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Research Report

Inheritance of Downy Mildew Resistance at Different Developmental Stages in Chinese Cabbage via the Leaf Disk Test

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Abstract. Downy mildew, caused by *Hyaloperonospora parasitica*, is a destructive disease of the Chinese cabbage (*Brassica rapa* ssp. *pekinensis)*. A rapid resistance/susceptibility test for *H. parasitica* was established by the inoculation of a leaf disk test. Four conditions were tested and the optimal condition was found when the inoculated leaf disk was placed into dark conditions at 16℃ for the first 24 hours after inoculation, followed by 5 days of light at 20℃ and 23℃ for 4 hours and 5 hours, respectively, dark at 16℃ and 12℃ for 3 hours and 12 hours, respectively, and a final temperature of 16℃ for 24 hours. There was a good correlation between the resistance levels of leaf disks and the resistance of seedlings or adult plants, which indicated that testing leaf disks should be the preferred methods to predict the resistance of adult plants. Using this method, downy mildew resistance was investigated in a double haploid (DH) population at four developmental stages. The results showed that the genetic pattern, which was deduced from the DH segregation, was relatively similar, but varied slightly during plant development.

***Additional key words*:** *Brassica rapa* L. ssp. *pekinensis*, disease resistance identification, double haploid population, *Hyaloperonospora parasitica*, in vitro test

Introduction

Downy mildew in *Brassicas* is caused by *Hyaloperonospora parasitica* (formerly of the genus *Peronospora*), which is a worldwide disease. The disease causes severe epidemics on different crucifers, including *Brassica* vegetables such as the cauliflower (*B. oleracea* var. *botrytis*), broccoli (*B. oleracea* var. *italica*) and Chinese cabbage (*B. rapa* ssp. *pekinensis*). Plants can be attacked at any stage from seedling to adult or flowering stages in the fields that have cool climates with frequent dew formation. In China, this occurs most frequently in the spring and autumn seasons. Most *Brassica* growers prefer chemical fungicide to control the downy mildew because it is a fast and effective one among control methods. However, the applications of fungicides have potential to cause adverse effects, such as environmental damage, food safety due to chemical residues on vegetables, or the development of pathogen resistance to the chemicals. In *H. parasitica*, resistance to other fungicides has been shown to occur (Molina et al., 1998) and breeding has become the most efficient and cost-effective means of managing outbreaks of this disease.

Classical screening for sources of resistance in *Brassica* to downy mildew can be performed either in the field or in the laboratory. In the latter, screening is performed by the inoculation of cotyledons or true leaves of seedlings with spore suspension of *H. parasitica* and assessing the level of resistance 7 days later (Niu, 1983; Williams, 1985). This screening strategy appears to be efficient as some plants identified at the cotyledon stage are resistant through to maturity; the prediction of adult plant resistance based on cotyledon stage resistance would save much time and labor. To date, no correlation has been found between cotyledon stage resistance tested in the laboratory and adult plant resistance in the field for several *B. oleracea* accessions (Coelho et al., 1998). Dickson and Petzoldt (1993) advised that the older plants should be tested so that the adult stage resistance could be better evaluated as there was no correlation between the two stages. Another problem of the classical cotyledon test is that susceptible seedlings die

when infected with a virulent isolate and cannot be studied at other later developmental stages.

Pathogensity tests using leaf disks have been developed and successfully used in several pathosystems, including the tomato (*Lycopersicon esculentum*) (Goth, 1997), melon (*Sphaerotheca fuliginea*) (Cohen, 1993), banana (*Mycosphaerella fujiensis*) (Hadrami et al., 1998), grape (*Plasmopara viticola*) (Brown et al., 1999), and apple (*Venturia inaequalis*) (Bénaouf, 1998). This method would be useful as a single plant would be used to test at different developmental stages, and this plant would not die because of the leaf disk test even if it was susceptible to the disease.

DH plants were produced via microspore culture. Because of their genetic homogeneity it is possible to evaluate the performance of DH lines in replicated trials thereby giving more robust data for quantitative traits. The leaf disk test is a promising approach that has been used in a genetic analysis by testing different stages of a downy mildew infection in each plant of a DH population in which the resistance trait was known to only occur in some members of the population. The aim of the present study was to develop the pathogenicity test using leaf disks for *H. parasitica* in the Chinese cabbage. On the basis of this method, the resistance to downy mildew of a DH population could be evaluated at the seedling, rosette, head formation and flowering stages, and the test could confirm whether resistance at these four developmental stages is controlled by different genes.

Materials and Methods

Plant Material

Ten accessions, including five susceptible accessions (B1, B4, B6, Ba8, and B18), one moderately susceptible accession (B17), one moderately resistant accession (B2), and three resistance accessions (B8, B10, and B12), were used in this screening method. In order to ascertain the nature of the genetic control of resistance to *H. parasitica* at different stages, a DH population that included 100 microspore-culture-derived DH lines from a cross between two *B. rapa* lines, 91-112 (susceptible to downy mildew) and T12-19 (resistance to downy mildew), was used in this study. The plants from the 91-112, T12-19, and 100 DH lines were grown in plastic-covered greenhouses in three replicates.

Inoculum Preparation

The isolate was maintained on seedlings of the Chinese cabbage variety "A8 JIAN", which is highly susceptible to downy mildew. Fresh spores used for inoculation were brushed from the young leaves of the susceptible maintenance stock with a writing brush and deposited into sterile distilled water. The spore suspension was filtered through two layers of muslin to remove plant debris and conidiophores. The spore concentration was determined using a hemocytometer and adjusted to 5×10^5 spores/mL for both the seedling and leaf disk tests. The seedlings and leaf disks were inoculated by spraying 50 μL inoculum on each seedling and leaf disk.

Classical Artificial Inoculation at the Seedling and Rosette Stages

The seeds of ten accessions were sown in $9 \times 9 \times 9$ cm^3 pots filled with a peat-based compost and covered with vermiculite. Growth room temperatures were controlled at 20-25℃ for daytime and 12-17℃ during the night. At 2 weeks and 8 weeks after sowing, plants were inoculated by spraying them with a conidial suspension on their abaxial sides. Immediately after inoculation, seed pots were covered with a polyethylene (PE) film. The pots were placed in a conditioning room and maintained at a relative humidity (RH) of 80-90% before being subjected to dark treatment for 16 h. Then, the PE film was removed and the pots were returned to the growth room under the conditions previously described. Six days later, the plants were covered with PE film again for 24 h to maintain a near saturated humidity that was favorable for the initiation of sporulation. Leaves were individually evaluated according to the rating system described in Table 2 (the seedling stage test) and Table 3 (the rosette stage test).

Leaf Disk Test

Leaf disks were removed from the sufficiently developed true leaves at the seedling stage. The third set of fully expanded leaves were removed at the adult and flowering stages, as leaves at this age generally provide a more reliable rating than older leaves (Coelho et al., 2009).

Ten 20-mm diameter disks were cut and placed in the clear plastic plates (45 cm × 30 cm × 4 cm) with the adaxial disk on a 1% agar surface that contained 50 ppm of enzimidazole. A spraying process was used to infect the leaf disks. After inoculation, the plastic plates were sealed with PE film and kept in a growth chamber in the dark for 16℃ for 24 h. Afterwards, the leaf disks were held for under 4 different conditions for 5 days as described in Table 1. During this time, the relative humidity of > 95% was maintained with a humidifier, and the light intensity is 10,000 lux. At least 10 leaf disks per accession were tested and three replicates were conducted for each treatment. Seven days after inoculation, susceptible accessions exhibited profuse sporulation and disease assessment was made using a six-point scale described by Leckie (1996) (Table 2). The mean disease indices (DI) were calculated for each accession according to the formula $DI = (\sum nX/9N) \times 100$, where (X) is the disease rate of each plant, (n) is the number of plants

Table 1. Four environmental conditions of downy mildew infection on Chinese cabbage using leaf disks. The leaf disks were placed into dark conditions at 16℃ for the first and last 24 h after inoculation. Condition 1 was that followed by 5 days of light at 20℃ and 23℃ for 4 h and 5 h, respectively, dark at 16℃ and 12℃ for 3 h and 12 h, respectively. Condition 2 was that followed 5 days of light at 22℃ and 16℃ for 9 h and 3 h respectively, dark at 12℃ for 12 h. Condition 3 was 5 days under a 9 h photoperiod (17℃ dark and 22℃ light). Condition 4 was 5 days under a 9 h photoperiod (16℃ dark and 20℃ light).

Condition 1			Condition 2			Condition 3			Condition 4		
Hours	Temp.	Light/Dark	Hours	Temp.	Light/dark	Hours	Temp.	Light/Dark	Hours	Temp.	Light/Dark
24 h	16℃	Dark	24 h	16℃	Dark	24 h	16℃	Dark	24 h	16℃	Dark
4 h	20℃	Light	9 h	22℃	Light	9 h	22℃	Light	9 h	20℃	Light
5 h	23℃	Light	3 h	16℃	Dark	15 h	17℃	Dark	15 h	16℃	Dark
3 h	16℃	Dark	12 h	12℃	Dark						
12 h	12℃	Dark									
24 h	16℃	Dark	24 h	16℃	Dark	24 h	16℃	Dark	24 h	16℃	Dark

Table 2. Downy mildew interaction-phenotype classes used for cotyledon and leaf disk evaluation.

Class	Host	Pathogen
0	No host response	No sporulation observed
1	Light necrotic flecking	No sporulation observed
3	Heavy necrotic flecking	No sporulation observed
5	Any host response	Sparse sporulation observed
7	Any host response	Moderate sporulation and dispersed 1/3 to 2/3 of the whole
9	Any host response	Moderate to heavy sporulation dispersed over whole

Table 3. Downy mildew interaction and phenotype classes used for evaluation of rosette plants.

Class	Interaction Phenotype
0	No host reaction, no sporulation observed
1	Only one leaf from the whole plant with small (≤ 1 cm^2) sporulated lesions
2	Only one leaf from the whole plant with large (> 1 cm^2) sporulated lesions; or only two or more leaves with small sporulated lesions
3	One leaf from the whole plant with large sporulated lesions and one to three leaves with small sporulated lesions
4	One leaf with large sporulated lesions and four or more leaves with small sporulated lesions; or two or more leaves with large sporulated lesions

with the same rate, and (N) is the sample size evaluated for each line. Accessions were categorized as resistant according to a slight modification of Silué's principles (Silué et al., 1996), resistance (R: DI < 33.33), moderately resistant (MR: 33.4 < DI ≤ 44.44), moderately susceptible (MS: 44.45 < DI ≤ 55.56) and susceptible (S: DI > 55.56).

Data Analysis

Three inoculation replicates were conducted, with 10 plants or 10 leaf disks per replicate. Data were analyzed using Microsoft Excel 2010. A correlation analysis was used to determine whether there were any significant relationships between the leaf disks test and seedling test ratings.

Results

Development of a Leaf Disk Test

To develop a leaf disk test for the assessment of resistance to *H. parasitica* in the Chinese cabbage, four conditions were used to culture inoculated leaf disks (Table 1). In condition 1, the leaf disks of plants that were resistant to downy mildew remained fresh during the evaluation period. On the contrary, conidiophores and branches of the downy mildew pathogen were found parasitizing the leaf disks of plants that were susceptible to downy mildew (Fig. 1). Each variety/isolate combination showed results that were comparable to those of the seedling inoculation test (Table 4). Some accessions displayed the symptoms of the host-pathogen interaction under condition 2 and conidiophores and branches of downy mildew pathogen were seen under microscopy,

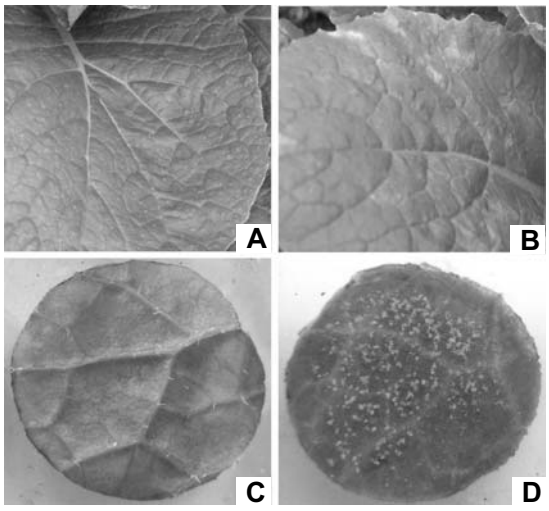

Fig. 1. The symptoms of inoculation with the seedling test and the leaf disk test. No host reaction was on the seedling leaf adaxial surface (A) and the abaxial leaf disks of B10 plant that were resistance to downy mildew during the evaluation period in condition 1 (C). On the contrary, severe symptoms of downy mildew on the upper leaf surface (B) and heavy condidiophores and branches dispersed on the adaxial leaf disks (D) of Ba8 plant that were susceptible to downy mildew.

but only sparse sporulation was observed on the leaf disks of highly susceptible accessions B6 and Ba8. When the inoculated leaf disk was placed under the third condition, all the tested leaf disks became yellow or dried and no symptoms were observed. Under the fourth condition, most of the leaves rotted and the pathogen, *H. parasitica,* could not grow mycelia; no symptoms were observed. Disease symptoms were evaluated by the visual examination (Fig. 2) and condition 1 was chosen to compare the screening procedures further as no significant differences were observed between the other two conditions in the leaf disk and seedling tests.

Fig. 3 showed the disease index (DI) of ten accessions at the seedling and rosette stages by two methods: the leaf disk test (under condition 1) and the seedling inoculation test. There was a good correlation between the resistance levels of leaf disks and the resistance of seedlings or rosette plants. This indicated that it was the preferred method for the prediction of resistance in rosette plants by testing leaf disks. Notably, accessions that were resistant at the seedling stage continued to be resistant at the rosette stage. Accessions that were highly susceptible at the seedling stage, such as

Table 4. Interaction phenotypes of ten *B. rapa* ssp. pekinensis accessions infected at the seedling stage with the isolate of *H. parasitica* with the seedling test and leaf disk test under four conditions.

	B1	B4	B6	Ba8	B18	B17	B2	B8	B10	B12
Seedling test	S	S	S	S	S	MS	MR	R	R	R
Condition 1	MS	S	S	S	S	MS	MR	R	R	R
Condition 2	R	S	MS	MS	MR	MR	R	R	R	R
Condition 3	Leaf yellowed or dried and unvisible symptoms									
Condition 4	Leaf yellowed and some become rotten									

R, resistance; MR, moderately resistant; MS, moderately susceptible; S, susceptible. The condition 1, 2, 3, and 4 were described in Table 1.

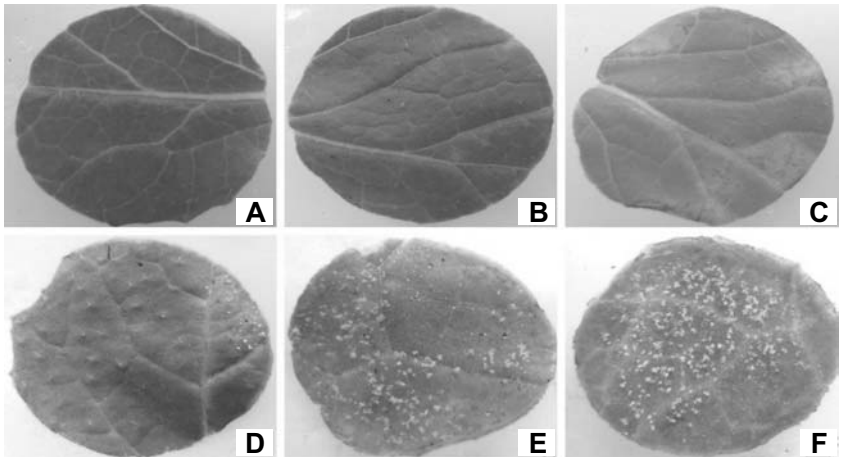

Fig. 2. Responses of Chinese cabbage leaf disks to infection with downy mildew. The disease index was determined depending on the size and numbers of necrotic spots and the degree of sporulation. A, No symptoms (index 0); B, Light necrotic flecking and no sporulation observed (index 1); C, Heavy necrotic flecking and no sporulation observed (index 3); D, Any host response and sparse sporulation observed (index 5); E, Any host response and moderate sporulation and dispersed 1/3 to 2/3 of the whole (index 7); F, Any host response and moderate to heavy sporulation dispersed over whole (index 9).

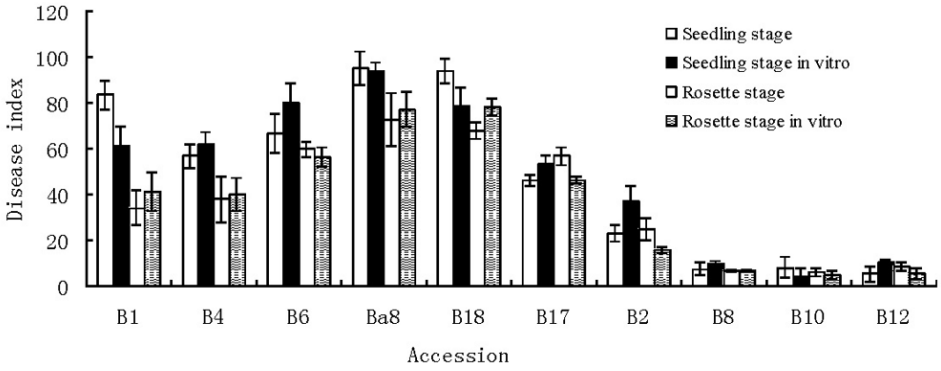

Fig. 3. Disease indices of the ten accessions at the seedling stage and rosette stage inoculated on intact leaves and leaf disks of Chinese cabbage with with *H. parasitica* spores suspension. Vertical bars indicate standard errors of the means.

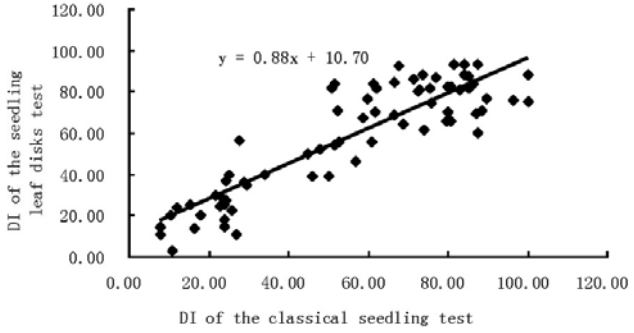

Fig. 4. The correlation between disease indices of the classical seedling test and the leaf disks test with 2 weeks old seedlings inoculated with *H. parasitica* spores suspension. R = 0.88 (r = 0.20, p = 0.05).

B1, had different susceptibilities at the rosette stage.

In addition, the resistance of a DH population that included 100 lines was evaluated at the seedling stage by the leaf disk test (under condition 1) and the seedling inoculation test. The results showed that the correlation coefficient between the DI of the leaf disk test and those of the seedling test was 0.88 (r = 0.20, p = 0.05), which was a highly positive correlation (Fig. 4).

Based on the results presented above, it was concluded that the condition 1 was optimal for the growth of the pathogen. Therefore, the proposed leaf disk test conditions were as follows: plants were placed in the dark at 16℃ during the first 24 h after inoculation, followed by 5 days in the light at 20℃ and 30℃ for 4 h and 5 h, respectively, and then dark conditions at 16℃ and 12℃ for 3 h and 12 h, respectively. The conditions during the seventh day were same as those on the first 24 h, with dark conditions at 16℃.

Downy Mildew Resistance in a DH Population at Different Developmental Stages

Using the leaf disk test described above, the downy mildew resistance was investigated in a DH population at four developmental stages: the seedling, rosette, head formation and flower stages. For the seedling and flowering stages, DI frequency distributions for downy mildew resistance in the DH population were continuous, and showed a typical bimodal distribution (Fig. 5). This suggested that a major QTL was involved in mediating the resistance to downy mildew during these two stages. However, at the rosette and heading stages, the DI frequency had a skewed or slightly disordered distribution (Fig. 5), which gave evidence that a major QTL and different sets of minor QTLs might play roles in downy mildew resistance. The average DIs of the population at the seedling and flowering stages were 56.44 and 48.64, respectively, which were higher than the average DI of the other stages measured (rosette stage: 40.76, heading stage: 40.58). This indicated that plants were more susceptible to downy mildew at the seedling and flowering stages.

Discussion

Yuen (1991) showed that inoculation of downy mildew isolates in vitro is a useful technique for screening downy mildew resistance in Chinese cabbage. This present study demonstrates that it is possible to reproduce Chinese cabbage-downy mildew interactions in leaves in vitro. The same responses were obtained from leaf disk inoculations as those obtained with the classical seedling inoculation test. Studies that have used a leaf-disk assay have been established in several other plant species, including grape (*Plasmopara viticola*) (Brown et al., 1999) and melon (*Sphaerotheca fuliginea*) (Cohen, 1993). Normally, it would take over 2 years to study the inheritance of downy mildew resistance during the adult stage under natural infection conditions in the field (Coelho et al., 2003a; Mahajan et al., 1995). The leaf disk method provides a more practical and rapid approach for the screening downy mildew resistance in adult stage plants in a large population.

Moreover, using this nondestructive method, we can sample

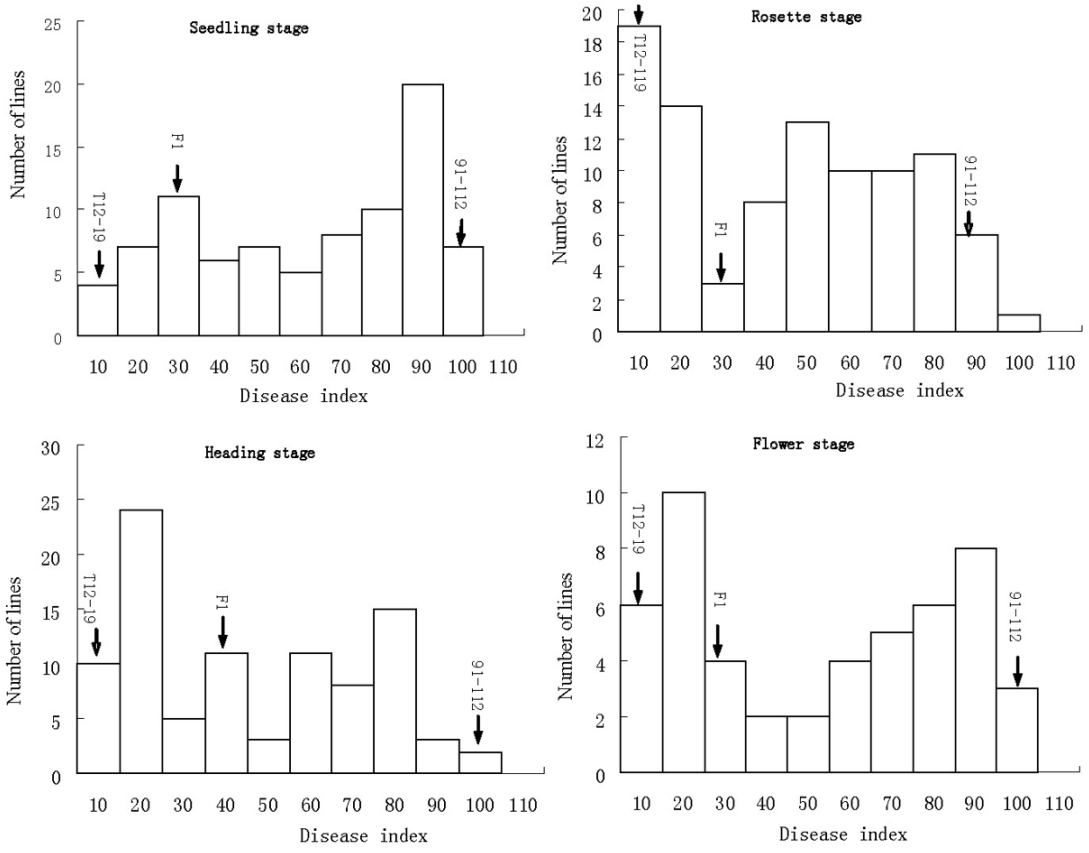

Fig. 5. Disease indices frequency distributions for downy mildew in the double haploid population. Arrows indicate the mean values for the two parents and the F1 generation.

and inoculate plants at multiple stages in the same plant. Therefore, one plant can serve as the source material for screening different isolates of the pathogen or other pathogens. In addition, this evaluation can be performed in the laboratory, and therefore, the likelihood that the inoculum could be inadvertently released into the commercial growing environment is very low. As described by Agnola et al. (2003), to obtain results that are comparable to those observed on seedlings using the classical cotyledon test, the leaf disks can be disinfected with 3% calcium hypochloride. In this research the non-disinfected leaf disks were used and the responses obtained with regards to the pathogen correlated well with those observed in seedling test. Similar results were obtained with other interactions, such as the grape (*Plasmopara viticola*) (Brown et al.,1999). The leaf disk test presented here will be very useful for the development of new *B. rapa* ssp. *pekinensis* cultivars with a high level of resistance to *H. parasitica* at different plant developmental stages.

Different genetic systems of resistance to downy mildew at the seedling and adult plant stages have been reported in *B. oleracea*. Dickson and Petzoldt (1993) showed that susceptibility to downy mildew at the seedling stage could change to resistance in broccoli grown under field conditions. Coelho and Monteiro (2003a) verified that cotyledon and adult plant resistance levels are very poorly correlated in *B. oleracea*. Monteiro et al. (2005) showed that the frequency of resistance at the cotyledon/flower and adult plant stages were fully independent, and inferred that the loci that govern resistance at different developmental stages belong to different linkage groups. Jensen et al. (1999) showed that broccoli plants that were resistant to downy mildew at the cotyledon stage effectively identified plants with high levels of resistance at subsequent developmental stages, which was also indicated by the findings of Wang et al. (2000). In Chinese cabbage, Yu et al. (2009) mapped a major QTL for downy mildew resistance at the seedling stage to the A8 linkage. Subsequently, Kim et al. (2011) identified the BrRHP1 resistance locus in adult plant stages which was located in the A1 linkage group. These different loci may have been identified as a result of the use of different materials, or it could be the case that a separate control locus operates at both development stages in the Chinese cabbage.

In this research, the leaf disk tests confirmed that the seedling stage resistance was controlled by a major QTL, which agrees with the findings of a study that used the classical seedling test (Yu et al., 2009), and a very similar genetic mechanism was found at the flower stage. However,

the segregation for resistance at the rosette and head formation stages changed slightly, and different sets of minor QTLs might play important roles in resistance to downy mildew. We deduced that the method that governed the genetic control of resistance was relatively similar between stages, with slight variations. This indicated that plants that are resistant to downy mildew at the seedling stage will maintain considerable levels of resistance at subsequent developmental stages. These findings require validation in future studies.

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