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Susceptibility of broccoli cultivars to bacterial head rot: *in vitro* screening and the role of head morphology in resistance

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Abstract

Head rot is a major disease of broccoli caused by the soft rot pathogens *Pseudomonas fluorescens* and *Erwinia carotovora*. Two *in vitro* pathogenicity tests were evaluated as a methods to identify broccoli cultivars susceptible or resistant to bacterial head rot. One test used mature heads excised from the plant and inoculated with squares of cotton lint which had been soaked in a bacterial suspension. The other test involved stab-inoculating axenically grown seedlings. With the excised head test, susceptible cultivars showed a black soft rot, whilst less susceptible or moderately resistant cultivars showed only watersoaking, or browning and slight softening of the tissue. No cultivar was completely resistant. Ten cultivars were tested, and their susceptibility ratings corresponded with previously recorded field data, with one exception. This laboratory test could be used to screen for susceptibility to head rot in broccoli breeding programmes. The seedling test distinguished differences in aggressiveness among bacterial isolates but not cultivar susceptibility. Increasing head size correlated negatively with disease resistance. Head shape, i.e. cultivars which showed a domed shape rather than a flat shape, was positively correlated with disease resistance. Thus small domed heads are more resistant to head rot than large flat heads. Other morphological characteristics, viz. floret prominence and number, and sepal stomatal number were not correlated with host resistance.

Introduction

Bacterial head rot of broccoli (*Brassica oleracea* var. *italica*), leads to crop losses of between 30% and 100% which represents a cost of £9.5 million annually for the UK industry (Campbell et al., 1995). Disease incidence and severity increase when head maturity coincides with periods of persistent wet weather (Canaday et al., 1987). Watersoaked lesions develop on the buds and then deteriorate into a brown/black soft rot. Head rot is an opportunistic disease caused by two genera of bacteria: fluorescent *Pseudomonas* spp. (*Pseudomonas marginalis* and *P. fluorescens*) and *Erwinia carotovora* subspp. *carotovora* and *atroseptica*; both groups coexist on broccoli heads and are capable of causing disease (Australia – Wimalajeewa, 1987; Canada – Hildebrand, 1986; UK – Brokenshire and Robertson 1986; UK – Robertson et al., 1993), although as a causal agent, *E. carotovora* may be more important in Scotland than in Australia and Canada (Darling, 1998). Field trials have shown broccoli cultivars to vary in their susceptibility to head rot, although none are completely resistant (Canaday et al., 1991; Robertson and Brokenshire, 1992; Robertson et al., 1993).

Pathogenicity tests which can be conducted in a glasshouse and/or a laboratory are desirable for a number of reasons: they are more cost effective on labour, materials, space and time and conditions can be closely controlled. These attributes are also of benefit to more detailed studies on the host/pathogen interaction. Further, if cultivar susceptibility and resistance can be demonstrated in the laboratory to be identical to field responses, then such tests would be useful for screening purposes in breeding programmes. In this paper,

two laboratory based pathogenicity tests are described and evaluated for determining cultivar susceptibility to head rot.

In one test, broccoli heads from mature plants were grown in a glasshouse to reduce the amount of saprophyte loading which may interfere with testing; field grown plants have far higher numbers of saprophytic bacteria than glasshouse heads (unpublished results). This test had been designed for distinguishing pathogenicity of different bacterial isolates (Harling et al., 1994) and its use has now been extended to determine differences in cultivar susceptibility to head rot. Field data on the susceptibilities of cultivars were compared to the data obtained in these laboratory based tests (Robertson et al., 1993; Campbell et al., 1995).

In the second test axenically grown broccoli seedlings were challenged by stab inoculation of the hypocotyl. A seedling test would offer even greater savings in time, space and materials over a mature excised head test to determine cultivar susceptibility and pathogenicity of isolates.

In addition to these tests, the importance of phenotypic characteristics in cultivar susceptibility to disease was assessed. Characteristics such as head doming, floret size and tightness (compactness), floret prominence, and head weight/diameter ratio vary considerably among broccoli cultivars. These characters may affect the duration of free water on the head, consequently influencing bacterial multiplication and penetration. Stomata, the site of bacterial infection (Hildebrand, 1989), may also influence disease susceptibility among different cultivars if numbers are greater in some cultivars than in others. To determine the importance of head morphology in susceptibility to disease, head characteristics of ten cultivars grown in the field were assessed and compared with disease ratings obtained previously (Robertson et al., 1993; Campbell et al., 1995). Robertson and Brokenshire (1992) found a correlation between the more domed cultivars and resistance to disease, but results were inconclusive with flatter shaped heads. Canaday (1989) found floret tightness, head doming and diameter to be positively correlated with disease resistance.

Materials and methods

Excised head pathogenicity test

Mature heads from glasshouse-grown plants were removed and inoculated by placing two 2×2 cm pieces of cotton lint (i.e. one upon the other), which had been soaked in a 1×10^4 cfu/ml suspension of the broccoli head rot pathogen *E. carotovora* subsp. *carotovora* isolate 5067 (previously determined as pathogenic by Campbell et al., 1995), on their surface. Heads were kept upright with sponge collars in closed 'Magenta GA7' vessels (Sigma Chemicals), with 20 ml of sterile distilled water (Figure 1a,b). Heads were incubated for five days at 20 °C (day)/10 °C (night) with a 16 h photoperiod in a Fisons environmental cabinet.

Fifteen heads of ten cultivars were evaluated. The susceptibilities of eight of these cultivars had been determined in field trials (Robertson et al., 1993; Campbell et al., 1995): susceptible cultivars were Skiff, Premium Crop and Corvet; less susceptible or moderately resistant cultivars were Shogun, Samurai, Marathon, Greenbelt and Dixie. The latter group all showed some disease in these field trials but disease was considerably less than in susceptible cultivars. In addition, two cultivars, Trixie and Headline, which had not previously been tested were included.

Results were recorded using a five point scale: 0 = no symptoms, 1 = watersoaking (loss of waxy bloom from florets), 2 = watersoaking and tissue browning but no rot, 3 = tissue browning and softening, 4 = extensive black soft rot. Categories 0, 1 and 2 were classed as resistant because of the absence of soft rot. Data were subjected to percentile angular transformation using arcsine; thus categories 0–4 above became 0% (category 0), 33% (1), 50% (2), 67% (3), 100% (4).

Seedling pathogenicity test

This method was adapted from Daniels et al. (1984). Broccoli seeds (six cultivars) were surface sterilised for 30 min in sodium hypochlorite (1% available chlorine), rinsed in sterile distilled H_2O, then placed on water agar plates for 48 h at 25 °C in darkness to germinate. Seedlings were transferred to replidishes ('Sterilin') containing Murashige and Skoog plant tissue agar (Murashige and Skoog, 1962), maintained in a humid environment (a sealed damp chamber) and incubated at 20 °C (day)/10 °C (night) with a 16 h photoperiod, until they had reached a height of 3–5 cm (ca. four days). Three inoculation methods were used. First a needle was dipped into a bacterial colony growing on a King's B (King et al., 1954) agar plate, then stabbed into the hypocotyl of the seedlings. Second, the cotyledons were stab inoculated and third, a loopful of bacteria directly from a colony was smeared onto the surface

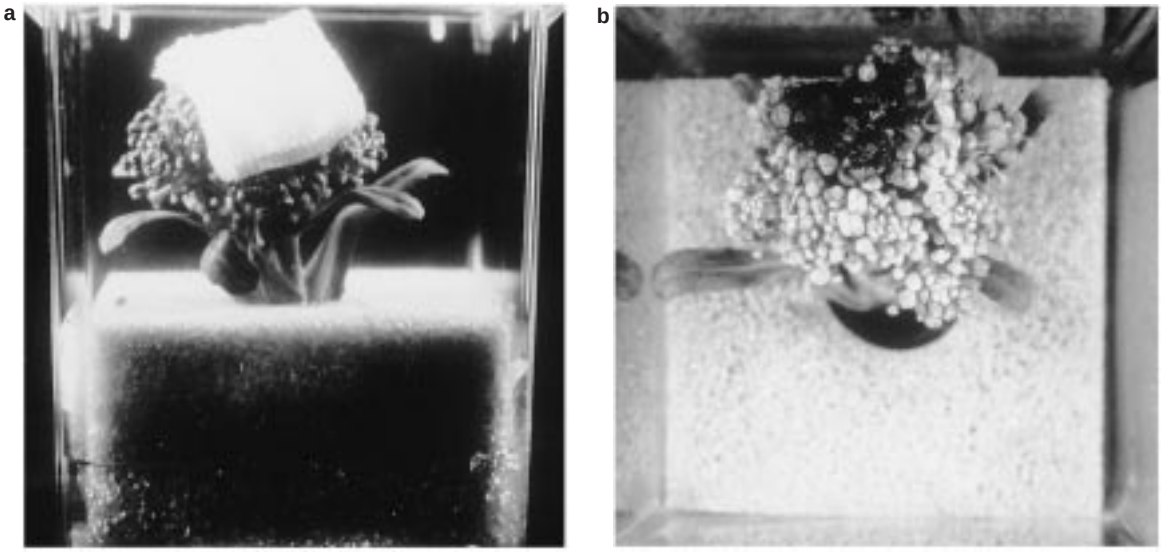

Figure 1. Excised head pathogenicity test for distinguishing cultivar susceptibility to bacterial head rot. (a) Portion of broccoli head inoculated with a square of cotton lint (2 × 2 cm), soaked in a bacterial suspension of *Erwinia carotovora*. (b) Top view of broccoli head with lint removed after 5 days, showing soft rot.

Table 1. Bacterial strains used in the seedling pathogenicity test

Isolate number	Species	Pathogenicity rating[a]
5067	*Erwinia carotovora* subsp. *carotovora*	Pathogenic
1065	*Erwinia carotovora* subsp. *carotovora*	Pathogenic
5038	*Pseudomonas fluorescens* (biovar IVb)	Pathogenic
5049	*Pseudomonas fluorescens* (biovar IVb)	Pathogenic
5024	*Pseudomonas fluorescens* (biovar Vb)	Non-pathogenic
5027	*Pseudomonas fluorescens* (biovar Vb)	Non-pathogenic

[a]All strains previously isolated from broccoli and pathogenicity ratings taken from Robertson et al., 1993 and Campbell et al., 1995.

of the hypocotyl or the cotelydons without wounding. Six bacterial strains were used (Table 1). The control involved wounding hypocotyls with a sterilised needle. Seedlings were incubated at 20 °C (day)/10 °C (night) with a 16 h photoperiod for five days. Three susceptible cultivars (Skiff, Corvet, Premium Crop) and three moderately resistant (Shogun, Samurai and Marathon) were tested. For each isolate/cultivar combination, 25 replicate seedlings were inoculated.

A four point scale was used to assess the results as follows: 0 = healthy, 1 = brown discolouration at inoculation point, 2 = brown discolouration with some limited tissue softening, 3 = spreading soft rot. Data were transformed using arcsine, giving transformed values of 0% (category 0), 21% (1), 47% (2), 100% (3).

Field trial to determine head morphological characters

Five moderately resistant (Shogun, Samurai, Marathon, Greenbelt and Dixie), three susceptible (Skiff, Premium Crop and Corvet) and two cultivars of unknown susceptibility (Trixie, Headline) were transplanted from module trays at four weeks old to field plots at Diamond Field, Auchincruive, Ayrshire (soil type: sandy clay loam). Ten test plants were sown for each cultivar, at a distance of 40 cm between the plants within the rows and 50 cm between the rows; a plot consisted of two rows of five plants each. A row of guard plants of the same cultivar surrounded each plot. A general fertilizer of 16 : 16 : 16 NPK was applied at a rate of 200 kg/ha two days after transplantation. The plot was protected by wire throughout the trial to reduce pigeon and rabbit grazing. Mature heads were excised for assessment after 8–10 weeks (depending on cultivar) following transplanting.

The following observations were made on each cultivar (ten replicate heads per cultivar):

1. Degree of head doming, i.e. whether inflorescences were triangular or flat in longitudinal section. Heads were rated on a five point scale where 0 = flat head, 1 = slight doming, 2 = moderate doming, 3 = rounded dome, 4 = peaked dome. Data were transformed using arcsine.
2. Bud number, i.e. number of florets per unit area of the inflorescence. Bud number within a 1 cm^2 area was recorded at three positions; one central and two peripheral regions of the head.
3. Bud prominence, i.e. the extent to which the buds protruded above the inflorescence surface, was recorded using a five point scale ranging from 0 = no protrusion, to 5 = highly protruded buds. Data were transformed using arcsine.
4. Head diameter (cm).
5. Stomatal numbers on floret sepals. Sepals were excised from florets of mature glasshouse grown heads, cleared in methanol for 2 h, washed three times in distilled H_2O, then stained in 10% safranin for 10 min. After washing again, the sepals were placed in 20% glycerol for 5 min before being mounted in glycerol and viewed using a light microscope. Number of stomata in each of 30 separate fields of view (at ×400 magnification) were recorded for each cultivar as six counts from each of five sepals.

Data for these morphological characters were compared with the disease resistance classifications for each cultivar using linear discriminant analysis (Genstat 5, Rothamsted Experimental Station, 1995), to determine their contribution towards disease resistance. This analysis finds the linear combination(s) of characters which best separates the cultivars by their disease response (resistant or susceptible). Comparisons were made both for resistance as determined in the field (Robertson et al., 1993; Campbell et al., 1995 – see above) and in the laboratory as reported in this paper.

Results

Excised head pathogenicity test

Significant differences in cultivar susceptibility were distinguished. Susceptible cultivars showed a soft black rot (see Figure 1a,b), whilst moderately resistant cultivars showed watersoaking or brown discolouration of tissue (the more resistant cultivars) or discolouration and some limited softening (the less resistant cultivars). Controls (inoculated with water) showed no symptoms or a slight loss of the waxy bloom on the florets.

Cultivars with a mean transformed score of 51% and above were classed as susceptible and included Skiff, Corvet, Shogun and Premium Crop (Table 2). Those at 50% and below were classed as resistant and included Greenbelt, Marathon, Samurai, Trixie, Dixie and Headline. According to the transformed disease score, a value of 50% or below means that the tissue showed no soft rotting, only discolouration, which

Table 2. Morphological characters of broccoli cultivars and disease in excised heads after inoculation with *Erwinia carotovora* subsp. *carotovora* strain 5067

Head characteristic	Cultivars										LSD $p = 0.05$
	P. Crop	Skiff	Corvet	Samurai	Shogun	Marathon	Dixie	Greenbelt	Headline	Trixie	
Bud prominence[a] (%)	33.3	46.7	48.3	40.0	31.7	28.3	41.7	46.7	78.3	33.3	10.60
Head diameter (cm)	10.6	9.1	10.4	6.4	6.2	6.8	7.8	7.4	7.2	4.9	1.80
No. of buds/cm^2 at side of head	24.5	51.0	40.1	64.4	77.2	77.1	40.4	42.7	41.4	42.8	10.44
No. of buds/cm^2 centre head	25.3	71.6	54.6	85.2	96.7	90.8	48.0	50.8	48.8	55.2	19.10
Head doming[b] (%)	33.3	51.7	44.1	63.3	83.3	68.3	68.3	75	71.7	76.7	15.10
No. of stomata/mm^2	153.6	176.0	nt[d]	141.8	123.5	126.5	81.8	131.0	153.6	117.3	18.20
% disease[c]	56	74	73	43	58	41	48	39	47	47	7.90

[a]Bud prominence 0% = not protruding above surface; 100% = protruding greatly above surface. Data transformed with arcsine.
[b]Head doming 0% = flat head shape; 100% = very domed head (triangular cross section). Data transformed with arcsine.
[c]Data transformed with arcsine 0 = no disease; 100% = black soft rot.
[d]Not tested.

possibly represents a defence reaction. The results reflected the resistance/susceptibility ratings observed under field conditions with the exception of Shogun which was more resistant in the field.

Seedling pathogenicity test

Only the *Erwinia* strains caused a soft rot of the hypocotyl and cotyledons in all cultivars (Table 3). The *Pseudomonas* strains, capable of producing disease on excised heads similar to the *Erwinia* isolates (Campbell et al., 1995), did not rot seedling tissues, but caused localised browning and some very limited non-spreading softening. Non-pathogenic strains also caused occasional tissue browning as did control (water) inoculations. Although there were differences in cultivar susceptibilities with the *Erwinia* isolates, cultivar susceptibility ratings did not correspond to field susceptibility data for mature heads or to the results of the excised head test.

The other inoculation methods on seedlings were unsuccessful in producing disease (data not shown). Cotyledon inoculations with wounding led only to tissue discolouration around the wound. Smearing the hypocotyls with a loopful of a bacterial colony led to some rot development, but not consistently so.

Head morphological characters and resistance

Significant differences among cultivars were found for all characters measured, i.e. head doming, size, side bud number, central bud number bud prominence and sepal stomatal number (Table 2). Head size was found to correlate with the degree of doming ($r = -0.89$; $p < 0.05$): flatter heads were larger. Linear discriminant analysis of these morphological data against the field- and lab resistance classifications of the cultivars was carried out. A discriminant score was assigned to each cultivar when compared against the field and lab classifications (Figure 2). Discriminant scores ranged from −11.172 for cv. Headline, to

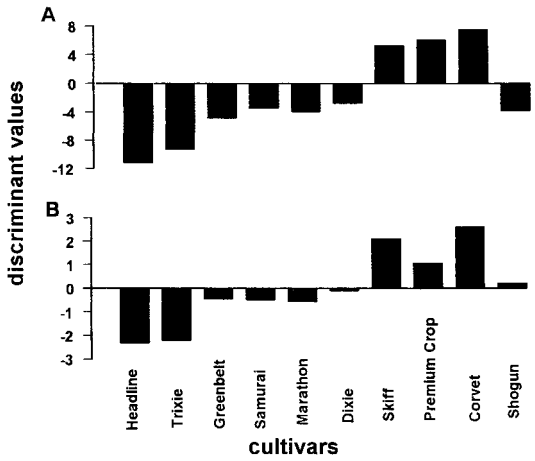

Figure 2. Histogram of discriminant scores for broccoli cultivars: (A) field classification, (B) laboratory classification. Increasing negative scores for cultivars indicate increasing disease resistance; increasing positive scores indicate increasing susceptibility.

Table 3. Response of seedlings of broccoli cultivars to stab inoculation with isolates of *Erwinia carotovora* or *Pseudomonas fluorescens*

Cultivar	% disease[a]							Mean of cultivar means
	Bacterial isolate[b]						Control	
	5067 *Ec*	1065 *Ec*	5038 *Pf*	5049 *Pf*	5024 *Pf*	5027 *Pf*		
Premium crop	81.6	93.6	36.8	36.9	28.1	38.3	3.1	45.4
Skiff	73.6	91.1	39.9	38	28.1	25	1.6	42.4
Corvet	41.1	44.2	38.3	58.6	20.2	32.8	0.0	33.6
Samurai	92.9	96.0	35.9	36.2	31.2	33.7	0.0	46.6
Shogun	69.1	76.2	34.3	46.7	26.6	29.7	6.2	41.2
Marathon	78.4	72.4	41.7	44.2	23.4	32.1	0.0	41.8
Mean of isolate means	72.8	78.9	37.8	43.4	26.2	31.9	1.8	

[a]Data transformed with arcsine 0 = no disease, 100% = spreading soft rot. LSD ($p = 0.05$), cultivars × isolates = 11.0%.
[b]For key to bacterial isolates see Table 1; *Ec = Erwinia carotovora, Pf = Pseudomonas fluorescens*.

+7.575 for cv. Corvet when morphological data were compared against the field classification, and −2.315 for cv. Headline to +2.596 for cv. Corvet against the lab classification, where increasing negative scores indicate increasing resistance and increasing positive scores indicate increasing susceptibility. Thus Headline was the most resistant and Corvet the most susceptible of the cultivars.

Correlation of head morphological characters against the field classification of resistance for these cultivars showed that both head diameter and degree of doming were the two characters which contributed most to resistance: larger heads were less resistant (head diameter $r = -0.937$, $p \leq 0.001$), and domed heads were more resistant than flat heads (doming $r = +0.900$, $p \leq 0.001$).

Correlations against the laboratory classification for resistance also showed significant values for head diameter ($r = -0.805$, $p \leq 0.01$) and head doming ($r = +0.701$, $p \leq 0.05$). No significant correlations were obtained for any of the other morphological characters.

Discussion

Results for the laboratory excised head test allowed differentiation between host resistance levels. The cultivars' susceptibility to disease reflected field trial data (Robertson et al., 1993; Campbell et al., 1995), with the exception of Shogun, which is resistant in the field. There is, however, recent evidence from growers that Shogun is becoming increasingly susceptible to disease, perhaps indicating resistance breakdown, and thus the laboratory test results for this cultivar may accurately reflect its current disease status.

Negative controls in this test did not rot, indicating that saprophytes already present on the heads did not cause disease: heads with low saprophyte populations are assured by growing plants to maturity in the glasshouse, and this avoids unwanted interactions of the inoculum with high numbers of saprophytes as may be seen in the field (Hildebrand, 1989). Surface sterilisation of heads prior to inoculation, which would be desirable, has not proved possible (data not shown).

The excised head test uses a fairly low bacterial concentration for inoculation and is straightforward to execute. Isolate pathogenicity can also be differentiated successfully (Harling et al., 1994). It has been observed that various types of lint do not give equally good results. Lint which is softer and 'fluffy' gives the best results. Because of the greater control over environmental conditions and the reliable provision of disease conducive conditions, this test has the potential for use as a laboratory screen for head rot susceptibility in broccoli breeding programmes.

The seedling pathogenicity test allows material to be produced quickly, requires little space and seeds can be surface sterilised before treatment. Results could distinguish between aggressiveness of bacterial isolates but not cultivar susceptibility. Cultivar susceptibility ratings did not correspond to field susceptibility data for mature heads (Robertson et al., 1993; Campbell et al., 1995). For example, Samurai was susceptible and Corvet resistant to *Erwinia* in the seedling test, but their actual response is vice versa in the field. Disease symptoms between sub-samples were also variable even with the most aggressive isolates. Daniels et al. (1984) found that stab inoculation of *Pseudomonas* spp. onto turnip seedlings gave unreliable symptom expression, which reflects the results found here with our strains of *P. fluorescens* and *E. carotovora*. *P. fluorescens* strains 5038 and 5049, pathogenic in the excised head test (Campbell et al., 1995), were only weakly pathogenic in the seedling test, a result which probably reflects the weaker pectolytic ability of *P. fluorescens* compared to *E. carotovora*. This test is unsuitable for distinguishing cultivar susceptibility in the present form. However, because of the unique architecture of the broccoli head, probably no test model for the disease which does not use heads will successfully reproduce the disease.

Linear discriminant analysis of the combined head morphological data against the field resistance categories of Robertson et al. (1993) and Campbell et al. (1995) correctly classified all the cultivars whose classification was known. In addition, two unknowns (Headline and Trixie) were classified as resistant. For the laboratory resistance categories, linear discriminant analysis correctly classified nine out of the ten cultivars. Shogun was the misclassified cultivar, although as discussed above, there are doubts about the recent field performance of this cultivar.

These comparisons suggest that resistance has a morphological basis. Correlations of all characters against both the field and lab classifications showed that head diameter and head shape (doming) were the characters which contributed most to resistance within the linear discriminant analysis: highly significant r values were obtained for these characters. Thus, larger heads are

more susceptible to disease, and domed heads are more resistant. Similar correlations were found by Canaday et al. (1991), in the USA, using a different set of cultivars. Thus, these two characters provide a basis for the selection of cultivars which are smaller and domed, provided these are acceptable attributes to the market. Both doming and size are not factors which play a part in the excised head test for cultivar resistance – despite this, differences are still apparent. This suggests that tissue resistance factors (physical/chemical barriers to infection) also contribute to resistance. The response of a cultivar in the field is a combination of gross morphological and tissue resistance factors.

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