five experiments could be well described by a generalized one-hit model which involves variation between plants with regard to the probability of infection.

Abbreviations: cv: cultivar; ECD: European Clubroot Differential set

Introduction

For studies on the control of clubroot, caused by the fungus *Plasmodiophora brassicae* Wor., or on the resistance of various host species to this disease, a reproducible test method is essential. The results of field tests are often difficult to reproduce, due to variation in inoculum density, temperature and soil moisture within and between experiments. Therefore several methods have been devised to inoculate seedlings in a glasshouse environment with resting spores of *P. brassicae*, offering a better control of climate and soil conditions and inoculum source.

Generally, the inoculum for glasshouse tests is prepared by macerating fresh or frozen clubs, the swollen roots characteristic of the disease, in water using an electric blender. The currently used inoculation methods are all variations of the root dip, slurry or pipette methods. In the root dip method [Johnston, 1968], roots of seedlings are dipped in a suspension of resting spores before planting in potting compost. In the slurry method [Toxopeus and Janssen, 1975], a resting spore suspension is mixed with potting compost. The resulting slurry is placed in holes in compost, and seedlings are planted in the slurry. In the pipette method [Lamers and Toxopeus, 1977; Voorrips and Visser, 1993], seeds are sown in potting compost, one seed per pot, and a defined quantity of

a resting spore suspension is pipetted in each pot. After inoculation the plants are grown for six to eight weeks at high soil moisture. The symptoms are generally assessed visually and classified in grades. A widely used grading system is described by Buczacki et al. [1975].

In most studies rather high inoculum densities have been used. In his review concerning the root dip and slurry methods, Dixon [1976] asserted that the inoculum densities used by different researchers varied from 10^5 to about 10^8 resting spores per plant. Voorrips and Visser [1993] applied $2 \cdot 10^7$ spores per plant using the pipette method, and Robak and Gabrielson [1988] considered 10^5-10^8 spores per plant necessary for consistent results using a similar test method. It has long been recognized that high inoculum densities are required to ensure consistent symptom development on susceptible plants under varying environmental conditions [e.g. Colhoun, 1958].

This report describes a series of experiments directed to the question as to whether the infection of clubroot-susceptible host plants by spores of *P. brassicae* can be described by a one-hit model, as originally formulated by Ridout et al. [1993] to describe infection of insects by viruses. The basic assumptions underlying one-hit models are that pathogen individuals act independently (*i.e.* without significant interaction during infection and pathogenesis) and that infection by one individual suffices to cause disease symptoms. Although interaction does occur between zoospores during clubroot pathogenesis [Tommerup & Ingram, 1971], the frequency of this interaction is not known. In the experiments described here, inoculations were performed with varying numbers of resting spores per plant. The number of resting spores available per plant appeared to be most precisely controlled with the pipette inoculation method which was therefore utilized in this study.

Materials and Methods

Cabbage cv Septa (Bejo Seeds b.v., Warmenhuizen, the Netherlands) was used as clubroot-susceptible host in all experiments. The inoculum used was a *P. brassicae* isolate obtained from an infested field at Brabant Experimental Station in the Netherlands and characterized as ECD 16/3/30 [Buczacki et al., 1975; Voorrips & Visser, 1993]. Resting spore suspensions were prepared from clubs stored at -20°C for up to 27 months as described by Voorrips & Visser [1993], and used for inoculation on the same day.

Two inoculation experiments (G1 and G2) were performed in a glasshouse as described by Voorrips & Visser [1993] in early spring (G1) and in winter (G2). Daylight was supplemented by 400 W SON-T lamps (45 $\mu E \cdot m^{-2} \cdot s^{-1}$, 14 $h \cdot day^{-1}$). Three similar experiments (P1, P2 and P3) were carried out in a phytotron chamber, at 22°C, with a photoperiod of 16 h at 110 $\mu E \cdot m^{-2} \cdot s^{-1}$ HPI-T illumination.

Each inoculation experiment consisted of a different series of inoculum densities, always including a control treatment with zero resting spores per pot and a treatment with at least 10^5 resting spores per pot; one seed was sown in each pot. The experiments were laid out in a randomized complete block design with two, three or four blocks per experiment and one replicate of each treatment per block. Replicates consisted of 42 pots each. Six weeks after inoculation the disease incidence was determined for each replicate as the percentage of well-developed plants showing symptoms, disregarding the variation in disease severity.

The validity of the basic and generalized one-hit model [Ridout et al., 1993] describing the relation between the mean number of inoculated resting spores and the proportion of diseased plants was examined. The Genstat5 command FITNONLINEAR [Genstat 5 Committee, 1987] was used to find parameter values yielding a minimum residual deviance for each experiment. The difference between the residual deviance of the one-hit models and of a general model without constraints on the mean probability of infection at each inoculum density was used to test the goodness of fit of the one-hit models. This difference of residual deviances follows approximately a χ^2_d distribution, where d is the difference in residual degrees of freedom between the one-hit and the general model [McCullagh & Nelder, 1989].

One of the assumptions implicit in the one-hit models is that resting spores in suspensions can be considered to be randomly distributed, resulting in a Poisson distribution of the number of resting spores inoculated per pot. This was verified in three freshly prepared suspensions by counting the number of resting spores in each of 100 or 200 unit cells of a Thoma haemocytometer. The volume of individual cells was 0.25 nl. The deviation of the counts from a Poisson distribution was tested by the Index of Dispersion test, with $(N\text{-}1)\cdot s^2/\bar{x}$ having approximately a χ^2-distribution with $(N\text{-}1)$ degrees of freedom.

Results and Discussion

Large between-test variation

The disease incidence treatment means of all inoculation experiments are shown in Fig. 1. Since no significant block effects were detected, the means were calculated as the total number of diseased plants divided by the total number of plants per treatment. Five replicates with less than 20 well-developed plants were rejected.

Among the experiments a large variation was observed in the incidence at similar inoculum densities (Fig. 1). The inoculum densities required to induce 50 % disease incidence varied from less than 10^3 resting spores·plant^{-1} in phytotron experiment P2 to approximately 10^5 spores·plant^{-1} in glasshouse experiment G2.

The low disease incidence in experiment G2 was possibly caused in part by more adverse conditions in the glasshouse in winter, even though minimum air and soil temperature were kept at 18 °C and 23 °C respectively, and daylight was supplemented with artificial light. However, probably not all variation between experiments was due to culture conditions. Differences in condition of the inoculum may also have been of importance. Since only small amounts of inoculum were prepared for each experiment, differences between individual clubs may have had a significant effect. This is supported by the fact that the differences between phytotron experiments were comparable to those between the glasshouse experiments.

Random distribution of resting spores in suspensions

If resting spores are randomly distributed in suspensions, as assumed in the one-hit models, the number of spores in a volume of suspension should follow a Poisson distribution. This was investigated using a haemocytometer. In three freshly prepared suspensions the number of resting spores per haemocytometer cell closely followed a Poisson distribution (Table 1). Also, in the freshly prepared suspensions resting spores did not adhere to each other. Therefore, the number of resting spores inoculated per pot using the pipette method may be considered to follow a Poisson distribution.

Table 1. Frequency distribution of resting spores of *P. brassicae* in three freshly prepared resting spore suspensions in unit haemocytometer cells, and test for deviation from a Poisson distribution.

Suspension	A		B		C	
spores per cell	obs.[3]	fit.[3]	obs.	fit.	obs.	fit.
0	0	0.1	0	1.1	30	34.9
1	0	0.7	4	5.9	68	61.0
2	1	2.3	22	15.3	53	53.2
3	6	5.3	21	26.3	27	30.9
4	14	9.2	32	33.9	17	13.5
5	15	12.9	41	35.0	4	4.7
6	13	15.0	29	30.1	1	1.4
7	12	14.9	26	22.2	0	0.3
8	7	13.0	11	14.3	0	0.1
9	13	10.1	6	8.2	0	0.0
10	7	7.0	3	4.2	0	0.0
11	6	4.4	2	2.0	0	0.0
12	3	2.6	1	0.9	0	0.0
13	3	1.4	2	0.3	0	0.0
14	0	0.7	0	0.1	0	0.0
Mean	6.97		5.16		1.74	
d.f.[1]	99		199		199	
I.D.[2]	103.4 n.s.		198.6 n.s.		184.5 n.s.	

[1] d.f.: degrees of freedom

[2] I.D.: Index of Dispersion; n.s.: deviation from Poisson distribution not significant ($P \geq 0.05$)

[3] obs. and fit.: observed and fitted frequencies of number of spores per haemocytometer cell (the fitted frequencies were obtained using the sample mean as Poisson parameter)

Validity of the basic one-hit model

The results were used to test the validity of the basic one-hit model, describing the relation between inoculum density and proportion of diseased plants. The expected proportion of infected plants (P_{inf}) is related to the mean number of resting spores inoculated per plant (n) as

$$P_{inf} = 1 - e^{-n \cdot \omega} \quad (1)$$

where ω is the (constant) probability of a spore infecting the host. This model is based on three assumptions: (i) the actual number of resting spores inoculated per plant follows a Poisson distribution; (ii) the probability that a spore infects the host plant is constant within each block in an experiment, and is not influenced by the inoculum density; and (iii) a plant will develop symptoms when infected by at least one spore. Assumption (i) was verified separately (Table 1). In this simple model, the effects of production and fusion of secondary zoospores and other aspects of the pathogenesis of clubroot are disregarded.

Table 2. Fit of basic one-hit model in two glasshouse (G1 and G2) and three phytotron (P1-P3) experiments with varying numbers of resting spores of *P. brassicae* inoculated per cabbage plant.

Test	ω[1]	d.f.[2]	residual deviance[2,3]	
G1	$2.95 \cdot 10^{-4}$	5	15.08	*
G2	$7.97 \cdot 10^{-6}$	7	31.48	**
P1	$6.44 \cdot 10^{-5}$	7	49.98	**
P2	$6.67 \cdot 10^{-4}$	7	3.53	n.s.
P3	$3.50 \cdot 10^{-4}$	15	69.61	**

[1] ω: estimate of the proportion of spores successfully infecting the host.

[2] differences of degrees of freedom and of residual deviance between the basic one-hit model and a model without constraints on the expected proportion of diseased plants at each inoculum density

[3] significance of lack of fit: $P \geq 0.05$ (n.s.), $P < 0.05$ (*), $P < 0.01$ (**).

Most of the experiments did not fit the basic one-hit model (Table 2). Only in experiment P2 was a non-significant ($P > 0.05$) residual deviance obtained. In this experiment however, only few data were obtained in the most informative range of inoculum density. In general, the disease incidence appeared to increase more gradually than predicted by the basic one-hit model.

Fitting the generalized one-hit model

In the generalized one-hit model considered here, assumption (ii) of the basic one-hit model was relaxed, to allow variation in susceptibility among host plants. The probability that a host plant is infected by an individual spore was considered to vary between plants according to a Beta distribution [Ridout et al., 1993]. The probability θ_j of a plant being infected when inoculated with j resting spores is given by Ridout et al. [1993] as

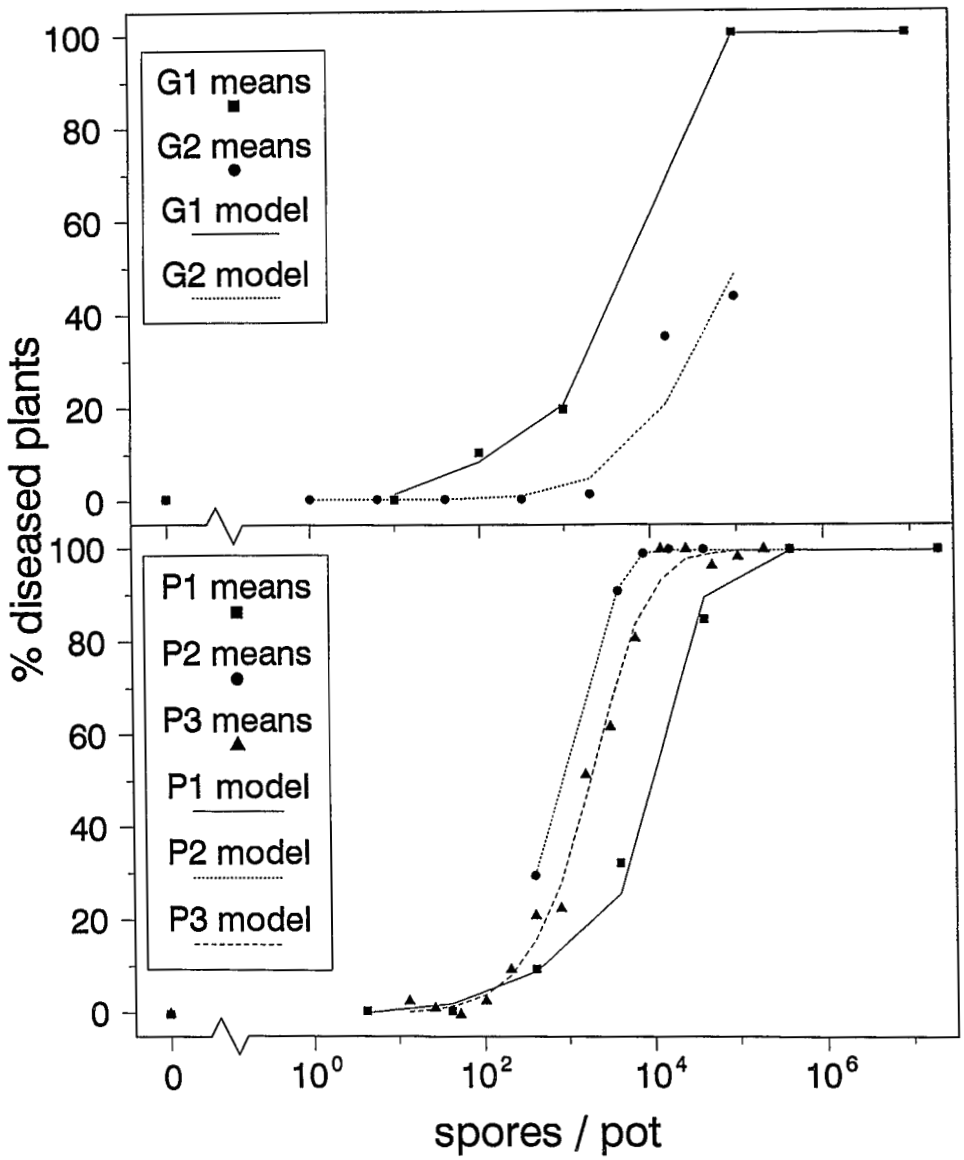

Figure 1. Observed disease incidence means and values predicted by the generalized one-hit model for the infection of cabbage plants inoculated with varying numbers of resting spores of *P. brassicae* in two glasshouse experiments. Top: glasshouse experiments G1 and G2; bottom: phytotron experiments P1, P2 and P3.

$$\theta_j = 1 - \frac{\prod_{i=1}^{j}[1-\omega+(i-1)\psi]}{\prod_{i=1}^{j}[1+(i-1)\psi]}, \quad j=1, 2, \ldots \qquad (2)$$

where ω is the mean probability that a plant is infected by an individual spore and ψ is a measure of the variability of ω between plants. If $\psi = 0$, no variation in susceptibility between plants is present and the basic one-hit model is obtained. If $\psi > 0$, variation in susceptibility occurs, and P_{inf} will increase more slowly with inoculum density than is predicted by the basic one-hit model.

Formula (2) and the Poisson distribution of the number of resting spores inoculated in each pot are not easy to compute for large numbers of spores. The Poisson distribution was therefore approximated by a Normal distribution with the same expectation and variance for expectations ≥ 56. Formula (2) was rewritten as

$$\theta_j = 1 - (1-\omega) \cdot \exp\left(\sum_{i=2}^{j} \ln[1-\omega+(i-1)\psi] - \ln[1+(i-1)\psi]\right) \qquad (3)$$

and approximated by

$$\theta_j = 1 - (1-\omega) \cdot \exp\left(\int_{1.5}^{j+0.5} \ln[1-\omega+(i-1)\psi] - \ln[1+(i-1)\psi]\, di\right), \quad j=1, 2, \ldots \qquad (4)$$

which is easily computed for any number of inoculated resting spores. The probability of a plant being infected (P_{inf}) was calculated by dividing the range of inoculated number of resting spores between $n-3\sqrt{n}$ and $n+3\sqrt{n}$ into 80 steps, multiplying the probability of each step by the θ_j for the midpoint of the step and summing over all the steps.

The generalized one-hit model fits the data well (Fig. 1, Table 3), in contrast to the basic one-hit model. This indicates that variation in the probability of infection between plants is an important factor in these experiments. The host, cv Septa, is part of the European Clubroot differential set (ECD, Buczacki et al., 1975) and generally considered to be susceptible to clubroot. Also in ECD tests with the clubroot isolate used in this study, cv Septa was always classified as susceptible [Voorrips & Visser, 1993]. However, all those tests were performed with high inoculum densities. It is possible that in cv Septa, an open-pollinated cultivar, a low level of resistance is segregating which becomes evident at low inoculum densities. However, the effects of possible genetic variation for susceptibility cannot be distinguished from those of variation in other factors, such as differences in soil humidity and soil packing between pots and in the spatial distribution of resting spores in the soil, and physiological differences between plants in the first weeks after germination.

The estimated mean probability of a spore infecting the host (ω) was remarkably low in all experiments (Table 3). In most experiments with single-spore inoculations [Buczacki, 1977; Haji Tinggal & Webster, 1981; Jones et al., 1982; Scott, 1985], about one percent

Table 3. Fit of generalized one-hit model in two glasshouse (G1 and G2) and three phytotron (P1-P3) experiments with varying numbers of resting spores of *P. brassicae* inoculated per cabbage plant.

Test	ω[1] (s.e.)	ψ[1] (s.e.)	d.f.[2]	residual deviance[2,3]	
G1	$1.07 \cdot 10^{-4}$ ($6.60 \cdot 10^{-7}$)	$5.23 \cdot 10^{-8}$ ($9.76 \cdot 10^{-9}$)	4	3.77	n.s.
G2	$2.50 \cdot 10^{-5}$ ($2.47 \cdot 10^{-8}$)	$8.60 \cdot 10^{-5}$ ($6.93 \cdot 10^{-8}$)	6	12.54	n.s.
P1	$5.53 \cdot 10^{-5}$ ($2.00 \cdot 10^{-8}$)	$1.40 \cdot 10^{-8}$ ($4.6 \cdot 10^{-11}$)	6	8.56	n.s.
P2	$6.15 \cdot 10^{-4}$ ($8.34 \cdot 10^{-5}$)	$9.70 \cdot 10^{-6}$ ($8.56 \cdot 10^{-6}$)	6	2.34	n.s.
P3	$4.63 \cdot 10^{-4}$ ($7.50 \cdot 10^{-6}$)	$2.15 \cdot 10^{-4}$ ($2.67 \cdot 10^{-5}$)	14	21.28	n.s.

[1] ω: estimate of the expected proportion of spores successfully infecting the host. ψ: estimate of magnitude of variation of ω between plants. s.e.: standard error of estimates

[2] differences of degrees of freedom and of residual deviance between the generalized one-hit model and a model without constraints on the expected proportion of diseased plants at each inoculum density

[3] n.s.: non-significant lack of fit ($P \geq 0.05$)

of the inoculations resulted in an infected plant, whereas in the work presented here the estimation of the proportion infecting resting spores was generally much lower. The higher infection frequency in single-spore inoculations was probably caused by the fact that the resting spore was placed in direct contact with the seedling root. The low infection rate is therefore in this case a consequence of the inoculation method, and not indicative of synergistic spore action [Garrett, 1970].

The good fit of the generalized one-hit model in each of the five experiments shows that the interaction between *P. brassicae* and its host can be modelled without assuming synergistic or competitive interactions among pathogen individuals. This suggests that interactions between pathogen individuals do not have a large influence on the probability of infection. Fusion of secondary zoospores has been observed [Tommerup & Ingram, 1971], although it is not known if this is a necessary condition for secondary infection. The results presented here suggest that either fusion is not necessary for infection, or that the number of dikaryotic spores formed by fusion is proportional to the number of resting spores inoculated.

If pathogen individuals do indeed act largely independently a new way to obtain genetically uniform pathogen isolates can be envisaged. At inoculum densities where only a small proportion of plants becomes diseased, the probability that those plants are infected by only one spore is large. Instead of manually isolating single resting spores, the much less laborious procedure of inoculating large numbers of plants with very dilute resting spore suspensions and harvesting the few diseased plants can be used.

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Chapter 5

Production, characterization and interaction of single-spore isolates of *Plasmodiophora brassicae*

Abstract

Out of 164 plants of clubroot-susceptible Chinese cabbage inoculated with single resting spores of *Plasmodiophora brassicae*, two plants developed clubroot symptoms. The two single-spore isolates (SSIs) extracted from these plants gave an identical reaction pattern on the European Clubroot Differential set (ECD) and seven doubled-haploid lines (DH-lines). Their reaction pattern differed from that of the original field isolate on four hosts: ECD hosts 06 and 07 were susceptible to the field isolate but resistant to both SSIs, while for DH-lines Bi and Pt the reverse was true. DH-line Pt was significantly less diseased by mixed inocula consisting of the field isolate and SSI-1 than by SSI-1 alone. It was concluded that the SSI-1 pathotype was a minor component of the field isolate, although it was isolated twice. The results also suggest that the alleviating effect of the field isolate in mixed inoculations with SSI-1 on DH-line Pt was due to induced resistance, rather than to competitive interactions.

Abbreviations: cv: cultivar; DH-line: doubled haploid line; ECD: European Clubroot Differential set; SSI: single-spore isolate

Introduction

Natural populations of *Plasmodiophora brassicae* Woron., the causal agent of the clubroot disease of cruciferous crops, consist of mixtures of pathotypes [Haji Tinggal and Webster, 1981; Jones et al., 1982a]. Isolates consisting of only one pathotype are more suitable for genetic studies of the pathogen, and for the study of genes conferring resistance to host plants. Since the resting spores of *P. brassicae* are haploid [Tommerup and Ingram, 1971], the progeny of one single resting spore may be assumed to be genetically homogeneous.

As *P. brassicae* is an obligate parasite, isolates derived from a single resting spore (single-spore isolates, SSIs) can only be obtained by inoculating host plant tissue with isolated resting spores. Several methods have been used to perform single-spore inoculations [Buczacki, 1977; Haji Tinggal and Webster, 1981; Jones et al., 1982b; Scott, 1985; Schoeller and Grunewaldt, 1986; Schulte, 1994]. Varying proportions of single-spore inoculations by these authors resulted in diseased plants.

The single-spore inoculations mentioned above were made with resting spores from various natural populations. Inoculum consisting of resting spores extracted from the inoculated plants again induced clubroot symptoms on susceptible plants, showing that the life-cycle of the pathogen can be completed by genetically uniform isolates. Contrasting differential pathogenicity was found among the resulting SSIs, even among those derived from the same natural population [Haji Tinggal and Webster, 1981; Jones et al., 1982b; Scott, 1985; Schoeller and Grunewaldt, 1986; Schulte, 1994]. This indicated that various pathotypes of *P. brassicae* are capable of survival as uniform isolates.

With the aim of obtaining genetically uniform isolates of *P. brassicae* for the study of resistance in *Brassica oleracea*, inoculations with single resting spores of a Dutch population of the pathogen were performed. Here the production and characterization of SSIs are reported. The SSIs displayed a differential pathogenicity not expressed by the original field isolate. Since suppression of differential pathogenicity in heterogeneous inocula can have serious consequences especially for resistance breeding, this phenomenon was further studied in mixed inoculations of one SSI and the field isolate on several host accessions.

Materials and Methods

Pathogen

A field isolate of *P. brassicae* was obtained from clubs of an unknown clubroot-susceptible cauliflower cultivar grown on an infested field of the Experimental Station Brabant at Breda, The Netherlands, and maintained on chinese cabbage (*B. rapa*) cv Granaat. This host cv has been widely used in clubroot research and is not known to carry resistance to this disease. The field isolate was characterized as ECD 16/3/30 [Buczacki et al., 1975; Voorrips and Visser, 1993].

Plant material

The 15 components of the European Clubroot Differential set (ECD; Buczacki et al., 1975) were obtained from H. Toxopeus (CPRO-DLO). ECD-host 08 segregated for resistance to the field isolate; for the tests reported here a uniformly resistant inbred line of this host was used.

Seven doubled-haploid lines (DH-lines) were obtained through microspore culture [Duijs et al., 1989]. DH-line Gr was derived from broccoli cv Greenia (Hammenhögs Frö AB, Hammenhög, Sweden), DH-line O7 from broccoli line OSU CR-7 [Baggett and Kean, 1985] and DH-line Pt from curly kale cv Petibor-F1 (Bejo Seeds b.v., Warmenhuizen, the Netherlands). The other DH-lines were derived from cabbage accessions: lines Bi and Bö from lines selected by I.R. Crute (HRI, Wellesbourne, U.K.) from the landraces

Bindsachsener and Böhmerwaldkohl; line Ch from a cross involving line 8-41 [Chiang and Crête, 1970]; line La from line Larson 8353 T [Nieuwhof and Wiering, 1963]. Cabbage cv Septa (Bejo Seeds b.v.), equivalent to ECD host 14, was used as a susceptible control.

Single-spore inoculations

Suspensions of resting spores of the field isolate of *P. brassicae* were prepared according to Voorrips and Visser [1993]. Each suspension was used during a period of 2-3 weeks and kept at 4 °C. Prior to use, an aliquot of suspension was diluted to a density of about 500 spores·ml^{-1}. A 2 μl droplet of the diluted suspension was pipetted on a microscope slide disinfected with 80 % ethanol. The droplet was scanned in overlapping parallel lanes at 300 x magnification. If one or zero resting spores were observed, the droplet was scanned again. Putative resting spores were re-examined at 450 x magnification. Droplets containing one or zero resting spores were used for single-spore or mock inoculations respectively. A 5 to 8 days old seedling of chinese cabbage (*B. rapa*) cv Granaat (ECD-host 05) was placed with its roots on the microscope slide to adsorp the droplet. Subsequently the seedling was transferred to a 1.5 ml eppendorf centrifuge tube, and 1 ml of sterile water was poured over the slide into the tube. After the seedlings had been incubated 48 h in a climate room (23 °C, 80 μE·m^{-2}·s^{-1}, 16 h day), they were potted in sterile compost in a glasshouse (18 °C). As a check for contamination, uninoculated seedlings were planted between the seedlings inoculated with one resting spore. The seedlings with mock inoculations were planted separately. After seven weeks the symptoms were evaluated, and clubs were harvested for propagation.

Resistance tests and propagation of isolates

For resistance tests and propagation of isolates the pipette inoculation method was used [Voorrips and Visser, 1993]. Symptoms were evaluated after six weeks on a scale of 0 - 3 according to Buczacki et al. [1975]. A disease index was calculated as the average symptom grade divided by 3, to yield a value between 0 (no symptoms) and 1 (all plants with severe symptoms).

Six tests were performed, designated A to F. The treatments (combinations of host accession and inoculum) used in each test are mentioned in Tables 1 to 5. Each treatment was tested in at least two replicates of six pots, the exact number of replicates depending on the amount of seed available. The non-parametric Kruskal-Wallis statistic (Siegel, 1956) calculated from the symptom grades of the individual plants was used to test for differences between treatments within each accession. Where this statistic proved significant ($P<0.05$), pairwise comparisons between treatments within each accession were made using the non-parametric Mann-Whitney (or Wilcoxon) U-test (Siegel, 1956).

Propagation of isolates was carried out on chinese cabbage (*B. rapa*) cv Granaat. Clubs were harvested, washed under tap water and surface-sterilized (15 s in 70 % ethanol, 20 min in 0.5 % NaOCl, six rinses in sterile water) before storage or further use.

Table 1. Reactions of the European Clubroot Differential set (ECD; Buczacki et al., 1975) inoculated with two single-spore isolates (SSI-1 and SSI-2) of *Plasmodiophora brassicae* and the original field isolate (F.I.).

Host accession	F.I.[1]		F.I.[2]		SSI-1[2]		SSI-2[2]	
	pl[3]	D.I.[3]	pl	D.I.	pl	D.I.	pl	D.I.
ECD01	35	0.00	36	0.00	34	0.00	35	0.00
ECD02	34	0.00	27	0.00	32	0.00	31	0.00
ECD03	33	0.00	27	0.00	34	0.00	32	0.00
ECD04	38	0.00	--[4]	--	--	--	--	--
ECD05	37	1.00	39	0.98	36	0.98	41	1.00
ECD06	40	1.00	33	1.00	35	0.00	36	0.00
ECD07	33	0.98	36	1.00	36	0.01	36	0.00
ECD08[5]	39	0.00	36	0.00	34	0.00	36	0.00
ECD09	36	0.00	36	0.01	36	0.00	35	0.00
ECD10	35	0.00	35	0.04	36	0.04	36	0.09
ECD11	24	0.13	32	0.53	33	0.28	34	0.35
ECD12	36	0.96	24	0.99	31	0.98	25	0.99
ECD13	36	1.00	35	1.00	35	1.00	35	1.00
ECD14	21	1.00	29	1.00	43	1.00	40	1.00
ECD15	36	0.52	28	0.18	31	0.48	27	0.59

[1] Earlier test results with the field isolate [Voorrips and Visser, 1993], included here for comparison

[2] Results of experiment B

[3] pl: number of plants assessed; D.I.: disease index (see Materials and Methods)

[4] --: no results due to germination problems.

[5] The original ECD08 segregated for resistance to the field isolate. The results in this table were obtained with an inbred line selected from the original accession.

Results

Production of single-spore isolates

Over a period of two months, 164 single-spore inoculations were performed. Only two plants inoculated with a single spore developed symptoms, both in the most severe grade. None of the 161 mock inoculated plants (inoculated with droplets in which no resting spore was observed) or of the 160 control plants separating the inoculated plants became diseased. The SSIs in the two diseased plants were designated SSI-1 and SSI-2. SSI-1 was propagated once and SSI-2 twice to produce sufficient inoculum for further experiments.

Characterization of field and single-spore isolates

With the 14 ECD hosts and seven DH-lines tested in experiments A and B, no differences in reaction were observed between SSI-1 and SSI-2 (Tables 1 and 2). Two ECD hosts (ECD06 and ECD07) were susceptible to the field isolate and resistant to the SSIs, while for two DH-lines (Bi and Pt) the reverse was the case, indicating contrasting differential pathogenicity between the field isolate and the SSIs. In the period between experiments

Table 2. Reactions of seven doubled-haploid lines of *Brassica oleracea* inoculated with two single-spore isolates (SSI-1 and SSI-2) of *Plasmodiophora brassicae* and the original field isolate.

Host accession	Field isolate				SSI-1				SSI-2			
	Exp. A		Exp. B		Exp. A		Exp. B		Exp. A		Exp. B	
	pl[1]	D.I.[1]	pl	D.I.	pl	D.I.	pl	D.I.	pl	D.I.	pl	D.I.
DH-line Bi	19	0.09	18	0.00	33	0.41	21	0.73	29	0.68	17	0.84
DH-line Bö	12	0.14	11	0.00		nt[2]	11	0.06		nt	12	0.00
DH-line Ch	27	0.00	14	0.00	13	0.00	16	0.00	15	0.00	16	0.02
DH-line Gr	23	1.00	18	1.00		nt	15	1.00		nt	18	1.00
DH-line La	38	0.04	18	0.00		nt	18	0.11		nt	17	0.12
DH-line O7	15	0.02	11	0.00	18	0.00	16	0.00	17	0.00	17	0.00
DH-line Pt	23	0.03	11	0.03	27	0.46	17	0.57	28	0.58	18	0.61

[1] pl: number of plants assessed; D.I.: disease index (see Materials and Methods)
[2] nt: not tested

A and B the field isolate and both SSIs were propagated on *B. rapa* cv Granaat. Host ECD04 germinated erratically in test B and the test results obtained with this host were not considered to be reliable.

Experiments with mixtures of field isolate and SSI-1

In experiment D, mixtures of the field isolate and SSI-1 in ratios of 1:4, 1:1 and 4:1 with a fixed total inoculum density of $2 \cdot 10^7$ spores·plant^{-1} were compared with the pure inocula at the same and at half the density (Table 3). In experiment C, only the 1:1

Table 3. Disease indices of *Brassica* accessions inoculated with mixtures of single-spore isolate SSI-1 and the original field isolate of *Plasmodiophora brassicae*.

	Inoculum (10^7 spores·plant^{-1})						
Field	1.0	2.0	1.6	1.0	0.4	0.0	0.0
SSI-1	0.0	0.0	0.4	1.0	1.6	2.0	1.0
Host accession							
Septa	1.00[1] (31)	1.00 (28)	1.00 (36)	1.00 (36)	1.00 (36)	1.00 (27)	1.00 (29)
ECD06	1.00 b (29)	1.00 b (30)	1.00 b (35)	1.00 b (35)	1.00 b (31)	0.01 a (30)	0.04 a (28)
ECD07	1.00 b (24)	1.00 b (22)	1.00 b (30)	1.00 b (31)	1.00 b (30)	0.00 a (24)	0.00 a (24)
DH-line Bi	0.00 a (24)	0.01 a (24)	0.16 b (27)	0.31 bc (29)	0.43 cd (25)	0.49 d (19)	0.39 cd (23)
DH-line Pt	0.04 a (19)	0.03 a (20)	0.65 b (31)	0.70 bc (30)	0.84 c (29)	0.77 bc (19)	0.85 c (20)

[1] The number of plants tested is indicated within brackets. The occurrence of letters behind the disease indices in a line indicates the presence of significant ($P<0.05$) inoculum effects. Within the same line disease indices followed by the same letter indicate that the effects of the corresponding inocula were not significantly different ($P \geq 0.05$).

Table 4. Disease indices of two *Brassica oleracea* accessions inoculated with mixtures of single-spore isolate SSI-1 and the original field isolate of *Plasmodiophora brassicae*.

		Inoculum (10^7 spores·plant^{-1})							
	Field	2.0	0.0	1.6	0.0	1.8	0.0	1.98	0.00
	SSI-1	0.0	2.0	0.4	0.4	0.2	0.2	0.02	0.02
Host accession	Exp.								
Septa	E	1.00[1]	1.00	1.00	0.96	1.00	0.95	1.00	0.94
		(36)	(42)	(37)	(40)	(47)	(39)	(44)	(40)
	F	1.00	1.00	0.99	0.99	1.00	0.99	1.00	1.00
		(22)	(24)	(24)	(24)	(27)	(28)	(27)	(29)
DH-line Pt	E	0.04 a	0.75 c	0.48 bc	1.00 d	0.21 ab	0.67 c	0.02 a	0.21 ab
		(15)	(12)	(20)	(9)	(8)	(14)	(16)	(14)
	F	0.00 a	0.78 d	0.33 c	0.61 d	0.29 bc	0.70 d	0.12 ab	0.59 cd
		(9)	(9)	(11)	(11)	(7)	(10)	(14)	(9)

[1] As in Table 3

Table 5. Disease indices of three *Brassica* accessions inoculated with mixtures of single-spore isolate SSI-1 and the original field isolate of *Plasmodiophora brassicae*.

		Inoculum (10^7 spores·plant^{-1})							
	Field	0.0	2.0	0.4	0.4	0.2	0.2	0.02	0.02
	SSI-1	2.0	0.0	1.6	0.0	1.8	0.0	1.98	0.00
Host accession	Exp.								
Septa	E	1.00[1]	1.00	0.99	0.97	1.00	0.96	1.00	0.97
		(42)	(36)	(37)	(35)	(32)	(33)	(42)	(39)
	F	1.00	1.00	0.95	0.98	1.00	0.98	1.00	0.97
		(24)	(22)	(14)	(19)	(15)	(20)	(18)	(21)
ECD06	E	--[2]	1.00	1.00	1.00	1.00	1.00	0.88	0.98
			(11)	(16)	(14)	(19)	(17)	(17)	(18)
	F	0.00 a	1.00 b	1.00 b	1.00 b	1.00 b	1.00 b	0.90 b	1.00 b
		(16)	(11)	(16)	(16)	(15)	(16)	(14)	(18)
ECD07	E	--[2]	1.00	1.00	1.00	1.00	1.00	0.98	1.00
			(17)	(19)	(18)	(18)	(18)	(18)	(18)
	F	0.02 a	1.00 b	1.00 b	1.00 b	1.00 b	1.00 b	1.00 b	1.00 b
		(16)	(15)	(14)	(17)	(18)	(18)	(17)	(16)

[1] As in Table 3.
[2] no results due to experimental mistake.

mixture was compared with both pure isolates, yielding very similar results (not shown). ECD06 and ECD07 were fully susceptible to all inocula containing the field isolate, and highly resistant to pure SSI-1 at both inoculum densities. DH-lines Bi and Pt showed

partial susceptibility to all inocula containing SSI-1. The inoculum mixture containing 20 % SSI-1 caused less severe symptoms on these lines than inocula with higher proportions of SSI-1.

In experiments E and F, mixed inocula of the field isolate and SSI-1 were compared with inocula consisting of the same absolute amount of either field isolate or SSI-1 (Tables 4 and 5). Table 4 shows the results with cv Septa and DH-line Pt; DH-line Bi was not included due to an insufficient supply of seed. The presence of the field isolate reduced the severity of disease in DH-line Pt, but not in cv Septa, compared to the corresponding inoculum without field isolate at all levels of SSI-1 tested. The disease severity of DH-line Pt inoculated with pure SSI-1 was not significantly related to inoculum density, although the least symptoms were observed at the lowest inoculum density. From Table 5, it is apparent that ECD06 and ECD07 were severely affected by all inocula containing even a hundredfold reduced amount of the field isolate. ECD06 showed slightly reduced symptoms when 99 % of the inoculum consisted of SSI-1; this reduction was not statistically significant ($P>0.05$).

Discussion

Production of single-spore isolates

Two out of 164 single-spore inoculations produced clubroot symptoms on the susceptible host cv Granaat. This demonstrated that no cooperative action of different pathotypes is required for successful infection. The success rate was comparable to most of the results reported before [Buczacki, 1977; Haji Tinggal and Webster, 1981; Jones et al., 1982b; Scott, 1985], but was significantly smaller than the 8 % and 66 % reported by Schoeller and Grunewaldt [1986] and Schulte [1994] respectively. Differences in success rate may be caused by the condition of the inoculum, as well as by different inoculation procedures. Attempts to quantify the proportion of infecting resting spores from the same batch of field isolate as used in this study were made earlier [Voorrips, 1996]. The estimated proportions varied from $3{\cdot}10^{-5}$ to $6{\cdot}10^{-4}$ in different experiments using the pipette inoculation method. The rate of infection by individual spores was therefore much higher with the single-spore than with the pipette inoculation method, showing that different inoculation procedures may indeed cause large differences in success rate.

Comparison of host specificity of field and single-spore isolates

No differences were observed between the two SSIs produced in this study in differential pathogenicity to all 21 hosts tested. Two major differences between both SSIs and the field isolate were found: ECD hosts 06 and 07 were susceptible to the field isolate but resistant to the SSIs, while remarkably for DH-lines Bi and Pt the reverse was true. The first type of difference was described before for other SSIs of *P. brassicae* [Haji Tinggal

and Webster, 1981; Jones et al., 1982b; Scott, 1985; Schoeller and Grunewaldt, 1986; Schulte, 1994]. However, the second type of difference observed here, where a SSI is pathogenic on a host resistant to the original field isolate, was only described by Jones et al. [1982b] and Schoeller and Grunewaldt [1986], although the latter authors did not confirm their result in a separate experiment.

Interaction of field and single-spore isolates

Tests E and F with inocula consisting of mixtures of SSI-1 and field isolate showed that DH-line Pt still reacted differently with mixtures consisting of only 10 % SSI-1 compared with the pure field isolate (Table 4). This suggests that the SSI-1 pathotype is not a major component of the field isolate. The fact that this pathotype was obtained as a SSI in both cases is therefore hard to explain. Either it can be considered as an improbable coincidence, or as evidence for some kind of selection for specific pathotypes operating during the SSI extraction process. One can speculate for example that not all pathotypes of *P. brassicae* in the field isolate are able to complete their life-cycle in a homokaryotic and homozygous condition. More SSIs from the same field isolate are needed to substantiate this finding.

DH-line Pt was partially susceptible to SSI-1 at inoculum densities down to $2 \cdot 10^5$ spores·plant^{-1} or less (Table 4). Addition of the field isolate to a final density of $2 \cdot 10^7$ spores·plant^{-1} resulted in clearly reduced symptom development. This shows that some other pathotype present in the field isolate, non-pathogenic on this line, either competed very successfully with SSI-1 for infection sites or other limited host resources, or rapidly induced resistance in this host genotype. A similar conclusion was reached by Jones et al. [1982b].

On ECD06 and ECD07 no alleviating effect of SSI-1 was observed (Table 5). Moreover, Voorrips [1996] showed that interactions between spores, competitive or otherwise, on a susceptible host do not have a large influence on the rate of infection by individual spores. Therefore the competition by non-pathogenic pathotypes for limited host resources is not likely to be the major cause of the interaction between the field isolate and SSI-1 when inoculated on DH-line Pt. This then leaves the possibility of induced resistance as an explanation of the observed effect. Reports of resistance to pathogens induced in plants by non-pathogenic micro-organisms are numerous [Madamanchi and Kuć, 1991]. In contrast to reported forms of induced resistance, where a lag period is required between application of the inducing and the pathogenic organism, the resistance described in this paper is induced by simultaneous inoculation with the inducing and the pathogenic pathotype. This may be explained by the fact that early contact between *P. brassicae* and its *Brassica* hosts in the root hair stages of infection is non-specific, whereas the specific resistant reaction develops later, in the root cortex [Voorrips, 1992].

Consequences of putative induced resistance

The possible occurrence of resistance induced by non-pathogenic pathotypes has important consequences for the strategy of breeding for clubroot resistance. The common practice of mixing isolates to screen breeding material for a broad resistance to clubroot may in fact reduce the level of infection compared to separate inoculation of the isolates, rather than enhance it. It will therefore be necessary to test also with separate isolates. Further, if a host genotype is resistant to a field isolate, this may be caused by induced resistance. Such resistance may break down by disappearance of inducing pathotypes as well as by the emergence of new pathotypes. Therefore, induced resistance may be less durable than constitutively expressed forms of resistance. On the other hand, cultivars capable of expressing induced resistance could conceivably benefit from the artificial application of an inducing pathotype to the field. A first priority for further studies on this subject is the isolation of resistance inducing SSIs.

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Chapter 6

Genetic analysis of resistance to clubroot (*Plasmodiophora brassicae*) in *Brassica oleracea*. 1. Analysis of symptom grades

Abstract

The inheritance of resistance to clubroot, caused by *Plasmodiophora brassicae*, in *Brassica oleracea* was studied in the F_1, F_2 and backcross progenies of four crosses between resistant and susceptible doubled haploid lines. The disease severity was scored visually on a 0-3 scale of symptom grades. These were analyzed qualitatively and quantitatively. The qualitative analysis involved the conversion of symptom grades to a classification as resistant or susceptible, and segregation ratios were used to test several simple genetic models. The quantitative analysis was based on a threshold model, in which symptom grades are considered to arise from splitting a continuous response range into disjoint intervals. This analysis was based on the maximum likelihood method, and several genetic models were evaluated.

Of the four resistances studied, one was shown to be largely determined by two complementary genes. Two other resistances were also shown to be probably controlled by two genes, but the mode of inheritance was not determined unambiguously. The fourth resistance appeared to be determined by more than two genes.

Abbreviations: cv: cultivar; DH: doubled haploid; ECD: European Clubroot differential set

Introduction

All vegetable crops belonging to *Brassica oleracea*, including cabbage, broccoli, cauliflower and others, are susceptible to the clubroot disease. This disease, caused by the fungus *Plasmodiophora brassicae*, occurs in all regions where these crops are widely grown. Clubroot is hard to prevent by cultural practices, and chemical treatments are not generally applicable (Voorrips, 1995). Resistant cultivars would therefore be desirable. Although some sources of resistance are available, the results of resistance breeding have been largely disappointing. For most of these sources only little is known about the genetic basis of resistance (Voorrips, 1995).

Between 1987 and 1994 a detailed study was carried out at CPRO-DLO to obtain more information on the genetics of resistances effective in The Netherlands. Many accessions of *B. oleracea* reported to be resistant to clubroot were tested with a Dutch isolate of *P. brassicae* (Voorrips & Visser, 1993). From resistant plants of the most promising accessions doubled-haploid lines (DH-lines) were obtained via microspore

culture (Duijs et al., 1992). Four resistant DH-lines, each derived from a different resistant accession, were crossed with a susceptible DH-line. Here the observation and analysis of segregation of resistance in progenies of these four crosses are presented.

One of the problems encountered in the genetic analysis of clubroot resistance in *B. oleracea* is the sometimes quantitative expression of the resistance. Disease symptoms are often classified into symptom grades. The analysis is either based on a distinction between "susceptible" and "resistant" plants according to the symptom grade, or the grades themselves are used as a measure of the level of resistance (Voorrips, 1995). The statistical tools usually employed in the analysis of symptom grades in clubroot literature are not really appropriate for this type of data, since they require normally distributed data with constant error variance. In this study two types of analysis of symptom grades are compared: a qualitative analysis and a quantitative analysis based on a threshold model (Jansen, 1990; Straathof et al., 1993). From the same material also quantitative measurements were obtained. The analysis of those data and the comparison with the analysis based on symptom grades are the subject of an accompanying article (Voorrips & Kanne, 1996).

Material and methods

Plant material

Four sets of populations involving four sources of clubroot resistance in *B. oleracea* were studied. Each set included a resistant and a susceptible doubled-haploid line (DH-line), their F_1, F_2 and respective backcrosses (Fig. 1). DH-line Gr, obtained through microspore culture (Duijs et al., 1992) from broccoli cv Greenia (Hammenhögs Frö AB, Hammenhög, Sweden) was used as the susceptible parent in all four sets. The four resistant parents were obtained via microspore culture from the following accessions:

- DH-line Ch from a cabbage line (accession 35890, Braunschweig gene bank, Germany) derived from a cross involving line 8-41 (Chiang & Crête, 1970);
- DH-line RD from a cabbage line derived from the double cross (Resistant Detroit x Golden Acre) x (Racin Market x Globe) (Nieuwhof & Wiering, 1962);
- DH-line Bi from a cabbage line selected by I.R. Crute (HRI, Wellesbourne, U.K.) from the landrace Bindsachsener;
- DH-line Pt from curly kale cv Petibor-F1 (Bejo Seeds B.V., Warmenhuizen, The Netherlands).

In all experiments some genetically uniform standard accessions were included, while in the experiments with the progenies derived from DH-line Bi also a population of DH-lines derived from the F_1 (Bi x Gr) was included. This population will be discussed in two further studies (Voorrips & Kanne, 1996; Voorrips et al., 1996).

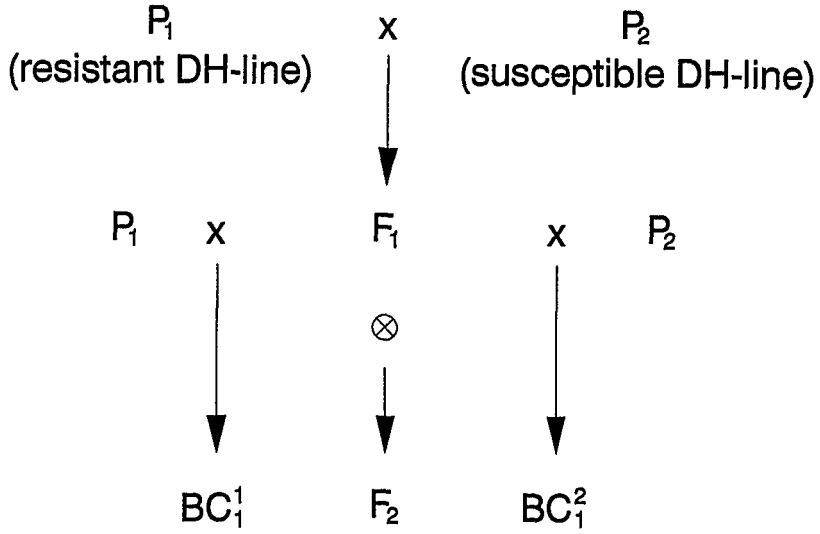

Figure 1. Schematic diagram of the populations studied. Four similar sets of populations were obtained. In each case P_2 was the clubroot-susceptible DH-line GR. The four resistant parents were DH-lines Ch, RD, Bi and Pt.

Pathogen

Two isolates of *P. brassicae* were used in the current study, designated "field isolate" and "SSI-1". The field isolate was obtained from an infested field at Brabant Experimental Station in the Netherlands and characterized as ECD 16/3/30 (Buczacki et al., 1975; Voorrips & Visser, 1993). SSI-1 was a single-spore isolate derived from this field isolate (Voorrips, 1996). The two isolates differed in pathogenicity towards several accessions, including DH-lines Bi and Pt. Suspensions of resting spores were produced from clubs stored at -20 °C according to Voorrips & Visser (1993).

Resistance tests

Resistance tests were carried out in a phytotron chamber, at 22 °C, with a photoperiod of 16 h at 110 $\mu E \cdot m^{-2} \cdot s^{-1}$ HPI-T illumination, according to Voorrips & Visser (1993). Briefly, one seed was sown per pot and $2 \cdot 10^7$ spores were applied to the soil. After six weeks, the root symptoms were evaluated as discussed below.

Seven experiments were performed: two each for the populations derived from DH-lines Bi (experiments K-105 and K-108) and Ch (K-106 and K-110), one for RD (K-102), one for Pt (K-103), and one for Bi and Pt together (K-112). In the experiments with the Ch and RD populations half of the blocks were inoculated with the field isolate and half with SSI-1; the experiments with the Bi and Pt populations were only inoculated with the field isolate since the parental lines were susceptible to SSI-1 (Voorrips, 1996).

Each experiment included eight blocks. In each block, all populations pertaining to the cross were included (P_1, P_2, F_1, F_2, BC_1^1 and BC_1^2), as well as several genetically uniform standard populations. In experiment K-112 each block included all populations from both the Bi and the Pt crosses. The exact numbers of plants evaluated per population depended on the available seed and germination rate. Further, weakly developed seedlings were not evaluated since these often fail to produce symptoms even when susceptible (Crute, 1986). The total numbers of plants evaluated are listed in the tables describing the results for each set of populations.

Symptom grades

In all experiments disease symptoms were assessed visually as follows (adapted from Buczacki et al., 1975). Grade 0: no symptoms; grade 1: small, separated globular or spindle-shaped clubs on tap root or side roots, not more than 2 mm in diameter; grade 2: intermediate symptoms; grade 3: clubs larger than 1 cm in diameter or affecting more than half of the root system. Symptoms of grade 3 always affected the tap root and the proximal part of side roots, and sometimes extended into the hypocotyl as well.

Qualitative analysis of symptom grades

The qualitative analysis was carried out to find evidence for simple models of the inheritance of resistance. For each experiment-inoculum combination attempts were made to distinguish plants in susceptible, resistant and, for some models, intermediate categories based on the symptom grade. The criterion was that the segregation (or lack

Table 1. Coefficients of parameters contributing to the genotypic values in monogenic and digenic models of clubroot resistance.

Genotype[a]	Parameters[b]						
	m	[d]	[h]	CR	DR	CS	DS
monogenic models							
A_1A_1	1	1	0				
A_1A_2	1	0	1				
A_2A_2	1	-1	0				
digenic models[c]							
$A_1A_1B_1B_1$	1	1	0	1	⅓	⅓	1
$A_1A_1B_1B_2$, $A_1A_2B_1B_1$	1	½	½	1	⅓	⅓	-⅓
$A_1A_2B_1B_2$	1	0	1	1	⅓	-1	-⅓
$A_1A_1B_2B_2$, $A_2A_2B_1B_1$	1	0	0	-⅓	⅓	⅓	-⅓
$A_1A_2B_2B_2$, $A_2A_2B_1B_2$	1	-½	½	-⅓	⅓	-1	-⅓
$A_2A_2B_2B_2$	1	-1	0	-⅓	-1	-1	-⅓

[a] Alleles A_1 and B_1 are inherited from P_1, the resistant parent, and alleles A_2 and B_2 from P_2, the susceptible parent.
[b] m: the expected mean of the F_∞-lines; [d]: the summed additive effects of all loci; [h]: the summed dominance effects of all loci (Mather & Jinks, 1982). Parameters for epistatic effects in digenic models: one of CR, DR, CS or DS (respectively Complementary or Duplicate gene effects for Resistance or for Susceptibility).
[c] The two loci in the digenic models are assumed to have equal additive and dominance effects.

thereof) in each population should fit the expected ratios for one of the following models: monogenic recessive, intermediate or dominant resistance, or digenic with unlinked complementary or duplicate genes. The categories did not necessarily correspond to the same symptom grades in all experiments, allowing some compensation for varying levels of disease severity between experiments and for varying levels of resistance between sources.

Quantitative analysis of symptom grades

A threshold model (Jansen, 1990; Straathof et al., 1993) was used to estimate the parameter values for a variety of genetic models in each experiment-inoculum combination. In the threshold model symptom grades are regarded as segments of a continuous response range which are separated by thresholds; the first threshold, separating grades 0 and 1 is defined at 0.0. The error term in the response is considered to be normally distributed with a constant variance set equal to 1.0. Several genetic models were fitted using this method, including a monogenic model, digenic models with two genes with equal effects, eventually including epistatic effects due to complementary or duplicate genes, and polygenic models without epistasis.

All models included the parameters th1 and th2 (the thresholds separating symptom grades 1, 2 and 3), the means of all standard populations, m (the expected mean of the F_∞-lines), [d] (the summed additive effects of all loci) and [h] (the summed dominance effects of all loci) (Mather & Jinks, 1982). The digenic models could further include a term describing the magnitude of the duplicate or complementary gene effect (Table 1). The polygenic model included parameters for variance components D, H and S(dh) (Mather & Jinks, 1982).

Log-likelihoods were estimated for each model. Likelihood ratio tests were used to investigate the effect of eliminating parameters from a model. Test statistics, twice the difference in log-likelihood between the full and the reduced model, were compared with a χ^2_d distribution where d is the number of eliminated parameters.

Results

The cross Ch x Gr The segregating populations derived from the F_1(Ch x Gr), along with the parents, F_1 and several standard genotypes were tested with either the field isolate or SSI-1 in two experiments, K-106 and K-110. The results and conclusions were similar for both isolates and for both experiments; only the results obtained with SSI-1 in experiment K-110 (the same objects as analyzed in Voorrips & Kanne, 1996) are discussed here (Table 2).

Considering simple genetic models with one or two unlinked resistance genes, the only adequately fitting model (P>0.05) involves two complementary resistance genes:

Table 2. Frequency distribution of symptom grades in populations related to the cross Ch x Gr tested with *P. brassicae* isolate SSI-1 in experiment K-110.

Population	grade 0	grade 1	grade 2	grade 3	total
P_1: DH-line Ch	50	2	0	0	52
P_2: DH-line Gr	0	0	1	39	40
F_1	12	35	21	1	69
F_2	17	28	2	46	93
BC_1^1	81	45	3	2	131
BC_1^2	0	11	2	49	62
standard 1	14	25	8	0	47
standard 2	0	0	0	43	43
standard 3	3	12	10	9	34

Table 3. Quantitative analysis of the symptom grade data of the Ch x Gr populations.

Model[a]		LL[b]	comparison[c]	d.f.[d]	P[e]
1	2 loci: [d], [h], CR	-46.1			
2	2 loci: [d], CR	-51.2	2 vs 1	1	***
3	2 loci: CR	-104.1	3 vs 1	2	***
4	2 loci: [d], [h]	-59.3	4 vs 1	1	***
5	1 locus: [d], [h]	-70.6	5 vs 4	0	-

[a] Parameters of 1-locus and 2-loci models are described in Table 1.

[b] LL: Log-likelihood of the best fitting solution.

[c] comparison: the numbers identifying the compared models.

[d] df: difference of degrees of freedom between the compared models.

[e] P: significance of the substitution of the simpler model for the more elaborate model: n.s., *, **, *** indicate $P \geq 0.05$, $P<0.05$, $P<0.01$, $P<0.005$, respectively.

genotypes $A_{Ch}.B_{Ch}.$ are resistant, all other genotypes ($A_{Gr}A_{Gr}..$ and $..B_{Gr}B_{Gr}$) are susceptible. This model predicts fully resistant F_1 and BC_1^1 populations and segregation ratios of 9:7 and 1:3 (resistant : susceptible) for the F_2 and BC_1^2 respectively. To obtain the best fitting ratios, the distinction between resistant and susceptible was made between grade 2 and 3.

Because the qualitative analysis indicated that two complementary loci might be involved in resistance, the quantitative analysis of symptom grades was also based on this assumption. In this analysis, the full model involving two loci with equal additive and dominance effects and epistatic effect CR (Table 1) was compared with reduced models omitting some of these effects. The two-loci model without epistasis was also compared with a one-locus model with additive and dominance effects (Table 3). For each model many solutions (combinations of parameter values) were found, all with virtually equal log-likelihoods. One solution for the model with additive and dominance effects and epistasis is shown in Fig. 2. The different solutions all had similar estimates for the threshold values separating the symptom grades and similar estimates for the genotypic values of the standard genotypes. They differed markedly for the estimates of m, [d], [h] and the epistatic effect. Therefore, the analysis could only be used to test the significance

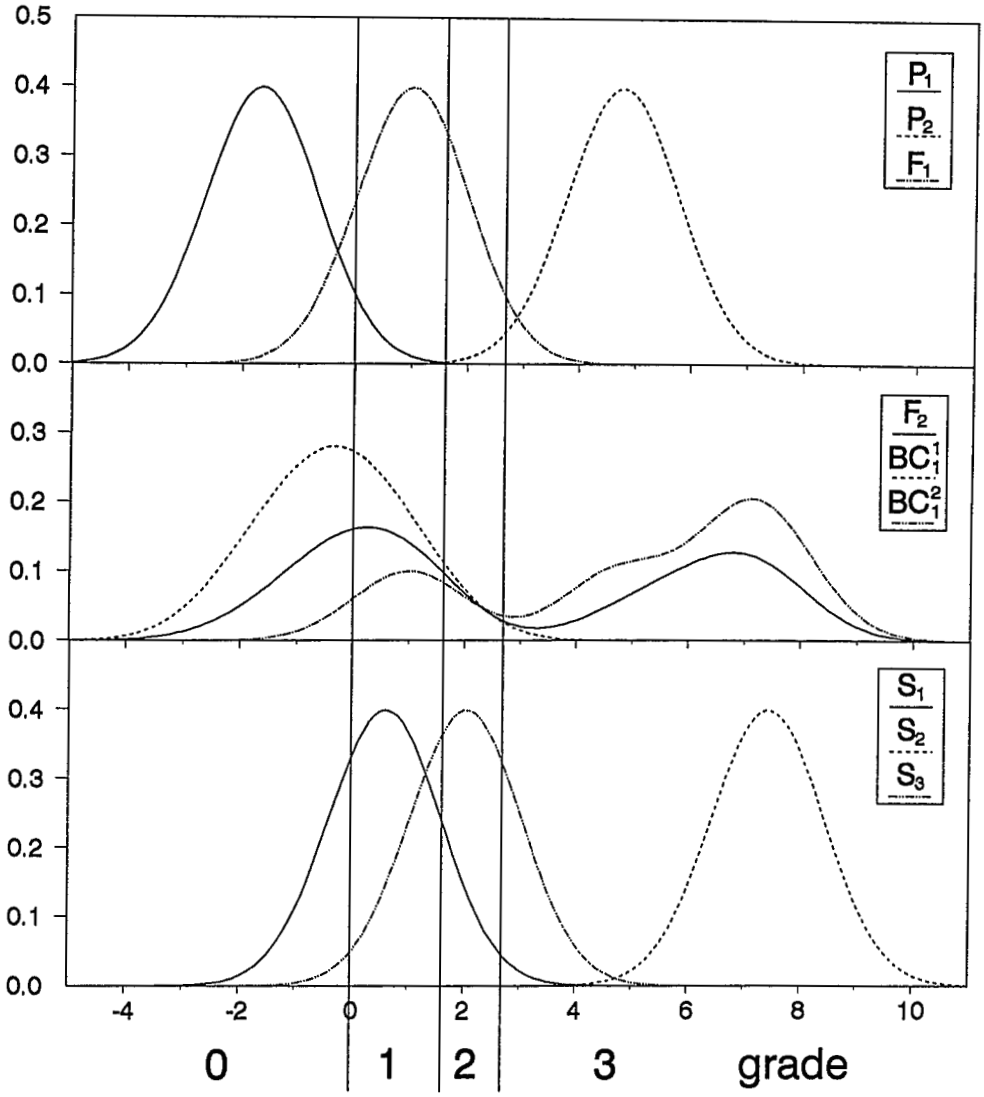

Figure 2. Example of a solution of the threshold model for the Ch x Gr populations (Table 2). The probability density graphs for P_1, P_2, F_1, F_2, backcross populations and three genetically uniform standard populations (S_1, S_2 and S_3) are shown with the following parameter values: thresholds separating the symptom grades at 0 (by definition), 1.70 and 2.71; m=5.86; [d]=-1.08; [h]=3.77; CR=8.62; genotypic means of the standard populations at 0.60, 7.44 and 1.84.

of the different effects, but not to estimate them. The epistatic effect and the [d] and [h] effects were significant. The fit of the two-loci model was much better than that of the one-locus model. The quantitative analysis of symptom grades therefore supports the simple model found in the qualitative analysis.

The cross RD x Gr The populations derived from the RD x Gr cross were tested with the field isolate and SSI-1 in experiment K-102. As for the previous cross, the results obtained with both isolates were similar, and only the results obtained with SSI-1 are presented (Table 4). Considering simple genetic models involving one or two unlinked resistance genes with qualitative effects, the only approximately fitting model involves one recessive resistance gene: $A_{RD}A_{RD}$ is resistant, $A_{RD}A_{Gr}$ and $A_{Gr}A_{Gr}$ are susceptible. This model predicts fully susceptible F_1 and BC_1^2 populations and segregation ratios of 1:3 and 1:1 (resistant:susceptible) for the F_2 and BC_1^1 respectively. To obtain the best fitting ratios, the distinction between resistant and susceptible was made between symptom grade 2 and 3, although several "resistant" plants were present in both the F_1 and the BC_1^2. Using a confidence level of 0.05 for chi-square test for the segregation ratios the deviation in the BC_1^1 was barely significant while the F_2 segregation fitted well. After correction for the frequency of "resistant" plants observed in the F_1 the model fitted in both the F_2 and the BC_1^1. However, the more or less intermediate scores in the F_1 and BC_1^2 had to be ignored for this interpretation.

Since the qualitative analysis suggested the presence of only one gene, the quantitative analysis was directed towards simple genetic models involving only one or two genes. In the two-gene models, epistasis due to complementary or duplicate genes for resistance or for susceptibility was taken into account (Table 5). The model involving

Table 4. Frequency distribution of symptom grades in populations related to the cross RD x Gr tested with *P. brassicae* isolate SSI-1 in experiment K-102.

Population	grade 0	grade 1	grade 2	grade 3	total
P_1: DH-line RD	2	3	0	0	5
P_2: DH-line Gr	0	0	0	10	10
F_1	1	1	5	39	46
F_2	4	13	35	186	238
BC_1^1	6	23	24	34	82
BC_1^2	0	0	2	83	85
standard 1	0	0	0	23	23
standard 2	1	4	17	13	35

Table 5. Quantitative analysis of the symptom grade data of the RD x Gr populations

Model[a]		LL[b]	comparison[c]	d.f.[d]	p[e]
1	2 loci: [d], [h], CR	-32.5			
2	2 loci: [d], [h], DR	-32.5			
3	2 loci: [d], [h], CS	-32.5			
4	2 loci: [d], [h], DS	-32.5			
5	2 loci: [d], [h]	-32.8	5 vs 1-4	1	n.s.
6	2 loci: [d]	-33.3	6 vs 5	1	n.s.
7	1 locus: [d], [h]	-39.8	7 vs 5	0	-
8	1 locus: [d]	-39.8	8 vs 7	1	n.s.

[a]..[e] as in Table 3

two loci with equal additive and dominance effects fitted better than the one-locus model; epistatic effects could not be demonstrated. The dominance effect was not significant in all models tested. The quantitative analysis therefore indicates a two-loci model without dominance or epistasis for the resistance present in DH-line RD, rather than the one recessive gene suggested by the qualitative analysis.

The cross Bi x Gr Since the resistant parent of this cross showed only limited resistance to isolate SSI-1, these populations were tested with the field isolate only in three experiments: K-105, K-108 and K-112. In experiments K-108 and K-112 also the root

Table 6. Frequency distribution of symptom grades in populations related to the cross Bi x Gr tested with *P. brassicae* field isolate in two experiments.

Population	grade 0	grade 1	grade 2	grade 3	total
Experiment K-108					
P_1: DH-line Bi	14	5	0	0	19
P_2: DH-line Gr	0	0	0	47	47
F_1	0	0	2	39	41
F_2	7	39	52	180	278
BC_1^1	51	109	53	55	268
BC_1^2	0	0	1	70	71
standard 1	2	27	13	5	47
Experiment K-112					
P_1: DH-line Bi	30	3	0	0	33
P_2: DH-line Gr	0	0	0	49	49
F_1	0	4	6	34	44
F_2	2	22	12	50	86
BC_1^1	31	28	13	20	92
BC_1^2	0	0	0	93	93
standard 1	8	43	29	10	90
standard 2	72	10	0	0	82
standard 3	1	0	2	47	50

Table 7. Quantitative analysis of the symptom grade data of the Bi x Gr populations.

Model[a]		LL[b]	comparison[c]	d.f.[d]	P[e]
Experiment K-108					
1	polygenic	-26.9			
2	polygenic, [h]=H=S(dh)=0	-29.5	2 vs 1	3	n.s.
Experiment K-112					
1	polygenic	-38.6			
2	polygenic, [h]=H=S(dh)=0	-43.1	2 vs 1	3	*

[a] In the full polygenic model (1) the components of means m, [d] and [h] and the three components of genetic variance D, H and S(dh) (Mather & Jinks, 1982) are estimated without constraints; in the reduced model (2) all parameters involving dominance effects are set equal to 0.

[b]..[e] as in Table 3

symptom weights were measured (Voorrips & Kanne, 1996); the symptom grade data of those two experiments are presented here (Table 6). The results of experiment K-105 were very similar to those of experiment K-108.

No simple qualitative genetic models involving only one or two resistance genes could be fitted. Therefore the quantitative analysis concentrated on fitting a polygenic model. The genetic variances of the segregating populations were assumed to be composed of the components D (variance due to additive effects), H (variance due to dominance effects) and S(dh) (interactions of additive and dominance effects) according to Mather & Jinks (1982). The full polygenic model and a model excluding all parameters associated with dominance ([h], H and S(dh)) were fitted to the data from both experiments (Table 7). Eliminating the effects of dominance from the models decreased the likelihood non-significantly in experiment K-108 and significantly in K-112, so dominance (of susceptibility over resistance) appeared to be important. The likelihood of the polygenic model with or without dominance was much higher than that of all models involving only one or two loci (not shown) suggesting that more than two loci are involved in this resistance.

The cross Pt x Gr As in the previous set of populations, the resistant parent was only partially resistant to isolate SSI-1, and all populations were therefore tested with the field isolate only in two experiments: K-103 and K-112. Qualitative analysis of both experiments yielded the same results; quantitative analysis of experiment K-103 was not possible because no standard populations with intermediate levels of resistance were available. Therefore only the data from experiment K-112 are presented here (Table 8).

Considering simple genetic models involving one or two unlinked resistance genes with qualitative effects, the only fitting model ($P \geq 0.05$) involved two duplicate genes for susceptibility. This model predicts fully susceptible F_1 and BC_1^2 populations and segregation ratios of 1:15 and 1:3 (resistant:susceptible) for the F_2 and BC_1^1 respectively. To obtain the best fitting ratios, the distinction between resistant and susceptible was made between grades 1 and 2.

The qualitative analysis indicated a relatively simple model. However, analysis of root symptom weight measurements in experiment K-112 showed no evidence for a simple genetic model (Voorrips & Kanne, 1996). Therefore, in the quantitative analysis of symptom grades simple models involving one or two loci with or without epistasis due to duplicate genes were compared with polygenic models (Table 9). The analysis indicated that the polygenic models did not fit better than the two-loci models, but that the two-loci models fitted much better than the one-locus model. Dominance and (in the two-loci models) all types of epistatic interactions were insignificant.

Analysis of symptom grades therefore suggested that the resistance in DH-line Pt is controlled by two genes. The exact mode of inheritance (duplicate genes or additive gene action) was not clear.

Table 8. Frequency distribution of symptom grades in populations related to the cross Pt x Gr tested with *P. brassicae* field isolate in experiment K-112.

Population	grade 0	grade 1	grade 2	grade 3	total
P_1: DH-line Pt	72	10	0	0	82
P_2: DH-line Gr	0	0	0	49	49
F_1	0	0	2	47	49
F_2	4	9	12	96	121
BC_1^1	2	9	6	15	32
BC_1^2	0	0	0	30	30
standard 1	8	43	29	10	90
standard 2	30	3	0	0	33
standard 3	0	4	6	34	44

Table 9. Quantitative analysis of the symptom grade data of the Pt x Gr populations.

Model[a]		LL[b]	comparison[c]	d.f.[d]	p[e]
1	polygenic	-29.5			
2	polygenic, [h]=H=S(dh)=0	-29.6	2 vs 1	3	n.s.
3	2 loci: [d], [h], CS	-31.5			
4	2 loci: [d], [h], DS	-31.6			
5	2 loci: [d], [h]	-32.8	5 vs 3,4	1	n.s.
6	2 loci: [d]	-32.8	6 vs 5	1	n.s.
7	1 locus: [d], [h]	-60.9	7 vs 5	0	-

[a] In the full polygenic model (1) the components of means m, [d] and [h] and the three components of genetic variance D, H and S(dh) (Mather & Jinks, 1982) are estimated without constraints; in the reduced model (2) all parameters involving dominance effects are set equal to 0. The parameters of the 1-locus and 2-loci models are described in Table 1.

[b]..[e] as in Table 3

Discussion

The analyses of symptom grade distributions in this study are based on the assumption that the alleles of the loci involved in the measured character segregate in the ratios predicted by Mendelian theory. When interpreting the results we should be keep in mind that skewed segregation of alleles has been shown to occur in *B. oleracea*. The frequency of skewed segregation of RFLP marker loci observed in intraspecific F_2 populations in *B. oleracea* is, however, rather small: 5 % (Slocum et al., 1990), 5-12 % (Kianian & Quiros, 1992) and 12 % (Landry et al., 1992). The magnitude of the deviations can vary much, depending on the selection pressure operating at the loci concerned. This means that our analyses should in general be valid, except where clubroot resistance loci might be linked with loci under selection pressure. This cannot be determined without further information.

Results of analysis of four resistant x susceptible crosses

The qualitative analysis of the cross Ch x Gr indicated the presence of two complementary resistance genes in DH-line Ch. The quantitative analysis corroborated this conclusion. Chiang & Crête (1970) analyzed the same types of segregating populations, where the resistant parent was line 8-41, one of the progenitors of our DH-line Ch. Based on a qualitative analysis of symptom grades, they also concluded that resistance was controlled by two loci. In their study, however, these appeared to act as duplicate genes for susceptibility. The results cannot be well compared however, due to differences in test methods, inoculum and pedigree of both parents. Therefore it is not clear whether the loci they have noted are the same as those described by us.

The qualitative analysis of the cross RD x GR indicated the presence of one recessive resistance gene in DH-line RD. The validity of qualitative analysis is dubious in this case, since the F_1 and BC_1^2 were not fully susceptible as expected in the qualitative model. Therefore the conclusion from quantitative analysis: two resistance genes, without epistatic interactions and without dominance effects merits more confidence.

Qualitative analysis of the cross Bi x Gr failed to produce evidence for one- or two-locus models. Quantitative analysis based on polygenic models provided evidence for dominance (of susceptibility over resistance). In this cross therefore the resistance appears to be determined by more than two loci. The segregation of resistance in the population of DH-lines derived from the F_1 of this cross however indicates a simple model of resistance (Voorrips & Kanne, 1996; Voorrips et al., 1996). Possibly some assumptions underlying the analyses presented here (*e.g.* Mendelian segregation of alleles of the resistance genes) are not valid in this cross.

Qualitative analysis of the cross Pt x Gr indicated two duplicate genes for susceptibility. Quantitative analysis confirmed the presence of two loci, but failed to show evidence for epistasis. The resistance segregating in this cross therefore appears to be largely controlled by two genes, but the exact mode of inheritance could not be determined.

Merits and problems of the two methods of analysis

The visual assignment of symptom grades is a fast method for recording disease symptoms. It has however the disadvantage of arbitrary thresholds, which cannot be consistently adapted to the overall level of disease expression in the experiment and which may vary depending on the observer, the previously scored plants and various unspecified factors.

A further disadvantage of symptom grades is their ordinal nature: common statistics such as means and variances cannot be used in statistical analyses. Two options are available to circumvent this limitation. Firstly, some threshold can be defined to divide plants into susceptible and resistant categories, and the fit of qualitative genetic models can be evaluated by chi-square tests on the frequencies of plants in each category.

Secondly, the full information of the symptom grades can be used by employing statistical methods designed for ordinal data.

The qualitative interpretation of symptom grades repeats the difficulties mentioned above associated with the specification of more or less arbitrary thresholds. If most plants were either completely free of symptoms or severely diseased, the distinction between resistant or susceptible could be easily made. However, intermediate levels of symptom expression occurred regularly in the segregating populations, although not usually in the parents. This makes the distinction more arbitrary, and also indicates that the qualitative analysis is at best only a rough approximation.

The quantitative analysis used here is based on the assumption of the presence of an underlying continuous scale on which all genotypes have a normal distribution with equal error variance; the four symptom grades are sections of this hidden scale separated by three threshold values. Two problems occur frequently when using this method of analysis. Firstly, when no genetically uniform populations are available with plants in more than two symptom grades, the thresholds cannot be well estimated and the results of the analysis are not informative. Secondly, the two extreme grades are infinite; therefore only a lower or only an upper limit can be calculated for the genetic value of a population with all plants in one of the extreme grades. This situation occurs frequently with the resistant and susceptible parents used in this study; in some cases this leads to a multitude of solutions with equal likelihoods. In spite of the latter problem, the log-likelihoods can still be used to compare genetic models, even if parameter estimates cannot be obtained.

The use of both approaches offers the possibility of mutual confirmation or disagreement of the conclusions. When both methods give similar results over several experiments, more confidence can be put in the conclusions. For the present work, such confirmation was obtained for the resistance in DH-line Ch: two complementary genes for resistance appear to be present. For DH-lines RD and Pt both methods of analysis indicated the presence of a small number of loci but no agreement was obtained on the exact mode of inheritance. For DH-line Bi the qualitative approach was not feasible, while the quantitative approach indicated a complex inheritance of resistance.

It seems likely that quantitative measurements of symptoms are better suited for genetic analysis of clubroot resistance than symptom grades. This question will be addressed in another study (Voorrips & Kanne, 1996).

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Chapter 7

Genetic analysis of resistance to clubroot (*Plasmodiophora brassicae*) in *Brassica oleracea*. 2. Quantitative analysis of root symptom measurements

Abstract

The inheritance of resistance to clubroot, caused by *Plasmodiophora brassicae* in *Brassica oleracea* was studied in the F_1, F_2 and backcross progenies of three crosses between resistant and susceptible doubled haploid lines. Fresh weights of affected (CW) and healthy (HRW) parts of the root system were measured and R = ln((HRW+0.5)/(CW+0.5)) was used as a measure of resistance. R was shown to have a normally distributed error term with a constant standard deviation over the entire scale. Analysis of means indicated the presence of two complementary resistance genes in one of the crosses. In the other two crosses analysis of means also indicated two-locus interactions, in these cases reducing the cumulative effects of loci homozygous for resistance or for susceptibility alleles. The numbers of segregating resistance genes in these two crosses could not be determined. The presence of epistatic interactions precluded analysis of variance; therefore in this case the measurements were not more informative than the symptom grades analyzed in a previous study.

R showed a high rank correlation (-0.85) with symptom grades assigned to the same plants. The results of genetic analyses of R were generally in agreement with those obtained earlier using symptom grades. Symptom grades can therefore be regarded as a reliable measure of symptom development, despite their more subjective nature.

Abbreviations: cv: cultivar; DH: doubled haploid; ECD: European Clubroot differential set

Introduction

The interaction between the fungus *Plasmodiophora brassicae*, the causal agent of the clubroot disease in crucifers, and its hosts leads to the formation of disease symptoms on the roots. These symptoms can vary from small, globular or spindle-shaped swellings on side roots to severe gall or club formation over the whole root system. In studies of the interaction the symptoms are commonly assessed on a disease severity scale of a small number of symptom grades. A widely used grading system is defined by Buczacki et al. (1975).

The use of a grading system has some clear advantages. It is the fastest way to evaluate symptoms, since no measurements need to be taken. Also, it does not involve

expensive or failure-prone devices. It has however the disadvantage of arbitrary thresholds between grades, which cannot be consistently adapted to the overall level of disease expression in the experiment and which may vary with the observer, the previously scored plants and various unspecified factors. A further disadvantage of symptom grades is their ordinal nature: commonly used statistics such as means and variances cannot be applied in statistical analyses.

An alternative way to assess clubroot severity involves measuring the symptoms. In addition, since vigorous plants can give rise to larger symptoms than weak plants, also the size of the plants has to be measured. Disease severity may then be expressed as some function of the symptom and plant size measurements.

In our work on the genetic analysis of the resistance to clubroot present in selected breeding lines of *Brassica oleracea* both approaches were assessed. In a series of experiments, symptom grades were evaluated in a number of populations derived from resistant x susceptible crosses (Voorrips & Kanne, 1996). In order to confirm the results of the analysis of those symptom grade data, and to permit the application of standard quantitative genetic analysis methods such as analysis of means and variances, symptom measurements and whole root system measurements were obtained as well in some of these experiments. As a measure of resistance a function of these data was derived such that the requirements for applying these quantitative methods (an approximately normally distributed error term with constant variance) were fulfilled.

Our research focused on three subjects: 1) assessment of the correlation between symptom grade data and the measure of resistance derived from the root system measurements, 2) genetic analysis of the clubroot resistance present in selected breeding lines based on this measure of resistance, and 3) comparison of the conclusions reached in this way with those obtained using symptom grades (Voorrips & Kanne, 1996).

Material & Methods

Plant material

Three clubroot-resistant doubled haploid lines (DH-lines) Ch, Pt and Bi, each derived from a different resistant accession, were crossed with susceptible DH-line Gr. DH-line Ch originated from a cross involving cabbage line 8-41 (Chiang & Crête, 1970), DH-line Pt from curly kale cv Petibor-F1 (Bejo Seeds B.V., Warmenhuizen, The Netherlands), DH-line Bi from a cabbage line selected by I.R. Crute (HRI, Wellesbourne, U.K.) from the landrace Bindsachsener, and DH-line GR from broccoli cv Greenia (Hammenhögs Frö AB, Hammenhög, Sweden) (Voorrips & Kanne, 1996). In each case, the F_1 was selfed to obtain a F_2 population, and the F_1 was backcrossed to P_1 (the resistant parent) and P_2 (the susceptible parent) to obtain the BC_1^1 and BC_1^2 populations respectively. Also, from the F_1

(Bi x Gr) a set of DH-lines was derived through microspore culture using the procedure described by Duijs et al. (1992).

Pathogen

Two isolates of *P. brassicae* were used in the current study, designated "field isolate" and "SSI-1". The field isolate was obtained from an infested field at Brabant Experimental Station in the Netherlands and characterized as ECD 16/3/30 (Buczacki et al., 1975; Voorrips & Visser, 1993). SSI-1 was a single-spore isolate derived from this field isolate (Voorrips, 1996). The two isolates differed in pathogenicity towards several accessions, including DH-lines Bi and Pt. Suspensions of resting spores were produced from clubs stored at -20 °C according to Voorrips & Visser (1993).

Resistance tests

Resistance tests were carried out in a phytotron chamber, at 22 °C, with a photoperiod of 16 h at 110 $\mu E \cdot m^{-2} \cdot s^{-1}$ HPI-T illumination, according to Voorrips & Visser (1993). In brief, one seed was sown per pot and directly inoculated with $2 \cdot 10^7$ spores. After six weeks, the root symptoms were evaluated (see below).

Three experiments were performed: one with the progenies derived from DH-line Ch (experiment K-110), one with those from DH-line Bi (K-108) and one in which the progenies derived from DH-lines Bi and Pt were tested simultaneously (K-112). Experiment K-110 with the Ch-populations consisted of four blocks inoculated with SSI-1. Experiments K-108 and K-112 consisted of eight blocks and were inoculated with the field isolate. In each block all populations: P_1, P_2, F_1, F_2, BC_1^1 and BC_1^2 were included. In the experiments with the populations derived from the cross Bi x Gr, the DH-lines derived from the F_1 were randomized over the whole experiment rather than over each block because neither the space nor the available seed allowed eight replications of each line in each experiment.

Root system measurements and symptom grades

For each plant, the fresh weights of the healthy part and the affected part of the root system were determined separately. The compost was removed from the root system under running tap water. Care was taken to recover as much of the root system as possible, but the finest roots were often lost, especially if decayed. The clubs were separated from the healthy roots; both were dried by squeezing between tissue paper and weighed separately on an electronic scale with 0.01 g precision. For statistical analysis, the function

$$R = \ln\left(\frac{HRW + 0.5}{CW + 0.5}\right)$$

was used as a measure of resistance, where HRW is Healthy Root Weight and CW is Club

Weight, both expressed in grams (g). Data of plants with a total root weight (HRW+CW) < 0.3 g were rejected. R was corrected for test and block effects before further analysis.

In addition, disease symptoms were assessed visually as described before (Voorrips & Kanne, 1996). Grade 0: no symptoms; grade 1: small, separated globular or spindle-shaped clubs on tap root or side roots, not more than 2 mm in diameter; grade 2: intermediate symptoms; grade 3: clubs larger than 1 cm in diameter or affecting more than half of the root system. Symptoms of grade 3 always affected the tap root and the proximal part of side roots, and sometimes extended into the hypocotyl as well.

Analysis of means

The means of the set of populations relating to each cross were analyzed according to a simple additive-dominance model without between-locus interactions and to three models incorporating two-locus interactions. The parameters involved in these models and the coefficients determining their contribution to the expected population means are given in Table 1; the models themselves are elaborated in the next section.

Table 1. Coefficients of parameters describing genetic effects contributing to the population means according to different genetic models.

	Parameters							
Population[a]	m[b]	[d][b]	[h][b]	[i][c]	[j][c]	[l][c]	[f][d]	g[e]
P_1	1	1	0	1	0	0	-1	1
P_2	1	-1	0	1	0	0	1	-⅓
F_1	1	0	1	0	0	1	0	1
F_2	1	0	½	0	0	¼	0	5/12
BC_1^1	1	½	½	¼	¼	¼	-¼	1
BC_1^2	1	-½	½	¼	-¼	¼	¼	0

[a] P_1: the clubroot-resistant parent, P_2: the clubroot-susceptible parent, BC_1^1 and BC_1^2: backcrosses of F_1 to P_1 and P_2 respectively.

[b] All genetic models define parameters m (the expected mean of the F_∞-lines), [d] (the summed additive effects of all loci) and [h] (the summed dominance effects of all loci) (Mather and Jinks, 1982).

[c] A general model incorporating two-locus interactions includes parameters [i], [j] and [l] (respectively the summed homozygote x homozygote, homozygote x heterozygote and heterozygote x heterozygote interaction effects over all pairs of loci) in addition to the m, [d] and [h] parameters (Mather and Jinks, 1982).

[d] A model describing two-locus interactions in pairs of two positive or two negative homozygotic loci involves parameter [f] (the summed effect of these interactions over all pairs of loci) in addition to the m, [d] and [h] parameters.

[e] A model involving two complementary resistance genes includes parameter g describing the interaction between these two genes, in addition to the m, [d] and [h] parameters.

Results

Measure of resistance based on healthy root weight and club weight

In order to obtain a quantitative measure of the level of resistance, healthy root weight (HRW) and club weight (CW) were measured in individual plants in addition to the assignment of symptom grades. A large proportion of small plants with a total root system weight (HRW+CW) < 0.30 g failed to develop symptoms even if they belonged to susceptible populations. Excluding these underdeveloped plants, a total of 3177 plants remained of which HRW and CW were determined. Among these plants, HRW ranged from 0.01 g to 7.45 g, CW from 0 g to 7.06 g and HRW+CW from 0.30 g to 9.34 g.

A function of the data (HRW and CW) was sought with a clear relation to resistance and a normally distributed error term with an approximately constant standard deviation over the entire scale. The distribution of the error term was checked with all genetically uniform populations: the doubled haploid parental lines, the F_1s and the set of DH-lines obtained from the F_1(Bi x Gr).

Functions based on CW alone were not considered, since the size of the clubs depends on the size of the plant as well as on its level of resistance (Crute, 1986). Functions CW/(HRW+CW), arcsin($\sqrt{}$(CW/(HRW+CW)), ln(CW/HRW), HRW/(HRW+CW), arcsin($\sqrt{}$(HRW/(HRW+CW)) and ln(HRW/CW) showed very small standard deviations at the extremes and larger ones at intermediate values. The function R = ln((HRW+0.5)/(CW+0.5)) showed a relatively uniform standard deviation in genetically homogeneous populations of varying levels of resistance (Fig. 1). Function R was therefore used in the remainder of this analysis. The values of R, corrected for test and block effects ranged from -2.38 to 2.37 in individual plants. The means of genetically uniform populations ranged from -1.33 (susceptible) to 1.57 (resistant), and the average sample standard deviation of R, calculated over all genetically uniform populations was 0.44. The

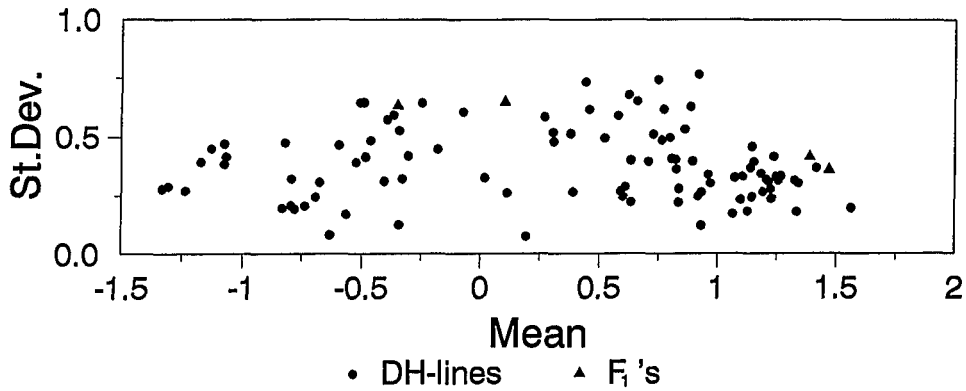

Figure 1. Standard deviations and means of the measure of resistance R in genetically uniform populations of *B. oleracea*: doubled-haploid lines and F_1's of crosses between doubled-haploid lines.

distribution of R in genetically uniform populations inoculated with the field isolate was tested for deviation from a normal distribution with this standard deviation using the Anderson-Darling statistic (Stephens, 1986). Of the 18 genetically uniform populations with at least 12 plants only two deviated significantly ($P<0.05$), which was considered acceptable. Therefore, R was treated as a quantitative character with a mean depending on the genotype and a normally distributed error term with constant standard deviation of 0.44.

Correlation of root measurements and symptom grades

In order to judge the reliability of the symptom grading, the correlation between the symptom grades and the measure of resistance R was calculated using Spearman's rank correlation coefficient (Siegel, 1956). In this case the R values were not corrected for test and block effects, because the corresponding correction was not possible for the symptom grades. Spearman's rank correlation coefficient over all 3177 plants with a total root weight $\geq$ 0.30 g was -0.85, indicating that both measures were fairly closely correlated. The distribution of R values in symptom grades 0 and 1 was similar, but clearly different from those in grades 2 and 3, although the total ranges of R values in grades 0, 1 and 2 overlapped considerably (Fig. 2). The variance of R values of plants in grades

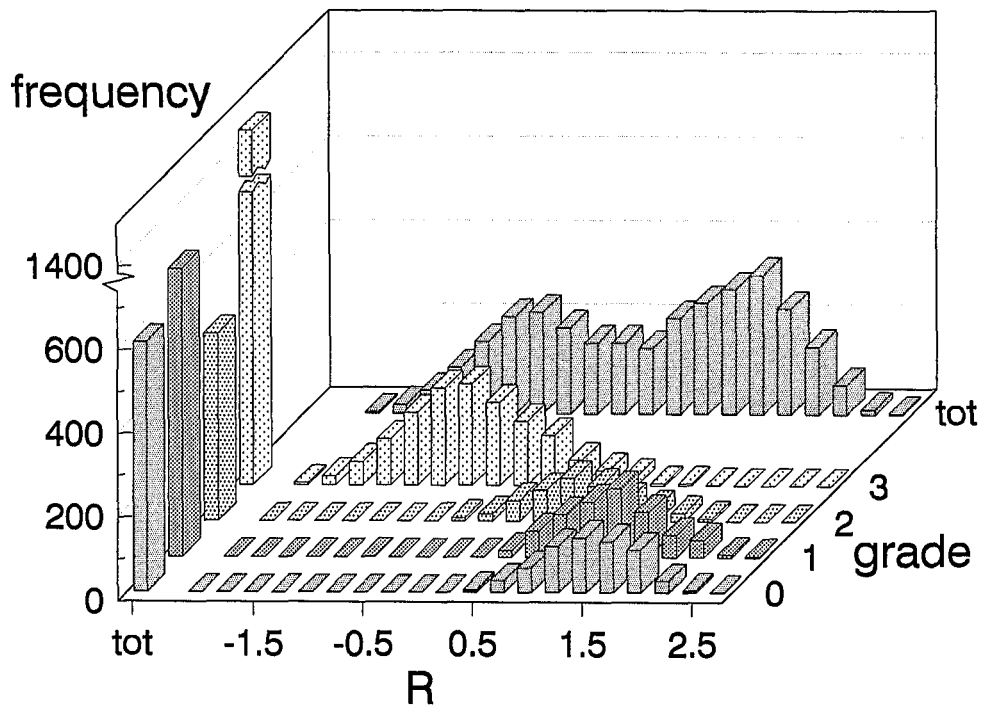

Figure 2. Joint frequency distribution of symptom grades and the measure of resistance R in individual plants.

0, 1 and 2 was comparable, but smaller than that of plants in grade 3. The large number of plants in grade 3 can therefore be partly ascribed to the relatively large width of that grade.

The difference between grades 0 (no symptoms) and 1 (slight symptoms) is not clearly reflected in the R values. On the resistant side of the scale, the symptom grades are therefore more informative than the R values. It is not clear whether this additional information is meaningful in practice, since the further development of disease symptoms and the production of resting spores after transplantation to the field were not studied in the measured plants.

Analysis of means based on root measurements

The cross Ch x Gr The means and variances of R of this set of populations were fitted to a basic additive-dominance genetic model, in which the genotypic value is obtained by addition of the effects of individual unlinked loci (Mather and Jinks, 1982). The

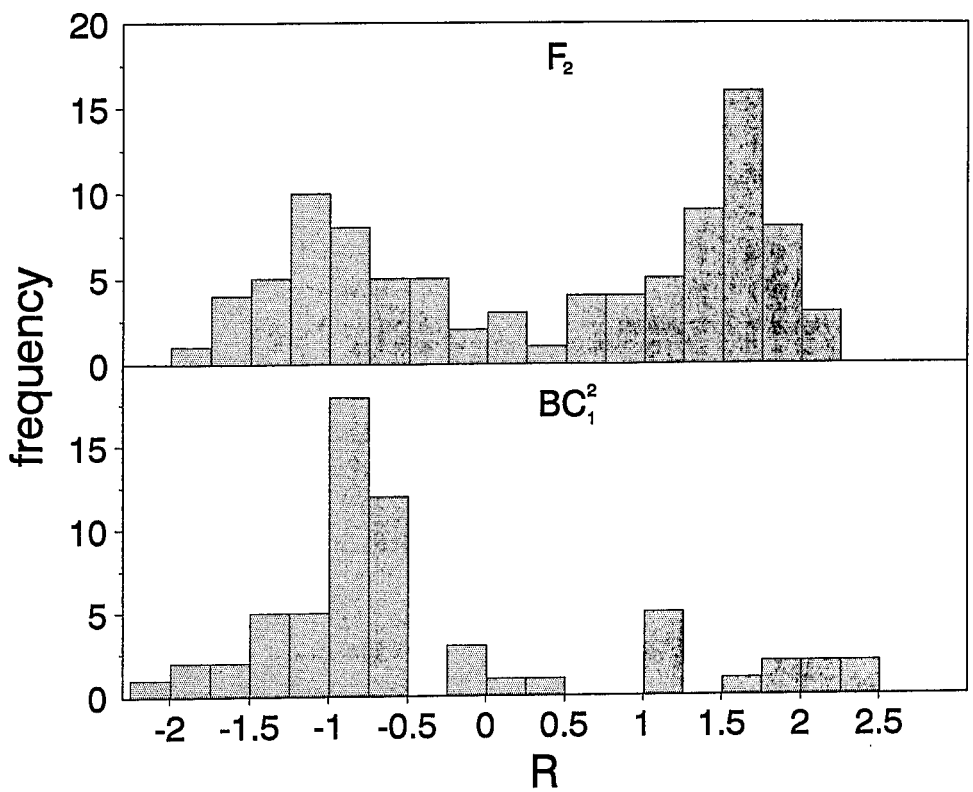

Figure 3. Frequency distribution of the measure of resistance R in an F_2 and a BC_1^2 population descended from the cross Ch x Gr.

Table 2. Analysis of means of the measure of resistance R of the Ch x Gr populations with a model describing the epistatic effect of two complementary genes.

population	n	var	mean		parameter	estimate	s.e.
			obs	exp			
P_1	52	0.078	1.23	1.26	m	-0.52	0.11
P_2	40	0.227	-1.08	-1.07	[d]	-0.06	0.25
F_1	69	0.130	1.47	1.52	[h]	0.20	0.24
F_2	93	1.601	0.34	0.35	g	1.84	0.35
BC_1^1	131	0.138	1.44	1.39			
BC_1^2	62	1.256	-0.39	-0.39		χ_2^2=4.80	

expected genotypic values of the populations pertaining to a single cross are composed as listed in Table 1. This model did not fit the observations, with a χ_3^2 value of 32.37.

The F_2 and the BC_1^2 showed a clearly bimodal distribution (Fig. 3). The segregation ratios in these populations (approx. 50:43 and 13:49 respectively) agreed well with the 9:7 and 1:3 (resistant : susceptible) ratios expected for a resistance determined by two unlinked complementary genes. This model was also indicated by the analysis of symptom grades in the same populations (Voorrips & Kanne, 1996). Further, the standard deviations within each of the peaks were approximately equal to that for genetically uniform populations (0.44). In this case therefore an enhanced model was fitted with a two-locus epistasis term g with coefficients +1 for genotypes $A_{Ch}.B_{Ch}$. and -⅛ for the other genotypes (Table 2). This model fitted well (χ_2^2=4.80, P≃0.1) (Table 2). Additive and dominance components were not significant and most of the variation between population means was described by the epistasis effect. However, a model excluding the additive and dominance effects did not fit (not shown). As an alternative, a six-parameter model including three types of two-locus interactions (Mather and Jinks, 1982; Table 1) was fitted (not shown). Parameter [l] was not significant, [h] and [i] almost significant and [d] and [j] were significant (P=0.05). A model including only interaction effect [j] in addition to parameters [d] and [h] did not fit (χ_2^2=18.34, P<0.005). A model including interaction effects [i] and [j] fitted well (χ_1^2=0.08, P>0.5), but at the cost of introducing one parameter more than in the model with two complementary genes mentioned above, and with a less obvious interpretation. The most meaningful interpretation is therefore that two complementary unlinked resistance genes largely determine the resistance present in DH-line Ch.

The crosses Bi x Gr and Pt x Gr The means and variances of R of the populations pertaining to the crosses Bi x Gr and Pt x Gr were also fitted to the basic additive-dominance genetic model. In these cases too, a significant lack of fit of the model was found, with χ_3^2 values of 100.84 and 14.65 respectively.

In both sets of populations all segregating populations showed an unimodal distribution. The lack of fit in these two cases was due to the fact that the observed

mean of each backcross population was considerably displaced towards the mean of the backcross parent compared to its expected position halfway the F_1 and the parental mean. Because the basic genetic model did not fit, the more elaborate six-parameter model mentioned above was fitted (not shown). This revealed that in these two sets of populations [j] (heterozygote x homozygote interaction) was the only significant interaction effect. The model including only the [j] interaction effect fitted well in both crosses (Table 3, Model A).

Although the meaning of the [j] effect is not easily interpreted, the model including this effect is equivalent to one where the genotypic value of extreme homozygotes is reduced by interactions [f] of pairs of positive or negative homozygotic loci (Table 1; Table 3, Model B). The mathematical equivalence of both models follows from the fact that the coefficients of parameter [f] in model B are equal to the differences of the coefficients of parameters [j] and [d] in model A (Table 1). The biological concept behind this model is that for any homozygous resistant locus the effect on the genotypic value is less when the remainder of the genotype contains more homozygous resistant loci. The same holds, *mutatis mutandis*, for homozygous susceptible loci. In other words, the more resistant (susceptible) a genotype is, the harder it becomes to increase the resistance (susceptibility) further.

Table 3. Analysis of means of the measure of resistance R of the Bi x Gr and Pt x Gr populations with two models involving two-locus interaction effects.

Bi x Gr populations										
	n	var	mean		Model A[a]			Model B[b]		
			obs	exp[c]	param.	est.	s.e.	param.	est.	s.e.
P_1	60	0.112	1.27	1.27	m	0.10	0.03	m	0.10	0.03
P_2	132	0.148	-1.08	-1.08	[d]	1.17	0.03	[d]	2.39	0.11
F_1	96	0.426	0.11	0.10	[h]	0.01	0.06	[h]	0.01	0.06
F_2	364	0.731	0.14	0.10	[j]	1.22	0.12	[f]	1.22	0.12
BC_1^1	360	0.511	0.98	0.99						
BC_1^2	164	0.268	-0.81	-0.79	χ^2_2=0.96			χ^2_2=0.96		
Pt x Gr populations										
	n	var	mean		Model A			Model B		
			obs	exp	param.	est.	s.e.	param.	est.	s.e.
P_1	82	0.137	1.42	1.42	m	0.17	0.03	m	0.17	0.03
P_2	132	0.148	-1.08	-1.07	[d]	1.25	0.03	[d]	2.38	0.30
F_1	50	0.409	-0.35	-0.31	[h]	-0.48	0.08	[h]	-0.48	0.08
F_2	121	0.824	-0.03	-0.07	[j]	1.13	0.30	[f]	1.13	0.30
BC_1^1	32	0.510	0.87	0.84						
BC_1^2	30	0.243	-0.96	-0.97	χ^2_2=0.54			χ^2_2=0.54		

[a] Model A: The basic additive-dominance model extended with two-locus interactions [j] between homozygous and heterozygous loci.

[b] Model B: The basic additive-dominance model extended with two-locus interactions [f] between two positive or two negative homozygous loci.

[c] The expected means for both models are identical.

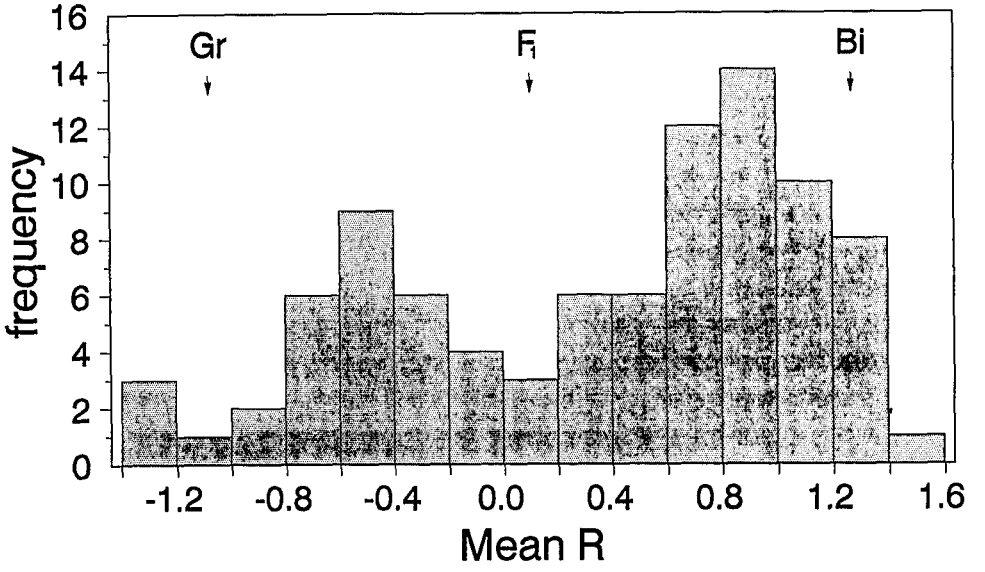

Figure 4. Frequency distribution of the line means of the measure of resistance R in a population of doubled haploid lines derived from the F_1 of the cross Bi x Gr.

From the F_1(Bi x Gr), also a population of DH-lines was derived. Root measurements were obtained from 91 of these DH-lines. Depending on the seed supply and germination, one to 16 plants per DH-line were measured except for one line which was used as a standard in many experiments; for this DH-line 179 plants were measured. From the numbers of plants per line and the error standard deviation of R per plant (0.44), the average error variance of the line means of R was calculated as 0.04. When the means and variance of the 91 DH-line means were included in either of the two four-parameter models, a χ^2_3 value of 10.64 ($P \simeq 0.025$) was obtained (not shown). In contrast to the other segregating populations, the means of the population of DH-lines showed a clearly bimodal distribution (Fig. 4). The size of the more resistant peak (59 DH-lines) was about twice that of the more susceptible peak (32 DH-lines). The variances within the peaks (approx. 0.12) were larger than expected (approx. 0.04) if the population within each peak were genetically uniform for resistance. Therefore, even allowing for possible distorted segregation ratios of resistance genes in the population of DH-lines, more than one gene must be segregating in this population.

Analysis of symptom grades indicated only two segregating resistance genes, with possible epistasis, in the cross Pt x Gr (Voorrips & Kanne, 1996). In the population of DH-lines from the F_1 (Bi x Gr) two genes for resistance were mapped on a genomic map (Voorrips et al., 1996). For these reasons, two-loci models similar to that developed for the cross Ch x Gr (see above) incorporating epistasis effects due to complementary or duplicate genes were fitted for these two sets of populations (not shown). However, none

of these models fitted in either set of populations at a confidence level of $P \geq 0.05$. The conclusions from those two studies could therefore not be confirmed by the analysis of means. The analysis of means presented above gives no indication of the number of segregating loci; it only indicates that epistasis is an important factor in both sets of populations.

Discussion

The measure of resistance R

The function R = ln((HRW+0.5)/(CW+0.5)) showed an approximately normally distributed error term with a constant standard deviation over the entire range. In addition, R proved to be well correlated with symptom grades, as expected for two measures of the same trait. Using R rather than symptom grades, the subjective influences interfering with the assessment of disease severity are eliminated. Further, correction for experiment and block effects becomes possible and quantitative genetic techniques based on normal distribution theory can be utilized. However, in this study an analysis of variance was not possible due to the importance of epistasis effects in all three crosses.

Results of analysis of three resistant x susceptible crosses

The analysis of means in this study, like all genetic analyses performed without genetic markers, is based on the assumption that the loci involved in the measured character segregate in ratios predicted by Mendelian theory. For F_2 populations in *B. oleracea*, the segregation of only 5 - 12 % of RFLP markers deviated significantly ($P<0.05$) from expected ratios (Slocum et al., 1990; Kianian & Quiros, 1992; Landry et al., 1992). In general, the analyses based on F_2 and presumably also backcross data should therefore be valid. In contrast, a large proportion of the loci in populations of DH-lines of *B. oleracea* deviate from the expected 1:1 ratio (Orton & Browers, 1985; Voorrips et al., 1996). This means that populations of DH-lines should not be used in conventional genetic analysis, at least in *B. oleracea*.

The cross Ch x Gr Two complementary resistance genes were demonstrated to operate in DH-line Ch. This agrees with the conclusion from the analysis of symptom grades in the same experiment (Voorrips & Kanne, 1996). The contrast between this conclusion and the genetic model of Chiang & Crête (1970) involving two duplicate genes for susceptibility was discussed in Voorrips & Kanne (1996).

The cross Bi x Gr From the distributions of R in the F_2 and backcross populations no conclusions concerning the number of segregating loci could be derived. No dominance appeared to be involved. However, two-locus interactions were found which reduced the

resistance or susceptibility of extreme genotypes. The bimodal distribution of the means of the DH-lines obtained from the F_1 indicated the presence of only few major resistance genes. The relative frequencies of both peaks in that population were not in agreement with those expected for various one- or two-locus models; elsewhere this is shown to be due to a distortion of segregation ratios (Voorrips et al., 1996).

From the analysis of symptom grades also no evidence for a simple genetic model was obtained. In one of the experiments (K-112) indications of dominance were found (Voorrips & Kanne, 1996). The conclusion from both the analyses of R and of the symptom grades is that the effects of dominance are small, and that the number of segregating major resistance genes remains unclear. This question is the object of a further study (Voorrips et al., 1996).

The cross Pt x Gr The results of the analysis of R were similar to those of the Bi-populations, the only exception being that the resistance in DH-line Pt was partly recessive. The indications for a simple genetic model with two segregating, possibly duplicate loci found by analyzing symptom grades (Voorrips & Kanne, 1996) were not confirmed in the current analysis. However, the number of plants in the informative BC_1^1 was very small (32); it is conceivable that a larger BC_1^1 would show evidence for segregation of a small number of genes.

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Chapter 8

Mapping of two genes for resistance to clubroot (*Plasmodiophora brassicae*) in a population of doubled haploid lines of *Brassica oleracea* by means of RFLP and AFLP markers

Abstract

A genetic map covering 615 cM in 12 linkage groups was assembled based on 92 RFLP and AFLP markers segregating in a population of 107 doubled haploid lines (DH-lines) of *Brassica oleracea*. The DH-line population was obtained through microspore culture from the F_1 of two homozygous parents: DH-line Bi derived from the cabbage landrace Bindsachsener, and DH-line Gr from the broccoli cv Greenia. Sixty-five percent of the loci, and in some cases complete linkage groups displayed distorted segregation ratios, a frequency much higher than observed in F_2 populations of the same species.

DH-line Bi was resistant to clubroot, caused by a Dutch field isolate of *Plasmodiophora brassicae*. Resistance in the DH-line population was determined in two ways: by assigning symptom grades to each plant, and by measuring the fresh weights of the healthy and affected parts of the root system of each plant. Using a multiple QTL mapping approach to analyze the fresh weight data, two loci for clubroot resistance were found which were designated *pb-3* and *pb-4*. The additive effects of these loci were responsible for 68 % of the difference between the parents and for 60 % of the genetic variance among DH-line means. Also indications for the presence of two additional, minor QTLs were found. Analysis of symptom grades revealed the two QTLs *pb-3* and *pb-4*, as well as one of the two minor QTLs indicated by analysis of the fresh weight data.

Abbreviations: AFLP: amplified fragment length polymorphism; DH-line: doubled haploid line; MQM mapping: multiple QTL models; RFLP: restriction fragment length polymorphism; QTL: quantitative trait locus

Introduction

In the past decade, molecular genetic markers have found their way into the study of *Brassica* genomes. RFLP maps have been published for *B. napus* (Landry et al. 1991; Ferreira et al. 1995b), *B. oleracea* (Slocum et al. 1990; Kianian and Quiros 1992; Landry et al. 1992) and *B. rapa* (Song et al. 1991; Chyi et al. 1992; Teutonico and Osborn 1994). These markers have also been used to clarify evolutionary and taxonomic relations within the genus *Brassica* (Song et al. 1988a, 1988b, 1990; Dias et al. 1991; Nienhuis et al. 1993). For plant breeders, the linkage of markers with morphological, physiological and

biochemical characters (*e.g.* Figdore et al. 1993; Kennard et al. 1994; Teutonico and Osborn 1994; Song et al. 1995) is useful, as well as markers closely linked to disease resistance genes. Linkage of genes for disease resistance with molecular markers have been described in *Brassica* species by Landry et al. (1992), Figdore et al. (1993), Ferreira et al. (1995a) and Dion et al. (1995).

Breeding for clubroot resistance in *B. oleracea* is difficult as expression of resistance is often quantitative and inocula generally consist of mixtures of pathotypes (Voorrips 1996). For these reasons, single plant evaluations of resistance are often unreliable, and indirect selection based on markers closely linked to resistance genes would be a great improvement. Indirect selection would also facilitate the incorporation of multiple resistance genes into cultivars. The study of the genetics of resistance can also greatly benefit from the linkage information provided by molecular markers.

In earlier reports we described classical genetic analyses of clubroot resistance in several resistant doubled haploid lines (DH-lines) of *B. oleracea* (Voorrips and Kanne 1996a, 1996b). For one of these lines: DH-line Bi, derived from the cabbage landrace Bindsachsener, the segregation data did not allow conclusions concerning the number and effects of segregating resistance genes. The present report deals with the molecular marker analysis of a population of DH-lines derived from the F_1 between resistant DH-line Bi and susceptible DH-line Gr, the construction of a molecular marker map of the *B. oleracea* genome and the mapping of QTLs for clubroot resistance.

Materials and Methods

Plant material

Clubroot resistant DH-line Bi was obtained via microspore culture from a cabbage line selected by I.R. Crute (HRI, Wellesbourne, U.K.) from the German landrace Bindsachsener. Clubroot susceptible DH-line Gr was similarly derived from broccoli cv Greenia (Hammenhögs Frö AB, Hammenhög, Sweden). From three plants of the F_1(Gr x Bi) a population of doubled haploids was obtained through microspore culture, according to the procedure described by Duijs et al. (1992). About $2.2 \cdot 10^7$ microspores from 218 flower buds yielded 390 embryos from which 120 flowering plants were regenerated, of which 107 produced seeds upon selfing.

Pathogen

The *P. brassicae* isolate used in this study was obtained from an infested field at Brabant Experimental Station at Breda, The Netherlands and characterized as ECD 16/3/30 (Buczacki et al. 1975; Voorrips and Visser 1993). Inoculum was prepared according to Voorrips and Visser (1993) from clubs stored at -20 °C.

Resistance tests

Resistance tests were carried out in a phytotron chamber, at 22°C, with a photoperiod of 16 h at 110 $\mu E \cdot m^{-2} \cdot s^{-1}$ HPI-T illumination, according to Voorrips and Visser (1993). One seed was sown per pot and inoculated with $2 \cdot 10^7$ spores. After six weeks the root symptoms were evaluated. Three experiments were performed in which both parents, the F_1 and the population of DH-lines were tested in eight blocks among a number of other populations not reported in this study (Voorrips and Kanne 1996a).

Symptom evaluation

In all experiments disease symptoms were graded visually: grade 0: no symptoms; grade 1: small, separated globular or spindle-shaped clubs on tap root or side roots, not more than 2 mm in diameter; grade 2: intermediate symptoms; grade 3: clubs larger than 1 cm in diameter or affecting more than half of the root system. Symptoms of grade 3 always affected the tap root and the proximal part of lateral roots, and sometimes extended into the hypocotyl.

In two of the three experiments also the fresh weights of the affected parts (the so-called clubs) and healthy parts of the root system of each plant were measured. From these two measurements: club weight (CW) and healthy root weight (HRW), each measured in g, the measure of resistance R = ln((HRW+0.5)/(CW+0.5)) was calculated. This value was shown in a previous study to correlate well with the symptom grade, and to have a normally distributed error term with an approximately constant error standard deviation of 0.44 (Voorrips and Kanne 1996b). The R-value was corrected for test and block effects before further analysis.

RFLP markers

All RFLP procedures were as described by Van der Beek et al. (1992, 1994). Genomic DNA was digested with restriction endonuclease *Eco*R1. Probes sized 0.35-2.5 kB were obtained from a *B. napus* embryo cDNA library and cloned into the *Pst*1 site of vector pBR322 (Harada et al. 1988) and made available by B.S. Landry (Agriculture Canada Research Station, St-Jean-sur-Richelieu, Canada). Cloned inserts were PCR amplified and radiolabelled (Bernatzky and Tanksley 1986).

RFLP markers were named after the probe (1NB8 to 4NF12) detecting them. If several polymorphic loci were detected with the same probe the loci were designated by appending different lowercase letters to the probe code. This nomenclature is similar to that followed by Landry et al. (1992), but identically named markers in their and our work do not necessarily refer to the same loci.

AFLP markers

AFLP (Amplified Fragment Length Polymorphism) is a recently developed molecular marker technique based on selective PCR amplification of restriction fragments (Vos et al. 1995).

Genomic DNA was digested with restriction endonucleases *Eco*R1 and *Mse*1. After ligation of double-stranded adapters to the ends of the restriction fragments preamplification was performed with primers specific for the *Eco*R1 and *Mse*1 adapters, including one selective nucleotide (underlined), followed by amplification with similar primers with three selective nucleotides (italics): 5' GAC TGC GTA CCA ATT C*A*A *C* 3', and 5' GAT GAG TCC TGA GTA A*C*C *T* 3'. Depending on the amount of amplified DNA after preamplification the reaction mix was diluted 1/20 or 1/40 in TE and 10 μl was used in the final amplification. The E35-primer was end-labelled with γ^{33}P ATP (Feinberg and Vogelstein 1983). Amplification fragments were separated on 5% denaturing polyacrylamide gels. The gels were dried and autoradiography was carried out with Kodak XOMAT AR X-ray film for 1-7 days at -70°C using intensifier screens. Segregating AFLPs were labelled A01 to A26 in order of decreasing fragment size.

Linkage analysis

The JoinMap 2.0 package (Stam 1993; Stam and Van Ooijen 1995) was used to assign markers to linkage groups and to calculate the most probable order and distances of markers within each linkage group. Map distances were based on Kosambi's (1944) mapping function. The basis for assigning markers to linkage groups was a LOD (log of odds) score $\geq$ 4.0 with one or more other markers in the group.

Quantitative trait locus (QTL) analysis

Since the error terms of the R value were approximately normally distributed (Voorrips and Kanne 1996b), the mean R values of the DH-lines could be analyzed with the Multiple QTL Model (MQM) method (Jansen 1994; Jansen and Stam 1994). This is a two-step method, where the first step consists of the selection of markers to be used as cofactors, and the second step consists of the estimation of the effects of putative QTLs throughout the genome after correction for the effects of the selected markers. In the first step, cofactors were selected from 38 markers distributed over the linkage map at approximately 25 cM intervals, the criterion being a significance of $P \geq 0.02$ for the marker effect on R. In the second step, a LOD-threshold of 2.7 was used to obtain a confidence level of 0.95 throughout the genome. This threshold was determined based on 1000 simulations with the actual molecular marker data, according to the method of Jansen (1994). The MQM analysis was carried out with a Genstat5 program developed by R.C. Jansen (CPRO-DLO, Wageningen, The Netherlands).

The error terms of the mean symptom grades of the DH-lines were not normally distributed and the mean symptom grades were therefore not suitable for MQM analysis. A non-parametric Kruskal-Wallis test was performed at each marker locus for significance of a QTL at that locus. In order to obtain a confidence level of 0.95 throughout genome, the tests per marker locus were considered significant if $P <= 0.001$. The Kruskal-Wallis

analysis was carried out using the MapQTL 3.0 package (Van Ooijen and Maliepaard 1995).

Since the segregating population consisted of DH-lines no dominance effects could be assessed using either analysis method.

Results

RFLP and AFLP markers

Sixty-six RFLPs between the parental lines were detected with 37 probes, using restriction endonuclease *Eco*R1. Three further probes (1NH12, 2NA11, 3NG10) did not detect any RFLPs. Five RFLP clusters without recombination were found (1NE1c/3ND12, 1NG9/2NA8a/3NB4b, 1NF2b/2NA1a, 1NF2a/1NH3a/2NF10 and 3NB4c/3NC3b). Because the *Eco*R1 restriction fragment sizes detected were unequal within all these groups these markers detected different but closely linked loci.

With one set of selective primers 26 AFLPs were detected. Five AFLP pairs without recombination were found. In four pairs (A11/A12, A13/A14, A17/A18 and A22/A23) both amplified fragments were inherited from the same parent. Since the sizes of both fragments in these pairs were very similar, it is possible that they represent double bands as reported by Vos et al. (1995), and not different markers. In one pair (A04/A05) each of the two parents contributed one amplified fragment.

Construction of the linkage map

Ninety-two markers were scored for 107 DH-lines. The grouping of the markers in linkage groups was determined at a range of LOD-scores. Preliminary mapping within the linkage groups at each LOD-score was performed. If gaps larger than 25 cM occurred within a group, or less than three markers were responsible for linking subgroups, the coherence of the group was checked by comparing the recombination between the markers on each side of the gap with their calculated map order. In this way, it was found that linkage groups assembled at LOD values ≥ 4.0 were coherent with one exception: groups 3 and 6 appeared to be linked based on the LOD-score (with 1NG1b on group 3 joined to 4NE11c on group 6) but the recombination values between markers in the two groups were not in agreement with the map order. Therefore, these two groups were separated (Fig. 1).

Eighty-seven of the 92 markers could be assembled into 12 linkage groups. The five remaining markers were not linked to any other markers. The linkage groups were numbered in decreasing order of map length (Fig. 1). The total map length covered by the 12 linkage groups was 615 cM.

Since the RFLP probes used were a subset of those used by Landry et al. (1992) for the construction of a *B. oleracea* map, it was possible in some cases to identify

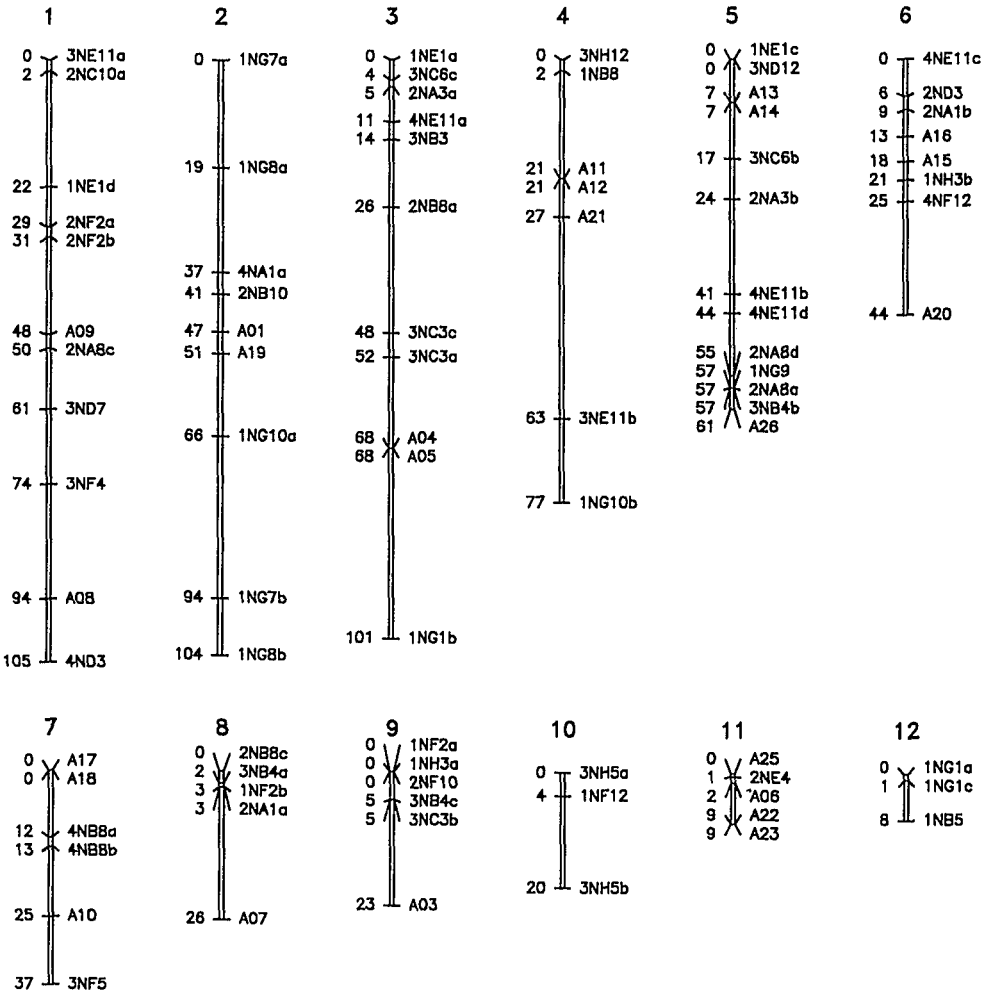

Figure 1. Linkage map of *Brassica oleracea* based on 92 RFLP and AFLP marker loci segregating in a population of 107 doubled haploid lines. Five markers (2NA8b, 2NB8b, 4NA1b, A02 and A24) were not linked to other loci.

corresponding linkage groups. Using the five single-copy probes 2NB10, 2NE4, 3NB3, 3NF4 and 4ND3 as common markers between the two maps, our linkage groups 1, 2 and 3 could be identified with their groups 5, 4 and 1 respectively. From the relative map positions of multicopy probe RFLPs, our linkage groups 5 and 12 could further be tentatively associated with two regions of their linkage group 3.

A high frequency of segregation distortion was observed. The segregation of 59 markers (64 %) deviated significantly ($P \leq 0.05$) from the expected 1:1 ratio. Almost all loci on linkage groups 3, 8, 9 and 12 segregated an excess of Bi alleles, and all loci in group 11 showed an excess of Gr alleles. On all linkage groups except group 10 single

loci or groups of loci showed segregation distortion. The most extreme segregation ratios were observed for markers 1NF2b and 2NA1a on linkage group 8 (93 Bi : 9 Gr : 5 missing) and unlinked marker 2NA8b (8 Bi : 96 Gr : 3 missing).

Mapping of genes for clubroot resistance

The severity of clubroot symptoms was measured by the R-value as well as by assigning a symptom grade. Means of the R-values and the symptom grades were calculated for all lines. The number of tested plants per line varied considerably due to the often low fertility of the DH-lines resulting in a very limited seed supply, as well as to a sometimes very slow or weak plant development. Data from plants with a total root system weight < 0.3 g were rejected, since these plants generally lacked the resources to develop clubroot symptoms (Voorrips and Kanne 1996b). For 91 DH-lines symptom measurements were available. One very fertile DH-line was used as a partially resistant standard genotype in the experiments; therefore many data (179) were available for this line. For the other 90 DH-lines the numbers of evaluated plants ranged from 1 to 16, with an average of 7.4. The variance of 91 DH-line means of R was 0.55. The error variance per plant, calculated from all DH-lines, the original F_1, the two parents and several other genetically uniform populations was 0.19 (Voorrips and Kanne 1996b). The mean error variance of DH-line means was calculated as 0.04, leaving an (additive) genetic variance of DH-line means of 0.51. The narrow-sense heritability in the DH-population was therefore 0.51/0.55 = 0.93.

The mean R-values of the DH-lines were analyzed using the MQM method. In the first step, three markers were selected as cofactors: 4NE11a, 2NA8c and 3NH5a on linkage groups 3, 1 and 10 respectively. From the total variance of DH-line means of 0.55, a residual variance of 0.18 was not absorbed by the cofactors. In the second step of MQM analysis, the LOD-scores of possible QTLs throughout the genome were determined. Two QTLs, designated *pb-3* and *pb-4* with LOD-scores above the threshold of 2.7 were found near two markers used as cofactors: 4NE11a and 2NA8c (Fig. 2). Near the third cofactor (3NH5a) and over a large part of linkage group 6 between marker loci 2NA1b and A20 elevated LOD-scores of about 2.0 were found, but these remained below the LOD-threshold value. The effects of substituting the homozygous resistant for the homozygous susceptible genotype were estimated 1.17 and 0.41 (50 % and 18 % of the difference between the parental line means) respectively for *pb-3* and *pb-4*; these two QTLs explained 54 % and 6 % respectively of the total genetic variance of the DH-line means. As one of the four possible homozygous QTL genotypes (with the Gr-allele of *pb-3* and the Bi-allele of *pb-4*) was scarcely represented among the DH-lines, a possible epistasis effect could not be estimated.

For *pb-3* and *pb-4* and for the possible QTLs near marker 3NH5 and on linkage group 6, the alleles conferring resistance were inherited from the resistant parent. The frequency distributions of mean R-values of DH-lines with the different genotypes at the

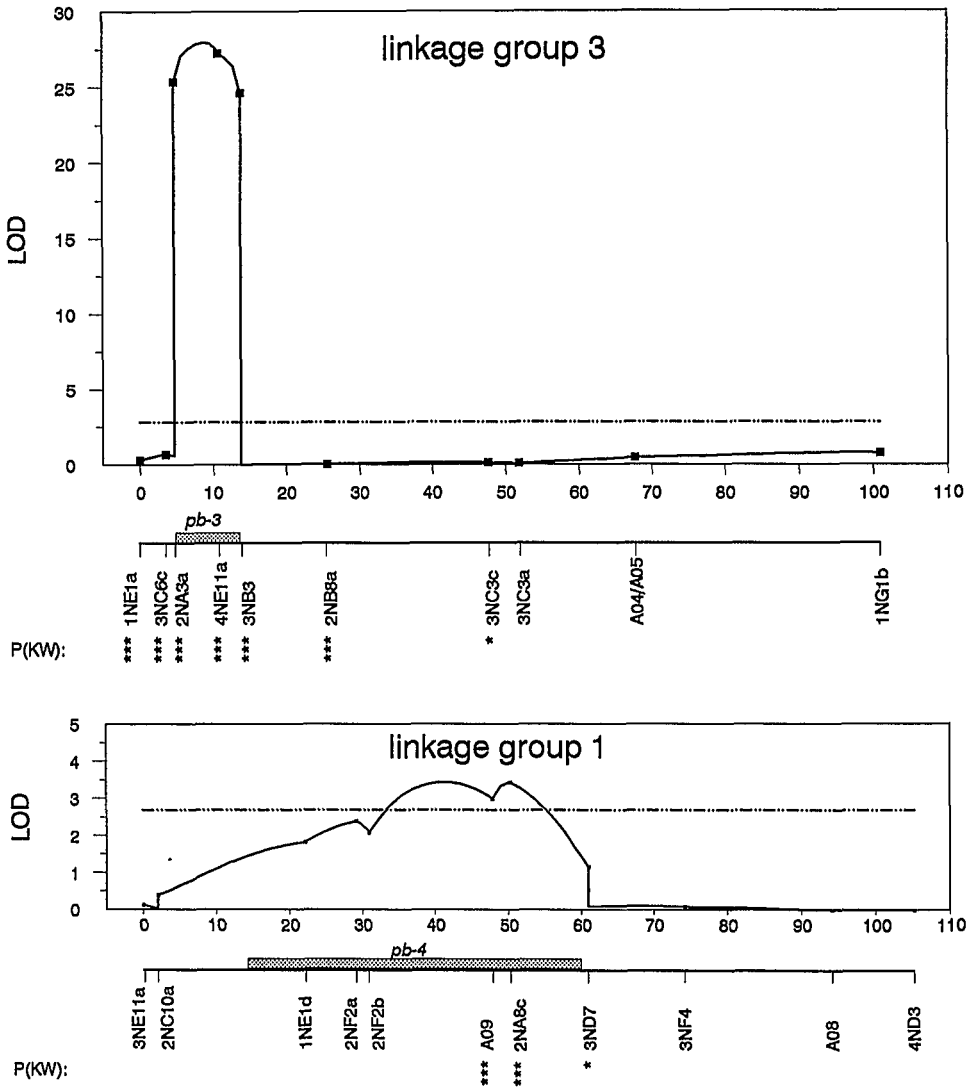

Figure 2. QTL likelihood maps for clubroot resistance on linkage groups 3 (*pb-3*) and 1 (*pb-4*). LOD-scores were obtained by MQM mapping of the measure of resistance R; the horizontal line represents the threshold LOD value of 2.7. Two-LOD support intervals are shown as grey boxes. The significance of Kruskal-Wallis tests P(KW) for mean symptom grades is indicated for each marker locus as * (P<0.01), ** (P<0.001) and *** (P<0.0001).

peak marker loci near *pb-3* and *pb-4* (4NE11a and 2NA8c respectively) are shown in Fig. 3A. Both 4NE11a and 2NA8c showed significantly distorted segregation ratios of 70:34:3 and 30:70:7 (Bi:Gr:missing) respectively.

QTL analysis of mean symptom grades of the DH-lines involved Kruskal-Wallis tests for the probability of finding a QTL linked with each marker locus. As expected,

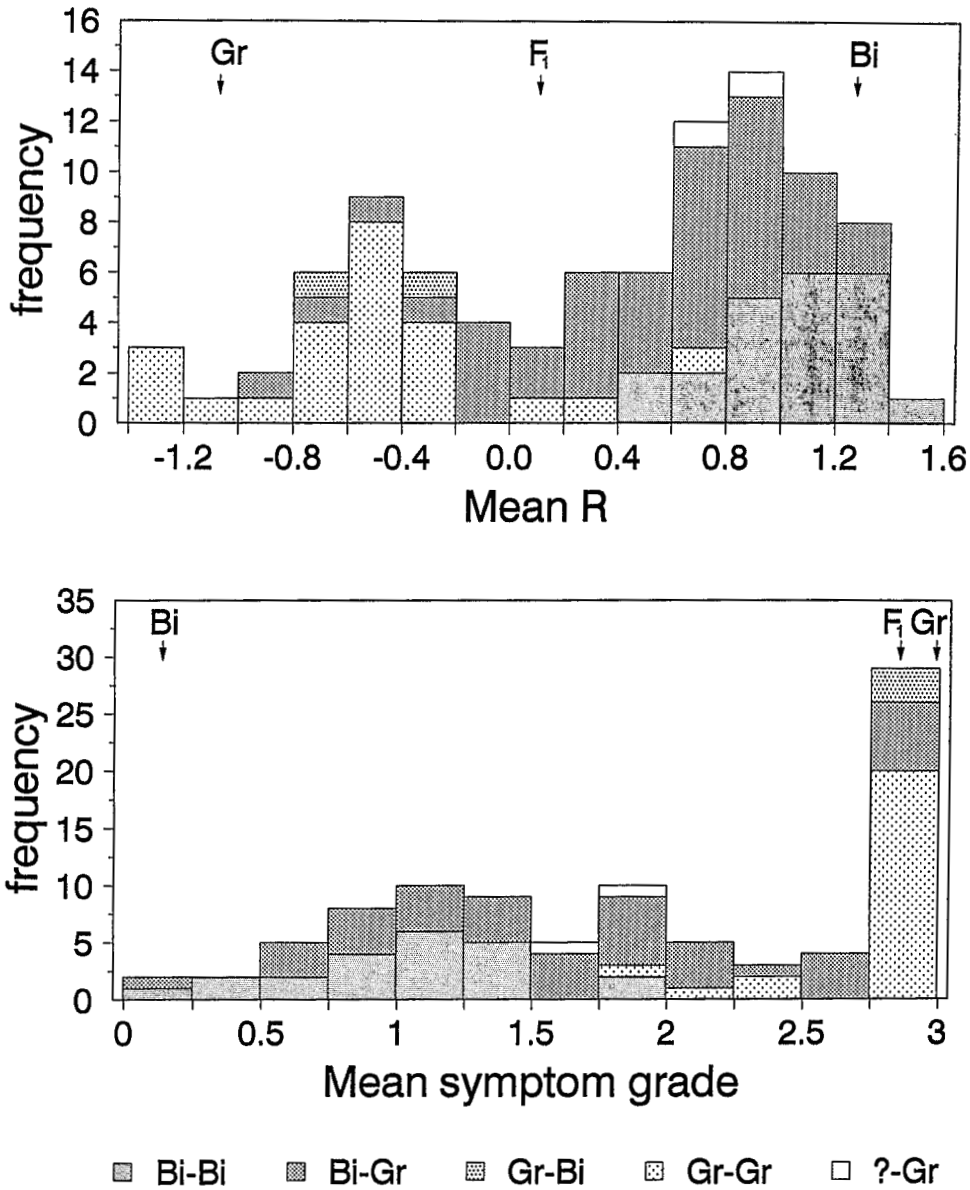

Figure 3. Frequency distributions of the line means of the measure of clubroot resistance R (top) and the symptom grades (bottom) in a population of doubled haploid lines, in relation to the genotypic composition (Bi or Gr allele) for the two marker loci 4NE11a and 2NA8c most closely linked to clubroot resistance loci *pb-3* and *pb-4* respectively.

significant effects were found for markers closely linked to *pb-3* and *pb-4* (Fig. 2). The frequency distribution of mean symptom grades of DH-lines with the different genotypes at loci 4NE11a and 2NA8c is shown in Fig. 3B. Like in the analysis of the R-values,

notable but not quite significant effects were detected at some loci on linkage group 6. The possible QTL near marker 3NH5a indicated by MQM mapping was not revealed by analysis of the symptom grades.

Discussion

The genetic map presented here is the first one based on RFLP as well as AFLP markers. For a DH-line population as used in this study, the dominant marker type of AFLPs is as informative as the codominant type of most RFLPs. The RFLPs were detected with cDNA clones and thus show homology with expressed sequences, while the AFLPs presumably represent a random sample from the genome. There was no obvious difference in the distribution of both types of markers over the genome, although AFLPs and RFLPs never mapped at the same locus.

The length of the linkage map, 615 cM, was less than the length of other genomic maps published for *B. oleracea*. This is most likely due to the smaller number of markers (92) in comparison to the other maps: 820 cM with 258 loci (Slocum et al. 1990), 747 cM with 108 loci (Kianian and Quiros 1992), 1112 cM with 201 loci (Landry et al. 1992).

Based on multicopy RFLP probes, our linkage groups 3 and 5 show homology over part of their length, presumably indicating an interchromosomal duplication. Recombination in the segment on linkage group 3 is apparently reduced in comparison with the homologous segment on group 5, as indicated by their different map lengths. Although these linkage groups are also homologous to linkage groups 1 and 3 of Landry et al. (1992) respectively, those authors found no evidence for this duplication due to an accidental lack of relevant polymorphisms in their mapping population (Fig. 4). Two other duplicated regions were found: 1NG7-1NG8 twice in linkage group 2, and 1NF2-3NB4 in groups 8 and 9. Also some closely linked duplications of the same probe were noted: 2NF2 in linkage group 1, 3NC3 in group 3, 2NA8 in group 5, 4NB8 in group 7, 3NH5 in group 10 and 1NG1 in group 11.

The frequency of distorted segregation, affecting 65 % of the loci is much larger than that observed in intraspecific F_2 populations in *B. oleracea*: 5 % (Slocum et al. 1990), 5-12 % (Kianian and Quiros 1992) and 12 % (Landry et al. 1992) of the RFLP marker loci respectively. In populations of DH-lines of *B. napus* 20-34 % of the marker loci showed skewed segregation (Tanhuanpää et al. 1994; Cloutier et al. 1995; Ferreira et al. 1995b). In contrast, Orton and Browers (1985) found significant deviations from the expected 1:1 ratios at all segregating isozyme loci in four DH-populations of *B. oleracea* (respectively 3, 2, 1 and 1 loci). The most extreme segregation ratios mentioned in Tanhuanpää et al. (1994), Ferreira et al. (1995b) and Orton and Browers (1985) were 9:26, 17:81 and 24:76 respectively, while we found even more extreme ratios (9:93 and 96:8). Presumably the increased frequency of distorted segregation ratios in *B. oleracea* DH-populations reflects

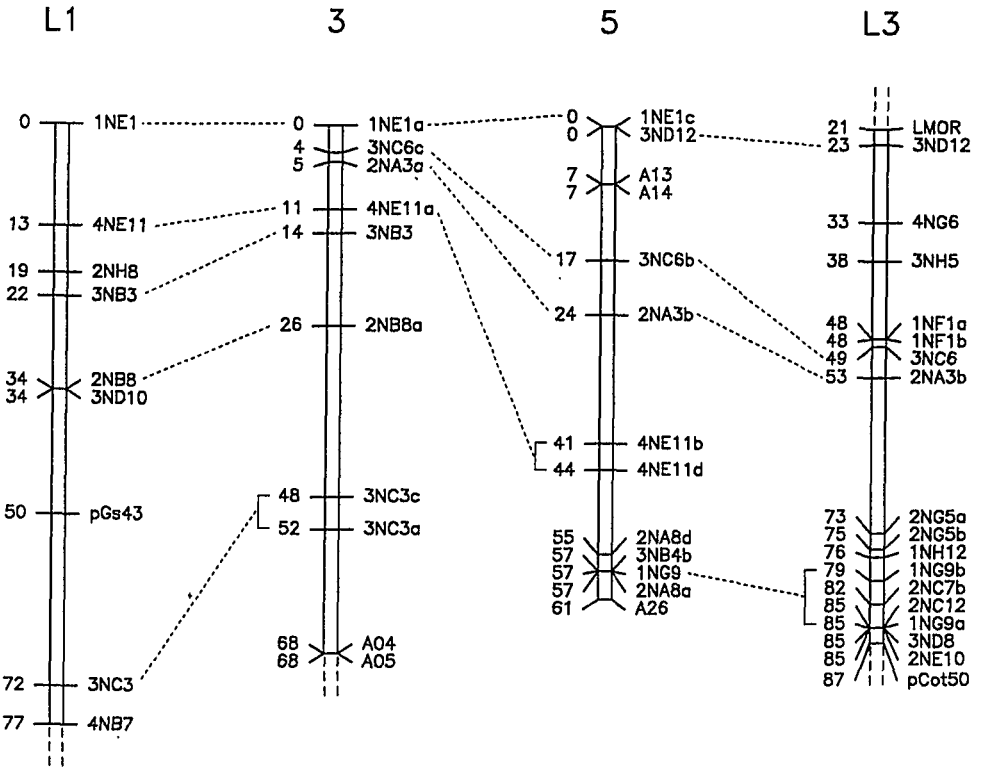

Figure 4. Duplication between linkage groups 3 and 5, and parts of the corresponding linkage groups published by Landry et al. (1992) (labelled L1 and L3) not showing this duplication.

selection pressure due to the microspore culture and plant regeneration process, and possibly also an increased selection pressure on deleterious recessive alleles in a DH-population compared to an F_2. In *B. oleracea* the regeneration of plants from microspores generally occurs at a lower frequency than in *B. napus*; this may account for the higher frequency of segregation distortion found in DH-populations in this species.

Voorrips and Kanne (1996a, b) studied segregation of clubroot resistance in populations descended from the same cross (Bi x Gr) that yielded the population of DH-lines discussed here. Neither the analysis of symptom grades, nor that of R-values determined for individual plants in F_2 and backcross populations showed evidence for simple inheritance of resistance. In retrospect, this is not surprising, since the QTL analyses presented here indicate that at least three QTLs are involved in the resistance of DH-line Bi. One QTL, *pb-3*, can be considered a major gene and is responsible for half of the total additive resistance effect in DH-line Bi. However, apart from *pb-3* and *pb-4* at least one further QTL must be present to account for the unexplained 32 % of the difference between the parental lines. Further, in the population of DH-lines both *pb-3* and *pb-4* showed significant segregation distortion. If this should also be the case in the F_2 or

backcross populations the genetic analyses carried out by Voorrips and Kanne (1996a, b) would be invalid. This illustrates two important advantages of QTL analysis: distorted segregation is easily observed and compensated for, and even a partial explanation of observed segregation of traits yields meaningful results.

Landry et al. (1992) were the first to report linkage of clubroot resistance genes with genetic markers. They studied an F_2-population from a cross between a cabbage breeding line resistant to race 2 (Williams 1966) and a susceptible rapid-cycling *B. oleracea* line. The parentage of the resistant line included *B. napus* cv Wilhelmsburger, resistant to races 2, 3, 6 and 7 as well as cv Badger Shipper, resistant to races 1, 3 and 6. The resistance tests were performed with a field isolate designated as race 2. Two QTLs for clubroot resistance were mapped (*CR2a* and *CR2b*) in linkage groups 6 and 1 respectively. Intriguingly, *CR2a* was linked to marker 2NA8, while our gene *pb-4* was linked to marker 2NA8c detected with the same probe. However, *CR2a* probably originates from *B. napus* based on the resistance of the progenitors of the segregating population, while *pb-4* originates from *B. oleracea*. The magnitude of the gene effects could not be compared as a different measuring scale was used in their study. Landry et al. (1992) estimated that *CR2a* and *CR2b* together explained 61 % of the variation for clubroot resistance in the F_2 population.

Figdore et al. (1993) studied the segregation of RFLP markers and clubroot resistance to race 7 (Williams 1966) in an F_2 from a cross between a susceptible cauliflower cv and broccoli line OSU CR-7 (Baggett and Kean 1985), which was resistant to race 7. They found strong evidence for one resistance gene, and possibly spurious indications for two other resistance genes. Treating symptom grades as quantitative data, they estimated that this gene accounted for 12.6% of the variance in the F_2. Since different markers were used in their study, the map position of this resistance gene cannot be compared with the positions of *pb-3* and *pb-4*.

The nomenclature of clubroot resistance genes in *B. oleracea* is not standardized at this time. Chiang and Crête (1970) hypothesized the existence of two recessive genes for resistance to race 6 (Williams 1966) which they labelled *pb-1* and *pb-2*. However, in a later study (1976) they found only evidence for one such gene. The work of Landry et al. (1992) was based on the same resistant parent used by those authors, but since they used another test method and another race of *P. brassicae* it is not surprising that they discovered different, in their case dominant, genes for resistance. Other authors have also presented evidence for mono- and oligogenic inheritance of clubroot resistance in *B. oleracea*, but have not labelled those genes (reviewed in Voorrips 1995). The approach of Landry et al. (1992) to name the genes after the race to which they confer resistance is confusing, because there is not yet a universally recognized nomenclature for races, and because one gene is likely to confer resistance to multiple races. Therefore, we prefer to follow the nomenclature system used by Chiang and Crête (1970), who added sequential numbers to the prefix *pb-*. Since the R-value of our F_1 was intermediate between the

parents and the mean symptom grade of the F_1 close to the susceptible parent, we use the recessive symbol to indicate the resistant alleles.

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General discussion

The clubroot disease, affecting cole crops such as cabbage, cauliflower and Brussels sprouts as well as many other cruciferous hosts, is widespread, affecting an estimated 10 % of the total cultivated area (Chapter 1). The disease was shown by Woronin (1878) to be caused by the fungus *Plasmodiophora brassicae*. In the almost 120 years since his publication considerable progress has been made in the understanding of the life cycle of this organism and the pathogenesis of clubroot in susceptible host plants. Very little is still known, however, about the genetic variation present within and between populations of this pathogen. Although the occurrence of differential pathogenicity has been described abundantly (Chapter 1) and has been assessed using various sets of differential hosts, all this information has not led to a better understanding of the genetic control of pathogenicity in *P. brassicae*. Similarly, although several sources of clubroot resistance have been found in *Brassica oleracea* (Chapter 1 and 2), the genetic control of many of these resistances has not been elucidated. Obviously resistance is generally not controlled by a single dominant gene.

Breeding for resistance to clubroot is a risky venture due to this lack of information. The development of cultivars incorporating resistance is difficult to plan economically while the genetic control of this character is not clear. A more serious problem is the impossibility to predict the usefulness of a new resistance, as long as the genetic variation of the pathogen in the region of interest is not assessed.

The principal aims of the study described in this thesis were to search for accessions of *B. oleracea* with resistance to Dutch isolates of *P. brassicae*, to develop breeding lines from these accessions homozygous for the resistance genes, and to study the genetic control of resistance.

The resistance test

Firstly, a seedling resistance test was developed which could be carried out year round under controlled conditions and with artificial inoculation (Chapter 2). The results of this test method were shown to correlate well with symptom development under normal field conditions in a heavily infested field. The test is easily applicable in breeding programmes, since it allows evaluation of the resistance of seedlings within six weeks after germination, and healthy cuttings can be recovered from resistant as well as susceptible seedlings for further evaluation and seed production. Advantages of this seedling test compared with other published seedling tests are the short duration, the precise control of the inoculum density applied to each plant, and the possibility to observe and measure the root system of each plant separately.

Screening of *B. oleracea* material

The test was used to evaluate the resistance of numerous accessions, previously described by others as resistant, to one representative Dutch clubroot isolate (Chapter 2). Among this group of reportedly resistant accessions the plant reaction varied from completely unaffected to severely diseased. The Dutch isolate differed therefore in pathogenicity from the isolates to which the susceptible accessions had proved to be resistant. This is not surprising in view of the large variation known to exist between populations of *P. brassicae*. Also within accessions variation for clubroot resistance was observed, as might be expected in this outcrossing species. Subsequent work to obtain breeding lines with high levels of resistance was focussed on accessions with relatively low disease indices in the screening experiments. These accessions therefore carried resistance to the Dutch clubroot isolate as well as to the isolate(s) used by other authors.

Root hair infection

The earliest stages of the pathogenesis of clubroot occur in root hairs. The development of the pathogen in the root hairs is easy to quantify microscopically. If clubroot resistance observed at the plant level were correlated with the degree of root hair colonization by the pathogen, the observation of these first infection stages could provide insight in the level and type of resistance of the host plant. Therefore the degree of root hair colonization by *P. brassicae* was studied in 13 accessions with varying levels of resistance to clubroot at the plant level (Chapter 3). Levels of root hair infection varied almost twentyfold among these accessions, indicating large genetic effects on the resistance to the early stages of pathogenesis. However, root hair infection was not correlated with development of clubroot symptoms. The most likely hypothesis is that even plants with very low numbers of infected root hairs still produce enough propagules of the fungus to become severely affected by the later stages of clubroot, if no further resistance is present. Therefore, resistance to root hair infection cannot be considered as an important component of clubroot resistance.

One-hit model for infection probability

The probability of a susceptible plant developing clubroot symptoms when inoculated with resting spores of *P. brassicae* was shown to fit a generalized one-hit model in a series of five experiments where plants were inoculated with varying numbers of resting spores (Chapter 4). This means that the infection process can be modelled quantitatively without taking into account interactions between *P. brassicae* individuals. Apparently, this

is in contradiction with the observation that secondary zoospores can fuse to form a dikarotic zoospore before invading the root cortex. It is possible that fusion of zoospores is not a prerequisite for successful infection and occurs only in a minority of the cases. However, even if fusions take place before each root cortex infection, it can be argued that these interactions need not influence the infection probabilities predicted by the one-hit model, assuming that this is the only stage where interactions take place.

If no interactions take place before the fusion of secondary zoospores, the number of secondary zoospores will be proportional to the number of inoculated resting spores. In general, the frequency of fusion products would be expected to vary with the square of the concentration of secondary zoospores if the probability of two spores encountering each other were a limiting factor. However, if the local concentration of secondary zoospores were high enough, each spore would encounter others. This may actually be the case, since secondary zoospores are released from the root hairs and probably remain in the direct vicinity of the root. In that case the number of fusion products would be proportional to the number of secondary zoospores, and thus to the number of inoculated resting spores. If no further interactions occur, the number of successful infections is proportional to the number of fusion products. Therefore, while the generalized one-hit model appears to be a correct description of the infection probability, this does not mean that spores do not normally interact.

The large variation in infection probability between tests is a warning against the idea that the infestation of soil samples could be quantified using a bioassay with susceptible host plants. Even among the three experiments conducted in the same climate chambers under identical conditions, the infection probability per resting spore varied by more than a factor 10.

Interaction between pathotypes in the field isolate

Single-spore isolates (SSIs) have been produced in this study by isolating single resting spores and inoculating those onto a susceptible host plant (Chapter 5). Two SSIs were obtained in this way from a Dutch field isolate. Tests with 21 differential hosts failed to discriminate between the two SSIs. Four differential hosts did discriminate however between the SSIs and the original field isolate (Chapter 5). Two host accessions, susceptible to the field isolate, were completely resistant to both SSIs. This suggests that genes for pathogenicity on those two host accessions occurred in the field population, but that the SSIs lacked those pathogenicity genes. Two other hosts however, while resistant to the field isolate, were partially susceptible to both SSIs. Inoculation experiments with different mixtures of the field isolate and one of the SSIs yielded two interesting results. Firstly, the pathogenicity of a mixture of the field isolate and the SSI on those two host genotypes was lower than that of an inoculum containing the same

amount of the SSI without the field isolate. Thus adding field isolate to the SSI decreases the pathogenicity or enhances plant resistance when inoculated onto these two host genotypes. Secondly, the pathogenicity of mixtures with more than 1 % - 10 % SSI was significantly different from that of pure field isolate, suggesting that the frequency of the pathotype represented by the two SSIs in the field isolate is less than 10 %. The first result indicates either competitive interaction between the SSI pathotype and other pathotypes present in the field isolate, or the induction of resistance in these hosts by other pathotypes in the field isolate. Induced resistance is the most likely explanation, because no competitive interaction effects were apparent in the other two differentiating host accessions. Although a generalization from only two cases is hazardous, the second result suggests that the SSI pathotype has a relatively high probability to be isolated in single-spore inoculation experiments. SSIs may therefore not be random samples from field populations but represent atypical genotypes especially suited to a homozygous lifestyle. In conclusion, the use of field isolates (or worse, mixtures of several field isolates) in resistance tests may conceal susceptibility to certain pathotypes in the mixture. On the other hand, not all pathotypes may be obtained as SSIs for separate testing. Neither tests with field isolates nor with SSIs can therefore answer the question whether a host is resistant to all pathotypes in a field. For future resistance breeding as well as for phytopathological studies it is thus essential to establish a classification system of races and of resistances based on characterized pathogenicity and resistance genes.

Classical genetic study of resistance

Chapters 6 and 7 are devoted to the genetic interpretation of the segregation of resistance in progenies from four resistant x susceptible crosses within *B. oleracea*. In each of these crosses both parents were doubled haploid lines (DH-lines) and therefore completely homozygous, enabling the most informative genetic analysis. Three types of genetic analysis were performed based on three ratings of plant resistance: (1) a qualitative rating as resistant or susceptible, (2) a rating on an ordinal scale of four symptom grades, and (3) a quantitative measure of symptom development. For parental DH-line Ch all three types of analysis yielded exactly the same result: two complementary resistance genes were present. In two other cases (parental DH-lines RD and Pt) the simple genetic models indicated by both analyses based on the symptom grades were not confirmed by the analysis of means, in the case of line RD because no weight measurements were available, and in the case of line Pt presumably because the number of measured plants was too small to yield clear results. For parental DH-line Bi the only indication of a simple inheritance of resistance was the fact that the population of DH-lines obtained from the F_1 showed a clearly bimodal distribution. Summarizing, in all four resistant parental lines indications were obtained for a small number of genes (1 or 2)

with a major effect on resistance, although only in the case of parental line Ch these indications were obtained from all three types of analysis. Specific information on the mode of inheritance of resistance was obtained only for DH-line Ch.

Although these classical genetic analyses have at least in one case yielded clear conclusions, an important drawback is that the genes identified in this way cannot easily be studied in other crosses or in interactions with other pathotypes. Since no linkage with marker genes is known and since the resistance genes are only characterized through their interaction with the *P. brassicae* isolates used in this study, it is not possible to determine their effect in interactions with other pathotypes. This is a general problem with classical genetic studies of all but the simplest genetic systems. Further, classical genetic studies rely on various assumptions concerning segregation ratios, main and epistatic effects and other aspects which cannot be directly verified and are in most cases not completely accurate.

Molecular markers for resistance

The use of molecular markers circumvents these problems. The molecular marker data not only show the actual segregation ratios in the genomic regions of interest but also allow compensation for the effects of segregation distortion. Also, the effect of each gene separately as well as its interactions with other identified genes can be assessed. Finally, since each gene can be identified by its position on the genomic map, its effects in interactions with other pathotypes can easily be studied. In Chapter 8, this technique has been used with a population of DH-lines, obtained from the F_1 of one resistant x susceptible cross. Classical genetic analysis of progenies of this cross allowed no clear conclusions concerning the genetic background of resistance. However, molecular marker analysis identified two genes explaining 60 % of the genetic variation for resistance in the DH-population and 68 % of the difference between the parents. Further, it showed segregation distortion in this population affecting large parts of the genome, including both identified resistance loci.

Since not all genetic variation was explained by these two genes, at least one additional resistance gene must be postulated. Even in the absence of segregation distortion in the F_2 and backcross progenies, a genetic model involving three or more genes with different effects could not have been supported by only phenotypic observations. Apart from the advantages offered by the fact that genes can be identified through linked markers, the molecular marker approach also enables analysis of more complicated genetic systems.

Perspectives for the genetic study of the *P. brassicae* - *B. oleracea* interaction

For the study of pathogenicity in *P. brassicae*, resistance genes identified by molecular markers will be the starting point. Homozygous differential hosts each with a different, identified resistance gene are available from the population of DH-lines mentioned above. These lines can be the basis of a more comprehensive set of differential host lines with identified resistance genes. The pathogenicity of single-spore or oligospore isolates and the field isolates from which they are derived can be rationally characterized with such a differential set. This will lead to insights in the genetic variability within and between pathogen populations. At the same time the specificity of resistance genes will become clear.

Research of Dekhuijzen (1979) and recently of Fuchs & Sacristán (1996) indicates that in resistance of *B. rapa* and *Arabidopsis thaliana* to clubroot hypersensitive responses play a role, suggesting that gene-for-gene action will be involved. If this is also the case in *B. oleracea*, pathogenicity genes can be identified through gene-for-gene relationships with known and mapped resistance genes. Another way to characterize pathogenicity genes of *P. brassicae* is likely to develop from work on the analysis of *P. brassicae* with molecular markers (Buhariwalla et al., 1995).

Perspectives for breeding for resistance to clubroot

Technically, breeding cole crops for resistance to a specific isolate of *P. brassicae* is feasible. Most studies indicate rather high heritabilities of resistance, and in several cases, including the four studied here, only limited numbers of (major) resistance genes appear to be involved. Where the major resistance genes are recessive intermittent generations of selfing are necessary to select for the homozygous resistant genotypes. Alternatively, if molecular markers are known to be linked with resistance genes, these can be used to select the heterozygous genotypes in backcross generations.

The real challenge lies in the genetic variation for pathogenicity between, and to a lesser extent also within, field isolates. The only well established fact is that considerable variation exists. However, due to the lack of genetically well characterized differential hosts almost nothing is known about the number of pathogenicity genes influencing this variation, nor about the geographic distribution of different pathotypes. Consequently the complexity of resistance needed in cultivars intended for cultivation in particular regions cannot be estimated in advance. Breeding for resistance may thus be easy or unsuccessful, depending on the variation of the pathogen in the region under consideration, but there is currently no possibility to estimate the probability of success.

This situation is beginning to change, as differential hosts with well characterized resistance genes are becoming available and advances in the genetics of pathogenicity in

the pathogen can be expected shortly. Past efforts to analyze the genetics of resistance and pathogenicity were often not carried to their full potential since they were generally either short-lived or isolated. The interest of the agricultural and scientific communities, although stimulated by the seriousness of the disease, has repeatedly wavered owing to the complexity of the problem. Currently there is a new wave of interest in this pathogen, fueled by the new possibilities offered by biotechnology. Let us hope that this wave of interest will be long and strong enough to produce the necessary results.

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Samenvatting

Knolvoet, een ziekte van de koolgewassen (*Brassica oleracea*) en andere kruisbloemigen, wordt veroorzaakt door de schimmel *Plasmodiophora brassicae*. Het is een belangrijke ziekte, waarmee wereldwijd naar schatting 10 % van het areaal koolgewassen besmet is. De mogelijkheden om de ziekte met cultuurmaatregelen te bestrijden zijn beperkt, en effectieve chemische bestrijdingsmiddelen zijn ofwel verboden omdat ze het milieu te zwaar belasten, ofwel te duur voor grootschalige toepassing. De veredeling van knolvoetresistente rassen van koolgewassen is daarom een interessant alternatief. Dit proefschrift behandelt een aantal aspecten van de interactie tussen *P. brassicae* en de koolgewassen die van belang zijn bij de veredeling op knolvoetresistentie.

Er werd een kiemplantentoets ontwikkeld. De ontwikkeling van symptomen in deze toets vertoonde een hoge correlatie met die in de veldsituatie. Deze toets is gebruikt om herkomsten van *B. oleracea* te vinden die resistent zijn tegen een Nederlands knolvoet-isolaat.

Resistentie tegen de vroege ontwikkelingsstadia van *P. brassicae*, optredend in de wortelharen, bleek niet gecorreleerd te zijn met resistentie tegen knolvoet. Waarschijnlijk zou alleen een absolute resistentie tegen deze vroege ontwikkelingsstadia de verdere vorming van knolvoetsymptomen voorkomen, maar een dergelijke absolute resistentie werd niet gevonden.

De kans dat een plant geïnfecteerd wordt door afzonderlijke sporen van *P. brassicae* werd bestudeerd in een reeks experimenten met verschillende inoculumdichtheden. De infectiekans kon goed beschreven worden door een zgn. "one-hit model" (één-treffer model), waarin interacties tussen sporen geen rol spelen. De infectiekans bleek sterk te variëren tussen experimenten, zelfs als deze in dezelfde klimaatkamer werden uitgevoerd.

Uit één veldisolaat van *P. brassicae* werden twee monospore-isolaten (SSI's, single-spore isolates) verkregen. Deze waren niet onderscheidbaar op basis van hun interacties met 21 differentiële waardplantherkomsten, maar ze bleken te verschillen van het veldisolaat in hun interacties met vier van deze herkomsten. Het pathotype van deze SSI's bleek slechts in lage frequentie voor te komen in de veldpopulatie. Het feit dat het toch in beide gevallen geïsoleerd werd suggereert dat niet alle pathotypen een gelijke kans hebben om als SSI geïsoleerd te worden. Resultaten van inoculaties met mengsels van het veldisolaat en één van de SSI's doen vermoeden dat in sommige waardplanten resistentie tegen knolvoetaantasting geïnduceerd kan worden door bepaalde pathotypes in het veldisolaat.

Uit vier koolherkomsten met knolvoetresistentie werden verdubbeld-haploïde lijnen (DH-lijnen) verkregen via microsporencultuur. Eén resistente DH-lijn uit elk van de vier herkomsten werd gekruist met een vatbare DH-lijn. Uit elke kruising werden de F_1, de F_2 en de reciproke terugkruisingspopulaties verkregen, en in één van de vier gevallen werd een populatie DH-lijnen verkregen via microsporencultuur van de F_1. In deze

nakomelingschappen werd de uitsplitsing van resistentie onderzocht en verscheidene genetische modellen werden getoetst. Klassiek-genetische analyses toonden aan dat in drie van de vier kruisingen één of twee hoofdgenen voor resistentie uitsplitsten. Op basis van de populatie DH-lijnen die uit de F_1 van de vierde kruising was verkregen werd een genetische kaart gemaakt van RFLP en AFLP merkergenen. Twee belangrijke resistentiegenen konden in deze populatie gekarteerd worden. Daarnaast werden aanwijzingen gevonden voor de uitsplitsing van minstens één extra resistentiegen in deze populatie.

Dit onderzoek laat zien dat merkergenen een zeer nuttig instrument zijn bij de veredeling van knolvoetresistente rassen. De waarde van elk gevonden resistentiegen moet geëvalueerd worden in specifieke teeltgebieden, aangezien populaties van *P. brassicae* kunnen verschillen in pathogeniteit.

Nawoord

Dit proefschrift is het resultaat van onderzoek dat heeft plaatsgevonden in de periode 1986 tot 1995 bij het voormalige Instituut voor de Veredeling van Tuinbouwgewassen (IVT) en het latere DLO - Centrum voor Plantenveredelings- en Reproduktie Onderzoek (CPRO-DLO). Naast de promovendus hebben verschillende mensen bijgedragen aan de totstandkoming van dit proefschrift. Enkelen wil ik hier bij name noemen.

Pim Lindhout is, eerst als collega en later vanuit zijn nieuwe positie aan de Landbouwuniversiteit, een belangrijke drijvende kracht geweest. Alle hoofdstukken dragen duidelijke sporen van zijn vele commentaar, maar belangrijker nog waren de stimulerende discussies, die zich niet beperkten tot het onderzoek.

Mijn promotoren, prof dr ir J.E. Parlevliet en prof dr ir P.J.G.M. de Wit hebben sinds 1991 interesse getoond voor dit onderzoek dat toch buiten hun directe onderzoeksgebied lag. Zij hebben alle hoofdstukken van dit proefschrift becommentarieerd voordat deze aan de diverse tijdschriftredacties werden aangeboden.

In de loop van dit project hebben Dirk Visser, Jos Kanne en Ria Jongerius een groot deel van het praktische werk voor hun rekening genomen. Dirk was in de eerste fase betrokken bij het ontwikkelen van de toetsmethode, de screening van geniteurs en de ontwikkeling van verdubbeld-haploïde lijnen daaruit. Jos was onder meer verantwoordelijk voor de zaadteelt en het toetsen van de splitsende populaties. Ria heeft al het RFLP- en AFLP werk uitgevoerd. Voor kortere periodes hebben ook Henri Noordman, Frans Bonnier en Martin Zevenbergen in dit project meegewerkt. De populatie DH-lijnen is deels afkomstig van microsporencultures van Yvonne Nöllen.

Wil het type onderzoek dat hier beschreven is een kans van slagen hebben, dan moeten de verzorging van het plantmateriaal en de voorzieningen in de kassen en op de proefvelden in orde zijn. Velen van het tuinpersoneel hebben zich ingespannen om één en ander goed te laten verlopen; in het bijzonder wil ik hier Peter Saat, Hans Janssen, Gerrit Terwoert, Johan Hulsman, Ab Wessels en Nettert van der Linde noemen.

Proeven leiden tot getallen, en getallen moeten op de juiste manier verwerkt worden om conclusies te kunnen onderbouwen. Hans Jansen, Ritsert Jansen en Johan van Ooijen zijn bij diverse hoofdstukken hierbij behulpzaam geweest.

Marieke Griffioen heeft geholpen bij het ontwerpen van de omslag van het proefschrift. De foto die zij bewerkt heeft is gemaakt door Dirk Visser.

Tenslotte hebben vele collega's uit de afdeling Groente- en Fruitgewassen maar ook daarbuiten met raad en daad klaargestaan, en bijgedragen aan een prettige werksfeer.

Curriculum vitae

Roeland Erik Voorrips was born in Chêne-Bougeries, Switzerland on 12 May 1957. In 1964 he moved to Eindhoven, The Netherlands, where he completed grammar school at the Van der Puttlyceum in 1975. He studied Plant Breeding at Wageningen Agricultural University and graduated *cum laude* in 1982, with Genetics and Plant Breeding as main subjects and Molecular Biology and Mathematical Statistics as minor subjects. During the next four years he was head of the Plant Breeding Laboratory of Royal Sluis seed company at Enkhuizen, where he was mainly involved with micropropagation and cell biology research. In 1986 he joined the Institute for Horticultural Plant Breeding (IVT) at Wageningen, which later became part of the DLO - Centre for Plant Breeding and Reproduction Research (CPRO-DLO). Here he became responsible for breeding research in the cole crops, including the study of clubroot which is the subject of this thesis. Recently the emphasis in his work was transferred to the solanaceous vegetables including tomato and hot pepper. He was invited speaker at the Eighth Crucifer Genetics Workshop (Canada, 1993) and member of the Scientific and Editorial Committees of the ISHS Symposium on Brassicas and Ninth Crucifer Genetics Workshop (Portugal, 1994).

Stellingen

1. Het is nu mogelijk om rassen van koolgewassen te veredelen met gespecificeerde combinaties van genen voor resistentie tegen knolvoet.
   dit proefschrift

2. Biotoetsen gebaseerd op de aantasting van jonge planten zijn ongeschikt om de knolvoetbesmetting van grondmonsters te kwantificeren.
   dit proefschrift

3. De suggestie van Buhariwalla et al. dat veldisolaten van *Plasmodiophora brassicae* geen complexe mengsels van pathotypen zijn is niet goed onderbouwd.
   H. Buhariwalla, S. Greaves, R. Magrath & R. Mithen, 1995. Development of specific PCR primers for the amplification of polymorphic DNA from the obligate root pathogen *Plasmodiophora brassicae*. Physiological and Molecular Plant Pathology 45: 83-94

4. Het toetsen van plantmateriaal met mengsels van veldisolaten om te selecteren op breed werkende resistentie tegen knolvoet is niet aan te bevelen.
   dit proefschrift

5. De proefopzet van Landry et al. geeft weinig vertrouwen in hun bewering dat zij ordinale gegevens kunnen omzetten naar een normaal verdeelde grootheid.
   B.S. Landry, N. Hubert, R. Crête, M. Chiang, S.E. Lincoln & T. Etoh, 1992. A genetic map for *Brassica oleracea* based on RFLP markers detected with expressed DNA sequences and mapping of resistance genes to race 2 of *Plasmodiophora brassicae* (Woronin). Genome 35: 409-420

6. Monospore isolaten van *Plasmodiophora brassicae* vertegenwoordigen wellicht bijzondere genotypen.
   dit proefschrift

7. Hoe belangrijker de statistische analyse, des te oninteressanter de conclusies.

8. "Thus ultimately the only justification for any transformation that may be used is that it works"
   K. Mather & J.L. Jinks, 1977. Introduction to biometrical genetics.

9. De veronderstelling van Chalmers dat het subjectieve bewustzijn geen invloed heeft op de fysieke wereld is aantoonbaar onjuist.
   D.J. Chalmers, 1995. The puzzle of conscious experience. Scientific American 273 (6): 62-68

10. Er zijn geen aanwijzingen voor het bestaan van buitenzintuiglijke waarnemingen of telekinese.

11. De borstcrawl verdient een prominentere plaats in het Nederlandse zwemonderwijs.

12. *Plasmodiophora brassicae* is een veelzijdig organisme: het kan het leven van zowel de plant als van de plantenveredelaar vergallen.

Stellingen behorend bij het proefschrift "Clubroot in the cole crops: the interaction between *Plasmodiophora brassicae* and *Brassica oleracea*" door R.E. Voorrips, 28 oktober 1996