THE LOCATION AND EXPLOITATION OF GENES FOR PEST AND DISEASE RESISTANCE IN EUROPEAN GENE BANK COLLECTIONS OF HORTICULTURAL BRASSICAS.

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Abstract

The objective of this project is to conduct a systematic evaluation of the distribution of diversity in *Brassica oleracea* with respect to resistance to three important parasites: *Peronospora parasitica* (downy mildew), *Albugo candida* (white blister) and *Brevicoryne brassicae* (cabbage aphid). A core collection of approximately 400 accessions representing the geographical, morphological and physiological diversity of the species has been selected for study. Protocols have been established to allow the rapid evaluation of phenotypic variation for response to all parasites and primary screening of the core collection is well advanced. Resistant individuals are being selected from within accessions expressing a high frequency of resistance. These are being crossed with a uniformly susceptible, self-compatible, rapid-cycling line to facilitate genetic characterisation and potentially easy gene-banking. Several putative new sources of resistance have been identified. Data will be linked to the European *Brassica* Database and together with seed will be made freely available to breeders and other researchers.

Keywords: Resistance, Downy mildew, *Peronospora parasitica*, Cabbage aphid, *Brevicoryne brassicae*, White blister, *Albugo candida*, *Brassica oleracea*, Core collection

1. Introduction

There is at present no genetically well-characterised resistance to the oomycete pathogens *Peronospora parasitica* (downy mildew) and *Albugo candida* (white blister) or to the insect pest *Brevicoryne brassicae* (cabbage aphid) in brassica crops of economic importance to European horticulture. The identification and utilisation of genes for resistance to these parasites would be of potential benefit in reducing the need for chemical controls thus reducing costs and decreasing adverse environmental effects. The objective of the described project is to evaluate a structured sample of the *Brassica oleracea* genepool for response to these three parasites with the aim of identifying and genetically characterising potentially useful sources of resistance.

The European *Brassica* database (Brass-EDB) has been used to select *B. oleracea* accessions for inclusion in a core collection to be screened for response to the parasites. The selection has been carried out on the basis of ecogeographical distribution and crop morphology so that as much diversity as possible is represented. Approximately 300 accessions have been selected from the genebanks at Horticulture Research International (HRI) and the Centre for Genetic Resources Netherlands (CGN), and a further 100 accessions have been obtained from other European germplasm collections.

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Screening for pathogen resistance is being carried out at HRI and Instituto Superior de Agronomia (ISA) and screening for aphid resistance is being carried out at HRI. Protocols have been developed for the rapid evaluation of accessions at the seedling stage to identify genotypes for further study. Seed quality has been an important criterion for selection of accessions for the core collection and tests have been carried out to ensure that the material supplied by the genebanks is suitable for use with the protocols developed.

2. Materials and methods

2.1. Production of seedlings for pathogen inoculation

Plastic inserts with module size 23 x 23 x 35 mm or 30 x 30 x 50 mm are filled with John Innes Seed compost or Levington F2 compost. A 10mm deep hole is made in each module and one seed sown per hole.The holes are filled and the surface of the compost is covered with vermiculite to a depth of approximately 5 mm. The plastic inserts are placed in a seed tray lined with capillary matting. The seed trays are placed on capillary matting in a glasshouse or growth room at a temperature of 20 ± 1°C with continuous illumination and left for 24 hours to allow the seeds to re-hydrate slowly, then watered well with a fine spray. The matting and vermiculite are kept moist but not waterlogged throughout an experiment. Seedlings are inoculated 7-9 days after sowing. One cotyledon of each seedling is marked by puncturing with the tip of a pair of fine forceps.

2.2. Screening seedlings for pathogen response

2.2.1. Preparation of *P. parasitica* inoculum

Cotyledons of maintenance plants bearing 1-2 day old sporophores are placed in distilled water and agitated to dislodge the spores. The spore suspension is filtered through two layers of muslin into centrifuge tubes and centrifuged at a speed of 2000 x g for 3 minutes. The supernatent is discarded and the pellet re-suspended in distilled water. This is repeated twice more and the pellet finally re-suspended in a known volume of distilled water. The spore concentration is measured using a haemocytometer and adjusted to 5 x 10^4 spores/ml. Inoculation of seedlings is carried out immediately.

2.2.2. Preparation of *A. candida* inoculum

Dry spores which have been collected from maintenance plants using a cyclone spore collector and stored at -70°C are used directly from the freezer. The spores are placed in a small Petri dish and 1-2 mls of distilled and filtered water are added. The spore suspension is placed in an incubator at a temperature of between 12°C and 16°C until the sporangia release zoospores (about 2 hours). The inoculum concentration is determined using a haemocytometer and is adjusted to 1 x 10^5 swimming zoospores/ml. The spore suspension is kept on ice and the inoculation of seedlings is carried out as soon as possible after inoculum preparation.

2.2.3. Inoculation of seedlings and incubation of the fungal pathogens at HRI

Two isolates of *P. parasitica* ("P005" and "P006") are used in each downy mildew screening experiment and one isolate of *A. candida* ("A001") in the white blister screening experiments. Two 10 ml droplets of inoculum of one isolate are pipetted onto each marked cotyledon and two 10 ml droplets of the second isolate (if used) are pipetted onto the second cotyledon of each seedling. A propagator top with the vents closed is placed over each seed tray immediately after inoculating the seedlings and the

seed trays are transferred to a growth room at a temperature of 16 ± 1°C with a 16 h photoperiod for the duration of the experiment. Scoring for downy mildew response takes place 4 and 7 days after inoculation, and for white blister response 9 and 12 days after inoculation. Selected seedlings are then transplanted into larger pots and placed in the glasshouse.

2.2.4. Inoculation of seedlings and incubation of the fungal pathogens at ISA

Two isolates of *P. parasitica* ("P501" and "P502") and two of *A. candida* ("A501" and "A502") are used in the experiments. Inoculation is carried out as at HRI and then the seed tray is placed in a sealed plastic bag (RH = 100%) in an incubator at a temperature of 16 ± 0.5°C in the dark for 24 hours. The plastic bag is removed and the seed tray is placed in the growth room under the conditions described in the protocol for production of seedlings. For the downy mildew experiments the seed tray is replaced in the plastic bag in the incubator as above on the 6th day after inoculation to induce sporulation. Scoring for response to the pathogen takes place 7 days after inoculation. For the white blister experiments the seed trays remain in the growth room and scoring takes place at 8 and 10 days after inoculation. Selected seedlings are then moved to a glasshouse.

2.3. Experiment design for screening for pathogen response

Two standard genotypes are used: a rapid-cycling line known to be susceptible to both the pathogens (CrGC3.4) and an accession known to be highly variable in its response (Thousand Head kale). These standards allow comparisons to be made betweeen results obtained in the two laboratories, and results obtained in different tests at each laboratory. At HRI replicate experiments comprising 3 blocks of 15 seedlings of each of 22 accessions from the core collection plus the two standards are carried out giving a total of 90 seedlings/accession evaluated for each pathogen. At ISA 30 accessions plus the two standards are evaluated at a time and these are split into 8 blocks of 5 seedlings giving a total of 40 seedlings/accession for each pathogen.

2.4. Scoring the response of seedlings to the pathogens

Scoring systems have been developed to take into account both the host plant response and the growth of the pathogen as measured by the amount and type of sporulation. The combination of these characters gives six interaction phenotype classes for each host/pathogen combination which are used to categorise the seedlings as shown below.

2.4.1. Scoring system for response to *P. parasitica*

Host Response	No	Yes				
Necrotic Flecking		(F)	F			
Diffuse Necrosis				dN		
Chlorosis			ch			
Tissue Collapse						tc
Pathogen Growth	No			Yes		
Type of Sporulation				(SS)	(S),SS	S
Interaction Phenotype [a]	**NN**	**(F)N**	**FN**	**SS**	**CS**	**HS**

[a] Interaction phenotype classes.

NN = no host response, no sporulation.
(F)N = light necrotic flecking [(F)], no sporulation.
FN = heavy necrotic flecking [F], no sporulation.
SS = any host response, sparse sporulation confined to point of infection [(SS)].
CS = any host response, sparse dispersed sporulation [SS] or moderate sporulation confined to point of infection [(S)].
HS = any host response, moderate to heavy sporulation dispersed over whole cotyledon [S].

2.4.2. Scoring system for response to *A. candida*

Host Response	No	Yes				
Necrotic Flecking		(F)	F			
Chlorosis			ch			
Tissue Collapse						tc
Pathogen Growth	No			Yes		
Type of sporulation				mp	FP,MP	LP,CP
Interaction Phenotype [b]	**NN**	**(F)N**	**FN**	**S1**	**S2**	**S3**

[b] Interaction phenotype classes.

NN = no host response, no sporulation.
(F)N = light necrotic flecking [(F)], no sporulation.
FN = heavy necrotic flecking [F], no sporulation.
S1 = any host response, minute pustules on upper surface of cotyledon [mp].
S2 = any host response, few or numerous small pustules on lower surface of cotyledon [FP,MP].
S3 = any host response, large scattered [LP] or coalescing pustules [CP] on lower surface of cotyledon.

2.5. Screening for response to cabbage aphid

2.5.1. The provision of *B. brassicae* for experiments

The *B. brassicae* cultures are maintained in the Insect Rearing Unit at HRI, Wellesbourne. The cultures are kept in 40 x 40 x 40cm plastic cages fitted with fine nylon backs. The cage fronts are fitted with a 35 x 35cm door made from fine nylon material which is secured with velcro. Aphids are reared on *B. oleracea* var. *gemmifera* cv. 'Oliver'. Stock plants of 'Oliver' are grown singly in 7.5cm plastic pots containing Levington M2 compost and placed in plastic drip trays in the cages. A single, mature, apterous (wingless) female was removed from the stock population and transferred to an 'Oliver' plant. After 21 days aphids produced by this aptera were transferred to four 21-28 day-old 'Oliver' plants in each cage. Every 21 days the aphids are sub-cultured onto fresh plants in order to build up large numbers of insects for experiments. A total of 6 cages provide adequate material for an evaluation experiment.

2.5.2. Glasshouse based rapid-screening experiments

Each experiment is carried out in a 5 x 3 x 3m cage constructed from a Dexion frame covered with a fine nylon mesh fitted over an aluminium mobile bench in a glasshouse. The mobile bench is also covered with the same material to prevent insects and other pests from moving into or out of the cage. Temperatures in the glasshouse range from 15°C to 25°C during the day and are kept above a minimum of 15°C at night. The glasshouse compartment is fitted with supplementary illumination to provide a 16h day during the winter period (November to March inclusive). There are 24 accessions from the core collection in each experiment and each one is sown in a 48 module seed tray filled with a 1:1 mixture of Levingtons M2 and John Innes composts. Excess seed is sown in small trays which are also placed within the cage. The seed is allowed to re-hydrate slowly for 24 hours and the trays are then watered sparingly for the duration of the experiment. Seedlings from the small plastic trays are used to fill gaps in the 48 module trays. 21 days after sowing seed the plants are inoculated with aphids taken from the Wellesbourne clone of the *B. brassicae* culture. Aphids on stock plants are counted and leaf sections cut off and placed in a plastic box to provide a batch of 100 aphids for inoculation. Twenty-four boxes are prepared for each experiment. Each tray of test plants in an experiment is inoculated in turn. The aphid-infested leaf sections and any free aphids within each box are transferred to the test plants. After a few days all aphids have moved onto the test plants and the leaf sections are removed.
Twenty-one days after inoculation the experiment is assessed. Each seed tray is examined and assigned to one of the following categories:

1 = No insects, no damage
2 = Slight damage, light infestation, less than 10 aphids per plant.
3 = Moderate damage, medium infestation, 10-100 aphids per plant
4 = Severe damage, heavy infestation, > 100 aphids per plant

Two test cages are present in the glasshouse and experiments are begun at 21-day intervals. Thus, two experiments are running at any one time. As soon as an experiment is finished a decision is made as to whether any plant material should be kept for seeding or further experimentation. All unwanted material is disposed of and the cage cleaned out. The cage walls, ceiling and floor are sprayed with alcohol to kill any remaining insects.

3. Results

The core collection is fluid and is still being updated as material becomes available from other collections or is regenerated. It is expected to eventually comprise 400 acessions. Screening for response to the parasites is ongoing and the results will be made widely available via the Brass-EDB. The data will be recorded as "number of plants in each interaction phenotype class" for the responses to the pathogen isolates, and a single score of 1-4 for the response to cabbage aphid. The pathogen response data can be used to generate histograms (Fig. 1) which allow the comparison of the responses of different accessions and the identification of isolate specific interactions.
A number of plants in categories (F)N, FN, CS or S1 (depending on the pathogen) have been selected as putative sources of resistance and are being crossed with the rapid-cycling line CrGC3.4 used as the susceptible control in the screening experiments. This will allow an analysis of the genetic control of the resistances to be carried out on a short time scale due to the rapid-cycling characteristics which enable a single generation to be completed in about three months. Location of resistance genes in a rapid-cycling line is intended to facilitate conservation and utilisation of these genes.

4. Discussion

The use of non-numerical scoring systems to describe the response of *B. oleracea* accessions to the fungal pathogens has two advantages over the numerical systems used by previous workers. Firstly, there are no value judgements made about the phenotypes identified, each different interaction phenotype may indicate the presence of a gene or genes with different modes of action, and secondly, the system can easily be extended by the addition of a suitable code if other types of host response or pathogen growth are observed. The standardisation of protocols as much as is practicable and the use of the same control genotypes in experiments at both HRI and ISA means that realistic comparisons can be made of a large data set.

The development of a glasshouse-based protocol for the rapid-screening of accessions for response to cabbage aphid will enable much larger numbers of genotypes to be screened than has previously been possible. This should allow the identification of sources of resistance in the *B. oleracea* genepool which may otherwise have been missed in smaller scale tests. Any promising accessions will be evaluated more rigorously in laboratory tests and will be included in field experiments.

It is hoped that the results will give an overall picture of the diversity present in the cultivated *B. oleracea* genepool for response to the three parasites. It will then be possible to investigate in more detail components of the genepool which show the greatest variation for parasite response and thus potentially identify a wider range of resistance genes which can be used in breeding programmes. It is also anticipated that the approach adopted will be applicable for other important parasites of *B. oleracea*.

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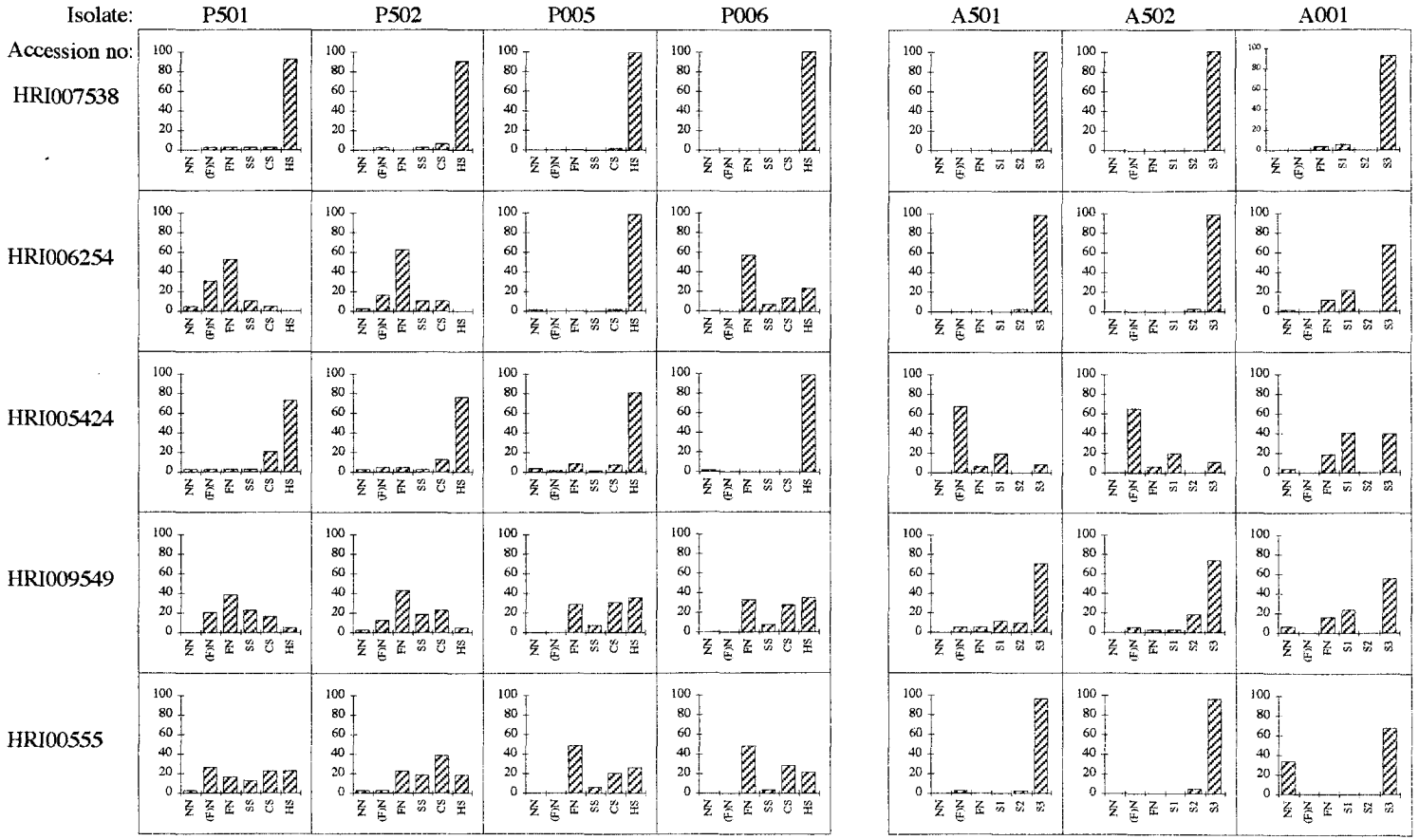

Figure 1. Histograms showing % of plants in each interaction phenotype class when inoculated with four downy mildew isolates and three white blister isolates.