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

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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.

Additional keywords: Brassica campestris, nutrient solution culture, plasmodium, zoospore.

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* (Webb, 1949; Kole and Philipsen, 1956). 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 cheesecloth, and diluted with deionized water to a density of 10^7 spores/ml.

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

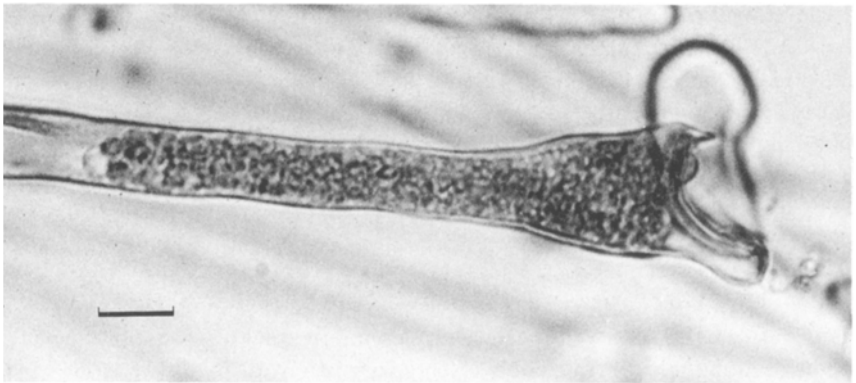

Fig. 1. Root hair with multinucleate plasmodium stained with aniline-blue, 7 days after inoculation with resting spores of *Plasmodiophora brassicae* in a nutrient solution. The bar represents 10 µm.

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 logarithmic link function; the variance function was assumed to be pro-

portional 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).

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.

Accession[a]	Plant resistance					Severity of root hair infection	
	Scale				Disease index	Mean	Confidence interval ($P = 0.95$)
	0	1	2	3			
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[c]	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

[a] Accessions are identified by the source of the resistance they carry. The *B. oleracea* accessions consist of three 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).

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

[c] 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.

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 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, both in the control and 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.

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

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	

* $P < 0.005$

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. However, the level of resistance to root hair infection found in this study 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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