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Isolation of mutants exhibiting altered resistance to *Sclerotinia sclerotiorum* from small M_2 populations of an oilseed rape (*Brassica napus*) variety

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Abstract

Two small M_2 populations, consisting of 39 and 50 plants, respectively, obtained by EMS-mutagenesis of an inbred line derived from oilseed rape cv. Linetta, were screened for altered leaf response to artificial inoculation with *Sclerotinia sclerotiorum*. In both experiments, the M_2 population exhibited greater variation and a lower mean infection value than the parental population; individuals in the most resistant class were obtained only from the M_2 population. Parent–progeny analysis of disease response scores revealed significant regressions only for the mutagenised population, with narrow-sense heritabilities of 0.75–0.83, compared to 0.14–0.22 for the parental population. When larger populations (approximately 600 individuals per population) were screened, similar results were obtained. Mutants with significantly greater resistance than the most resistant 'Linetta' line were obtained at frequencies of 1.7% (from an M_2 population size of 593) to 5.1% ($n = 39$). The altered leaf response to *Sclerotinia* in selected mutant lines was positively correlated with stem response to artificial inoculation. Detailed analysis of one mutant (HH-1), with significantly higher *Sclerotinia* resistance than the parent, demonstrated that HH-1 was more resistant to artificial stem inoculation than four commercial varieties tested, including cv. Briol, which is reported to exhibit high levels of resistance in the field. Field trials in moderately- and heavily-infested soils showed that HH-1 exhibited significantly greater resistance to natural infection than 'Linetta', with percentage plant deaths of 5.3% (compared to 22.4% in the parental population) and 13.6% (47.3%) under moderate and high inoculum pressure, respectively. The seed yield of HH-1 was significantly higher than that of the parent population under a heavy *Sclerotinia* infestation; in the absence of *Sclerotinia*, the yield difference between the two populations was not significant. The implications of these results are discussed in respect of a re-evaluation of the efficacy of mutagenesis for the isolation of agronomically valuable micro-mutants.

Introduction

From the late 1970s, the area sown to oilseed rape (*Brassica napus* ssp. *oleifera*) increased dramatically in EU member states as a result of production incentives provided under the Common Agricultural Policy. These developments lead to a shortening of crop rotations, with a consequent build-up of persistent soil-borne pathogens, such as the fungus *Sclerotinia sclerotiorum*. Stem rot of oilseed rape, caused by *S. sclerotiorum*, is a particular problem in wet years. In 1991, for example, the incidence of stem rot in the UK increased from less than 12% (averaged over the previous five years) to 49% of crops (Hardwick et al., 1993).

In early summer, apothecia develop from sclerotia in the soil and release ascospores. These spores

colonise fallen petals which have attached to the stem and racemes, providing the fungus with nutrients necessary to effect infection of the stem (McLean, 1958). Lesions girdle the stem, causing wilting, premature pod ripening and hence pod shatter and loss of seed, reduced individual seed weight and, in severe cases, plant death (Cox et al., 1983). Sansford (1995) estimated yield reduction due to *Sclerotinia* infection at 0.0146 t/ha per 1% stem affected at pod ripening; this value doubled when main racemes were affected. Stem rot can cause crop losses of up to 60% (Jellis et al., 1984; Yarham and Giltrap, 1989).

Fungicides are effective in reducing severe yield losses (Morrall and Dueck, 1982); there was an increase in the percentage of UK oilseed rape crops sprayed to control *Sclerotinia* from 9% in 1991 to 44% in 1993 (Turner and Hardwick, 1995). Given the very wide host range of the causal organism (Purdy, 1979), crop rotation is relatively ineffective against stem rot. Commercial varieties of oilseed rape lack any significant level of physiological resistance to *S. sclerotiorum* (Morrall and Dueck, 1982; Sweet and Pope, 1992) and variability in disease response between cultivars in the field is believed to be due largely to disease escape as a consequence of altered plant architecture. Moderate *Sclerotinia* resistance has been reported in several related species, including *Brassica nigra*, *B. juncea* and *Sinapis alba* (Morrall and Dueck, 1982; Kolte, 1985; Quinlan, 1992). Novel approaches to the introduction of increased *Sclerotinia* resistance into oilseed rape include the development of transgenic plants expressing traits such as oxalate oxidase activity (Thompson et al., 1995) directed at degrading oxalic acid, a pathotoxin of the fungus (Godoy et al., 1990).

Induced mutagenesis has proved to be a successful technique for introducing disease resistance genes into crop varieties (Konzak, 1956; Maluszynski et al., 1995), but the adoption of mutagenesis as a tool in the breeding of resistant crop varieties has been restricted by reports of extremely low frequencies of resistant mutants in M_2 populations (e.g. McKenzie and Martens, 1974; Harder et al., 1977) due to frequencies of major gene disease-resistant mutations in the order of 1 in 10^4–10^7 (International Atomic Energy Agency, 1977). On the other hand, it is widely accepted that micro-mutations in minor genes, causing small quantitative changes in phenotypic characters, occur at much high frequencies (IAEA, 1977). There have been several published reports in which induced mutants exhibiting increased partial (quantitative) disease resistance have been isolated from small (often $n < 100$) M_2 populations at percentage frequencies (Hänsel, 1971; Varghese, 1985; Worland and Law, 1991; Kinane and Jones, 1996). Such results should encourage re-assessment of the use of mutagenesis in resistance breeding programmes.

Against a non-specialised necrotrophic pathogen such as *S. sclerotiorum*, any resistance would probably be quantitative in nature. Successful isolation of *Sclerotinia*-resistant mutants from small M_2 populations of oilseed rape could introduce potentially valuable genes into the *B. napus* gene pool. This study also permits evaluation of the efficacy of small-population M_2 screening for the isolation of desirable micro-mutants from a heterogeneous crop (oilseed rape), as opposed to the homozygous material which has been used in similar studies to date (e.g. wheat; Kinane and Jones, 1996).

A preliminary report of this research has been published in a conference proceedings (Mullins et al., 1995).

Materials and methods

Plant material

Most of the work described here used an inbred line of spring oilseed rape (*B. napus*) cv. Linetta obtained by three generations of forced self-pollination. Selection started with a single seed; the resulting plant was selfed. Individual plants from this selfed seed were, in turn, self-pollinated, and progeny rows of each plant were assessed visually. One selfed seed from the most uniform population was selected at random, and the entire process was repeated once more. For varietal comparisons, the spring oilseed rape varieties Forte, Printol, Briol and Starlite were used in glasshouse and field trials. Glasshouse-grown plants were grown at a density of 2 per 17.5 cm pot of peat-based potting compost containing both rapid- and slow-release fertilisers. The minimum temperature in the glasshouse was 18 °C and the photoperiod (natural light supplemented by 400 W sodium vapour lamps) was 16 h.

Fungus material

Two isolates of *S. sclerotiorum* were used: isolate OSR 1 was obtained from infected *B. napus* plants and isolate JA 3 from infected plants of Jerusalem artichoke

(*Helianthus tuberosus*). Unless stated to the contrary, all glasshouse studies were conduced with OSR 1 and all field studies with JA 3.

Cultures were initiated from sclerotia collected from infected *B. napus* plants and stored at room temperature. Sclerotia were surface sterilised (70% ethanol for 60 s), placed (1 per plate) on glucose asparagine yeast extract agar (GAY) (2% (w/v) glucose, 0.1% (w/v) potassium dihydrogen orthophosphate, 0.5% (w/v) potassium chloride, 0.5% (w/v) magnesium sulphate heptahydrate, 0.001% (w/v) ferric sulphate, 0.2% (w/v) asparagine, 0.2% (w/v) yeast extract, 2% (w/v) agar) (Mylchreest, 1985) and incubated at 22 ± 2 °C under a 16 h photoperiod, using white fluorescent tubes (photosynthetically active radiation, PAR, 220 µmol photons s^{-1} m^{-2}). After 3–4 days incubation, subculturing took place with a plug (5 mm in diameter) from the advancing edge of the culture being transferred to a fresh GAY agar plate; such cultures were the source of inoculum for artificial inoculation studies.

Artificial inoculation (leaf)

At growth stage 2.5 (Sylvester-Bradley and Makepeace, 1984), the third-formed true leaf was removed and trimmed to occupy a 9 cm petri dish containing a disc of Whatman no. 1 filter paper soaked with distilled water. Mycelial plugs (5 mm diameter) from 3-day old *S. sclerotiorum* cultures were placed on the adaxial surface of the leaf (two per leaf, either side of the mid-rib, avoiding major veins). Lesions were measured (as the mean of two diameters measured at right angles to one another) after 24 h at 22 °C under a 16 h photoperiod. For the purpose of presentation, leaf lesion diameter was classified into a 6-point system: Class 1: 0–0.4 cm; Class 2: 0.5–0.9 cm; Class 3: 1.0–1.9 cm; Class 4: 2.0–2.9 cm; Class 5: 3.0–3.9 cm; Class 6: 4.0 cm and above. With the exception of studies of individual plants in the M_2 and 'Linetta' comparisons, 15 plants of each genotype were inoculated.

Artificial inoculation (stem)

The stem inoculation technique was based on the method described by Brun and Renard (1983). Lengths of softwood (40 mm × 2 mm × 2 mm; matches minus the 'heads') were autoclaved in malt extract solution (2 g l^{-1}) and then placed on 1-day old *Sclerotinia* cultures on GAY agar. After incubation for 10 d at 22 °C (under a 16 h photoperiod) a match, coated in mycelium, was removed from the culture and placed in a shallow cut (made with a sterilised needle) in the oilseed rape stem (at growth stage 2.5), 10–15 cm above ground level. Horizontal spread of the lesion around the stem was measured after 2 weeks incubation under glasshouse conditions. Fifteen plants were inoculated for each genotype.

Mutagenesis

One hundred seeds of the 'Linetta' inbred line were pre-soaked on moist filter paper for 16 h in the dark at 20 °C before being immersed in 100 ml of a vigorously aerated solution of 0.03% (v/v) ethyl methane-sulphonate (EMS; Sigma Aldrich Chemical Co., Poole, UK) for 8 h; this concentration had previously been determined to be optimal using the method described in IAEA (1977). The EMS-treated seed (the M_1 generation) was rinsed under running tap water for 3 h and then sown in peat-based compost, the plants grown to maturity in the glasshouse, forcibly self-pollinated and the resulting seed from the M_1 plants bulked to produce the M_2 generation.

M_2 population screening for Sclerotinia *response*

This study was conducted three times with different numbers of plants of the M_2 and 'Linetta' populations: study A: 39 M_2, 37 'Linetta'; study B: 50 M_2, 48 'Linetta'; study C: 593 M_2, 604 'Linetta'. In each study, the experiment was organised in a completely randomised design. Each plant was scored for leaf response following inoculation with isolate OSR 1. Each plant was then self-pollinated and the leaves of 15 selfed progeny of each of 12 M_2 and 13 'Linetta' plants (studies A and B) and 12 M_2 and 12 'Linetta' plants (study C) were inoculated with OSR 1, and the mean progeny score was determined for each plant. To allow for differences in average infection response because the parent and progeny scores were necessarily not obtained at the same time, the values for individual parent plants were expressed as a percentage of the mean parent value (i.e. mean of 12 M_2 plants or 13 'Linetta' plants in study A); a similar approach was used to express the corresponding progeny values. To calculate the narrow-sense heritability (h^2) for the variation of *Sclerotinia* response in the M_2 (or 'Linetta') population, the parent–progeny regression was conducted; the regression coefficient equalled the narrow-sense heritability of *Sclerotinia* response.

Artificial inoculation of oilseed rape varieties

Six spring oilseed rape populations (the varieties Linetta, Forte, Printol and Briol and the 'Linetta'-derived mutants HH-1 and HH-13) were investigated, in a completely randomised design. Stem inoculation was carried out on 15 plants of each genotype. Percentage plant death and horizontal spread of the lesion ('stem girdling', expressed as percentage of the stem circumference affected by the lesion) were recorded weekly over a 4-week incubation period under glasshouse conditions.

Natural infection studies

The 1993 field trial was carried out on a site (Fota Estate, Cobh, Ireland), which had been infested with sclerotia of *S. sclerotiorum* isolate JA 3 during the previous year and planted with a *Sclerotinia*-susceptible variety of Jerusalem artichoke (*Helianthus tuberosus*), resulting in the presence of a moderate inoculum pressure (Cassells and Walsh, 1995). The spring-sown trial included 'Linetta' and the mutants HH-1 and HH-13, with the experiment sown in a replicated randomised block design (five replicate plots per line) at a density of 170 seeds per m^2 with each plot measuring 2 m × 2 m. Nitrogen, in the form of 7 : 6 : 17 N : P : K compound fertiliser with added sulphur, was applied to the seed bed at 40 kg ha^{-1}. A further 70 kg ha^{-1} of nitrogen with added sulphur was applied at growth stage 1.5 (Sylvester-Bradley and Makepeace, 1984). Pest control (principally of aphids) was achieved with application of Metasystox-55 (active ingredient: osydemeton-methyl; Bayer) and the plots were hand-weeded on a regular basis. *S. sclerotiorum* infection was estimated (from the stem base to the first node) by the employment of a scoring system incorporating six classes:

Class 1: no lesions visible
Class 2: appearance of lesions on stems, with largest lesions encircling less than 1/10 of the stem circumference
Class 3: largest lesion encircling from 1/10 to 1/3 of the stem circumference
Class 4: largest lesion encircling from 1/3 to 1/2 of the stem circumference
Class 5: largest lesion encircling from 1/2 to 3/4 of the stem circumference
Class 6: plant death

S. sclerotiorum infection of the main raceme was scored using the same system.

The second field assessment (1994) was conducted on an adjacent artificially-infested site in which the same (susceptible) variety of *H. tuberosus* had been planted for the two previous seasons, resulting in a high level of *Sclerotinia* infestation. The oilseed rape lines examined were 'Linetta', HH-1 and the varieties 'Briol', 'Forte' and 'Printol', with the cultural treatments, plot sizes and experimental design and the *Sclerotinia* assessment protocol being identical to that used in the 1993 trial.

The yield trial consisted of HH-1, 'Linetta' and the commercial variety 'Starlite', planted at a density of 170 plants per m^2, in a replicated randomised block design (2 m × 2 m plots), three replicates per genotype, with cultural treatments as described for the *Sclerotinia*-resistance assessments. After harvesting, pods were dried at room temperature and threshed manually; the seeds were dried at 60 °C for 2 days before weighing.

Statistical analysis

The data approximated to normal frequency distributions. For multiple comparisons, parametric ANOVA was conducted, with Fisher's Protected Least Significant Difference (LSD) Test.

Results

Isolation of mutants exhibiting altered Sclerotinia *response*

Artificial inoculation of detached leaves of individual plants from small glasshouse-grown populations of the M_2 and 'Linetta' populations (study A) revealed a change in the frequency distribution (Figure 1) of *Sclerotinia*-response in the M_2 population (mean lesion diameter ± SD, 1.98 ± 1.26 cm; $n = 39$) from that exhibited by the inbred 'Linetta' population (2.25 ± 1.05 cm; $n = 37$). The M_2 population expressed greater variation plus a shift in average plant response towards increased resistance. Six M_2 plants (15.4% of the total) were in the most resistant response class (Class 1) compared to none in the 'Linetta' population (Figure 1). The proportion of plants in the two most resistant classes (1 and 2) in the M_2 population (12/39) was significantly greater ($\chi^2 = 4.58$; 1 df; $p < 0.05$) than that from the 'Linetta' population (4/37).

Inoculation tests (Figure 2a and b) carried out on the leaves of selfed progeny of twelve individuals within

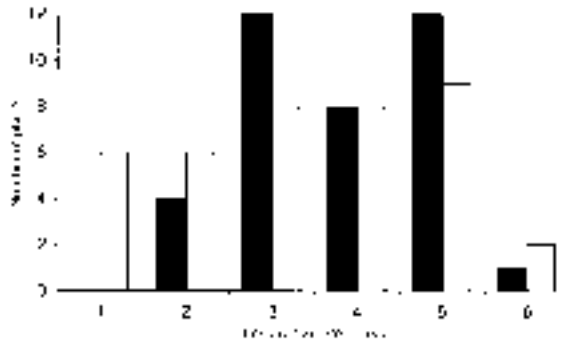

Figure 1. Frequency distribution of lesion diameter in 'Linetta' (■) and M_2 'Linetta' (□) following detached leaf inoculation with *S. sclerotiorum*. [a]Class 1: 0 – 0.4 cm; Class 2: 0.5– 0.9 cm; Class 3: 1.0 –1.9 cm; Class 4: 2.0 –2.9 cm; Class 5: 3.0 –3.9 cm; Class 6: 4.0 cm and greater.

the M_2 population selected to cover the full range of responses to *S. sclerotiorum* recorded a highly significant correlation with the corresponding parental values ($r = 0.94$, $n = 12$; $p < 0.001$) and a highly significant parent/progeny regression ($b = 0.75$, $F_{1,10} = 71.86$; $p < 0.001$) unlike the results from the parallel study on 'Linetta' ($r = 0.51$, $n = 13$; NS; $b = 0.22$, $F_{1,11} = 3.97$; NS). When selfed progeny from the six most resistant progeny lines from each of the M_2 and 'Linetta' populations were compared, two of the M_2 lines (5.1% of the original M_2 population) were significantly more resistant to *Sclerotinia* than the most resistant line selected from the 'Linetta' population (Figure 3).

When the small-population screening study was repeated on independently-selected batches of seed of M_2 ($n = 50$) and 'Linetta' ($n = 48$) (study B), the results for the M_2 population (mean lesion diameter 2.06 ± 1.21 cm; $r = 0.93$, $n = 12$; $p < 0.001$; $b = 0.83$, $F_{1,10} = 59.29$; $p < 0.001$) and the 'Linetta' population (2.31 ± 0.89 cm, $r = 0.30$, $n = 39$; NS; $b = 0.14$, $F_{1,11} = 3.21$; NS) confirmed the findings of the earlier experiment. There was no significant difference ($t = 0.94$) between the heritabilities of *Sclerotinia* response from the two M_2 populations.

The experiment was then repeated using moderate-sized populations of M_2 and 'Linetta' ($n = 600$ approximately; study C). The frequency distribution mean lesion diameter varied from 2.36 ± 0.41 cm ('Linetta') to 2.02 ± 0.58 cm (M_2). When progeny from the 12 most resistant plants from the two populations were analysed, 10 of the M_2 lines (1.7% of the original M_2 population) were significantly more resistant than the most resistant of the 'Linetta' lines (Figure 4).

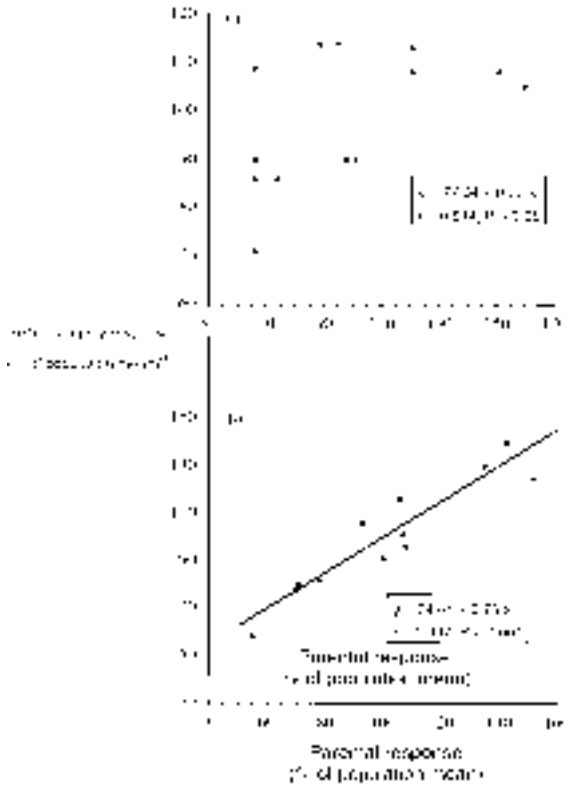

Figure 2. Parent–progeny regression for response (as lesion diameter) to artificial leaf inoculation with *S. sclerotiorum* for (a) 'Linetta' and (b) M_2 'Linetta'. [a]Because the parent and progeny plants were inoculated in different years, correction was made by expressing the individual values as percentage of the respective population mean.

Two mutants isolated from the first experiment, HH-1 and HH-13, which were significantly more and less resistant, respectively, to *S. sclerotiorum* than 'Linetta', were chosen for further study.

Responses to detached leaves of different isolates

When HH-1, HH-13 and 'Linetta' were inoculated with the Jerusalem artichoke isolate (JA 3) of *S. sclerotiorum*, the resistance rankings were identical to those obtained following inoculation with isolate OSR-1 obtained from oilseed rape plants (Table 1).

Responses to stem inoculation

When stems of the two 'Linetta' mutants and 'Linetta' were artificially inoculated with *Sclerotinia*, the results showed that the resistance responses of the three

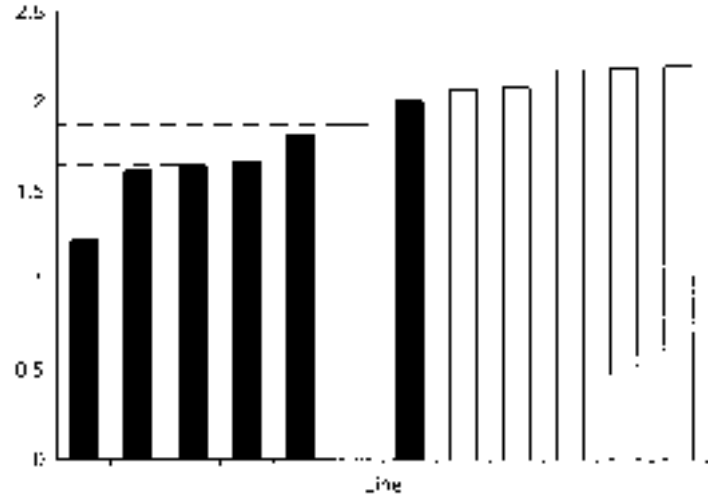

Figure 3. Comparison of the response to artificial *Sclerotinia* inoculation of selfed progeny from the six most resistant progeny lines of M_2 (■) and 'Linetta' (□) identified from the small population screening, study A (Figure 2). Dotted lines indicate LSD ($p < 0.05$).

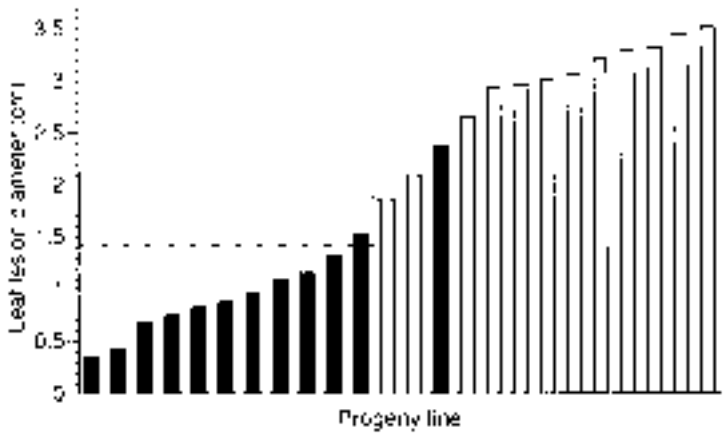

Figure 4. Comparison of the response to artifical *Sclerotinia* inoculation of selfed progeny from the 12 most resistant progeny lines of M_2 (■) and 'Linetta' (□) identified from the large population screening, study C. Dotted lines represent LSD ($p < 0.05$).

Table 1. Stem lesion sizes exhibited by 'Linetta' and mutant HH-1 following artificial inoculation with two isolates of *S. sclerotiorum*

Oilseed rape genotype	Lesion horizontal spread (cm)	
	Isolate OSR 1	Isolate JA 3
'Linetta'	1.58 b*	1.39 b
HH-1	1.12 a	0.95 a

*Any two samples with a common letter are not significantly different at the $p < 0.05$ level using Fisher's Protected Least Significant Difference Test.

lines (Figure 5a) were identical to those obtained following leaf inoculation (in order of decreasing resistance: HH-1, 'Linetta', HH-13). In a comparison of the three 'Linetta'-derived lines with three commercial European spring oilseed rape varieties, both 'Briol' (a cultivar reported to have high field resistance to *S. sclerotiorum* in Sweden; P. Gay, Semences Cargill, personal communication) and HH-1 exhibited zero plant death. 'Linetta' and 'Forte' were moderately infected while HH-13 and 'Printol' were heavily infected, with all the 'Printol' plants dying within

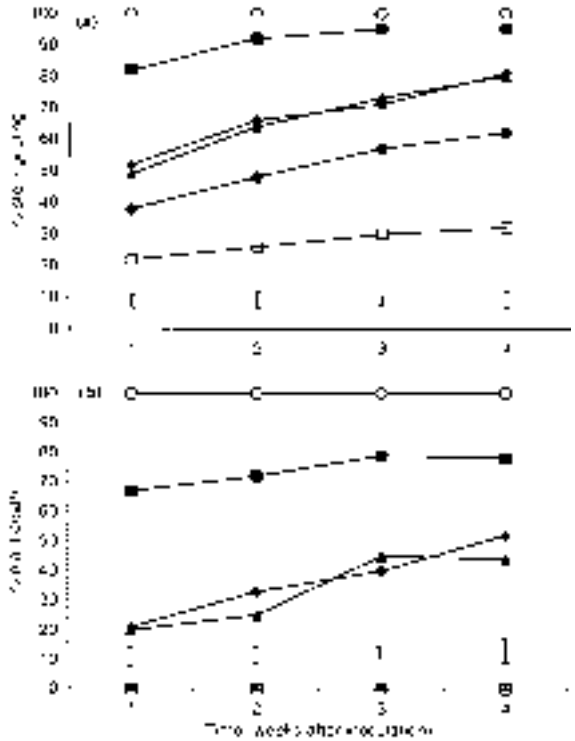

Figure 5. Response to artificial stem inoculation by *S. sclerotiorum* in terms of (a) percentage of stem circumference girdled by the lesion and (b) percentage plant death in six spring oilseed rape genotypes. Key: 'Linetta' (▲), mutants HH-1 (□) and HH-13 (■), varieties Briol (●), 'Printol' (◯) and Forte (◆). Error bars indicate LSD ($p < 0.05$) at each week.

1 week of inoculation (Figure 5b). A significant positive correlation was obtained between leaf and stem response of these six genotypes to artificial inoculation with *Sclerotinia* ($r = +0.92;\ n = 6;\ p < 0.01$).

When *Sclerotinia* response was expressed as percentage of the stem circumference affected by lesion girdling, the relative ranking of the six genotypes (Figure 5a) was the same as when they were based on percentage plant death (Figure 5b). Both 'Briol' and HH-1 developed lesions over the 4 weeks of the inoculation period with HH-1 producing significantly smaller and slower-developing lesions which stopped growing after 3 weeks (Figure 5a); the mean rate of increase (determined by regression analysis) of percentage stem girdling per week (±SD) for HH-1 was 3.8 ± 1.41%, compared to 8.8 ± 2.52% for 'Briol' ($t = 3.51;\ 6\ \mathrm{df};\ p < 0.05$).

Responses to natural infection in the field

In the first year (1993) of the field trials (in the presence of a moderate inoculum pressure), HH-1 exhibited significantly higher frequencies of plants (18.2%) which expressed no stem symptoms than 'Linetta' (0.25%) and HH-13 (0.18%) ($\chi^2(2) = 176.36,\ p < 0.001$) and significantly fewer dead plants (Table 2). Chi-squared analysis of the distribution of plants of each of the three 'Linetta' derivatives between the 'resistant' (Classes 1–3) and 'susceptible' categories (Classes 4–6) ($\chi^2(2) = 54.78,\ p < 0.001$) indicated that the differences in frequency distribution were highly significant. The resistance of HH-1 was also expressed in raceme tissue (Table 2).

In 1994, the field trials (at a site with a higher inoculum pressure, as indicated by the higher percentage plant death of 'Linetta' in the 1994 trial (47.3%) compared to the 1993 trial (22.4%)) were expanded to include the three commercial varieties previously investigated in glasshouse trials; the highly susceptible mutant HH-13 was omitted. HH-1 was confirmed to have high resistance to *Sclerotinia* (Figure 6), unlike 'Briol' which exhibited the highest frequency (more than 75%) of plant death of all the genotypes tested. Of the five populations compared, only HH-1 had plants represented in the most resistant class. When the 'Briol' data were omitted, the stem resistance ranking of the lines tested in the field was the same (in order of decreasing resistance: HH-1, Forte, Linetta, Printol) as that obtained from the glasshouse studies ($r = 0.875$).

When seed yields of 'Linetta', HH-1 and 'Starlite', the most widely grown spring oilseed rape variety in Ireland at the time, were compared in soil heavily infested with *Sclerotinia*, mutant HH-1 significantly out-yielded both the varieties (Table 3). Visual assessment indicated that 'Starlite' was more heavily infected than 'Linetta' and HH-1.

Table 2. Responses of stems and racemes of 'Linetta' and two mutant lines to natural infection by *S. sclerotiorum*

Genotype	Percentage plant death	Mean symptom class (stems)[a]	Mean symptom class (racemes)[a]
'Linetta'	23.0	3.21	4.48
HH-1	5.3	2.21	2.88
HH-13	28.7	3.44	4.83

[a]Class 1: no symptoms; Class 6: plant death.

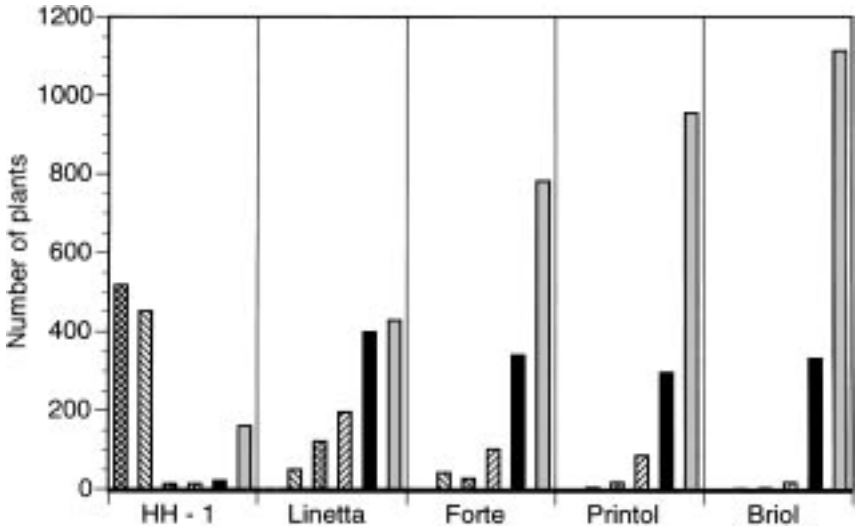

Figure 6. Frequency distribution of stem infection class in response to natural infection (high inoculum pressure) by *S. sclerotiorum* within populations of five spring oilseed rape genotypes. Key: Class 1 (▩, no lesions), Class 2 (▩, largest lesion diameter up to 1/10 stem circumference), Class 3 (▩, 1/10–1/3 stem circumference), Class 4 (▩, 1/3–1/2 stem circumference), Class 5 (■, 1/2–3/4 stem circumference), Class 6 (▩, plant death).

Table 3. Seed yields of 'Linetta', the mutant HH-1 and the commercial variety 'Starlite' in *S. sclerotiorum*-infested soil

Genotype	Seed yield ($g\,m^{-2}$)
'Linetta'	690 a*
HH-1	987 b
'Starlite'	622 a
LSD ($p < 0.05$)	241

*Any two samples with a common letter are not significantly different at the $p < 0.05$ level, using Fisher's Protected Least Significant Difference Test.

Discussion

The evidence for genetic variation for *Sclerotinia* response in the small M_2 populations (compared to the small 'Linetta' populations) is quite strong. In both experiments, the frequency distribution of *Sclerotinia* response in the M_2 population was significantly different from that in the 'Linetta' population, with the M_2 populations exhibiting greater variation (expressed as larger standard deviation values) than the corresponding 'Linetta' population. This increased variation was predominantly genetic in nature, with narrow-sense heritabilities of 0.75–0.83.

Although similar results were obtained in the two small-population studies, the high frequency with which mutant lines with increased resistance were isolated, combined with the small population sizes and the residual background variation in *Sclerotinia* response in 'Linetta', still suggested that the 'mutant' lines could actually be rare segregants within the 'Linetta' population, not identified in 'Linetta' because of the small-sized (possibly non-representative) sample which was analysed. To eliminate this possibility, the study was repeated using populations more than 10 times larger than had been used previously. The results confirmed the earlier findings, that mutants with significantly increased *Sclerotinia*-resistance could be isolated from small populations of M_2 plants.

The frequency with which mutants could be isolated with significantly greater resistance to *Sclerotinia* than the most resistant 'Linetta' line was high: 2/39 (5.1%) in experiment A, 10/593 (1.7%) in experiment C. The lower frequency obtained in experiment C reflects the fact that only 12 of more than 30 highly resistant M_2 lines were analysed in the progeny trials, due to space and labour constraints, and the fact that the population used in experiment A was small, where it is possible that an unrepresentative sample may have been taken by chance; the true frequency is presumably between these two extremes.

The mutagenesis programme used here employed the alkylating chemical EMS as mutagen; this chemical induces predominantly base substitution point mutations, reducing the risk of the high-frequency chromosome mutations associated with physical mutagens (IAEA, 1977). The appropriate mutagen dosage had previously been determined by conducting optimisation studies to reduce the risk of multiple mutations (IAEA, 1977; Yonezawa and Yamagata, 1977). The frequency of qualitative major gene macro-mutants obtained in the M_2 population were of the same order of magnitude as published values, e.g. 4.86×10^{-5} for the yellow seed trait (O'Brien, 1992) compared to 6×10^{-5} reported by George and Rao (1983). This result indicates that the higher-than-usual rates of isolation of resistant mutants in this study did not result from excessive dosages of mutagen.

The isolation of mutants exhibiting quantitative (partial) disease resistance at percentage frequencies has been reported for several plant–pathogen combinations, including barley–*Erysiphe graminis* f. sp. *hordei* (Varghese, 1985) and wheat–*Erysiphe graminis* f. sp. *tritici* (Worland and Law, 1991; Kinane and Jones, 1996). It has been reported that so-called 'micro-mutations' occur at a much higher frequency (up to percentage levels) than the 'macro-mutations' commonly reported from mutation breeding programmes (IAEA, 1977). Micro-mutations involve (in particular) minor genes which largely control the quantitative expression of phenotypic characters in an additive manner. Dozens, possibly hundreds of genes, most of which affect general metabolism rather than specifically disease resistance, may affect the expression of the oilseed rape–*Sclerotinia* interaction. Mutation in any one of these genes could result in a quantitative change in expression of the trait. By proposing the involvement of, say, 100 target genes, with a micro-mutation frequency in any one gene of the order of 1 in 10^4 or greater (IAEA, 1997), mutants exhibiting a quantitative change in gene expression could be obtained at percentage frequencies, as observed in this and similar studies.

The question remains, however, as to why was such a high frequency of mutants with increased resistance consistently (in three experiments) observed here, but in relatively few other studies? A common characteristic between this study and those of Varghese (1985), Worland and Law (1991) and Kinane and Jones (1996) was the small size of M_2 and M_3 populations screened; increased resistance of the oilseed rape mutants in this study could be due to the deletion of 'susceptibility' or 'resistance-suppressor' genes, as suggested by Worland and Law (1991). When small populations are screened, intensive evaluation of each plant is possible, with quantitative assessment of lesion size, pustule number etc. This facilitates the identification of micro-mutants with small changes in partial resistance. In the more classical mutagenesis programme, the received wisdom, that disease resistant mutants would be isolated at frequencies of 1 in 10^4 or less necessitated the screening of very large (10^4–10^7) (e.g. Harder et al., 1977; Yonezawa and Yamagata, 1977; Favret et al., 1983) M_2 populations. Consequently, evaluation of such large populations permits only cursory examination of each plant and would only identify individual M_2 plants with very high levels of quantitative resistance or with qualitative resistance, i.e. identifying only the rarer classes of resistant mutants. A mutation from susceptibility to complete resistance would usually involve a recessive-to-dominant change-of-function mutation in a major gene, the class of mutation with the lowest frequency (IAEA, 1977). Conventional-scale mutation breeding, therefore, represents a self-fulfilling prophecy: expecting a very low frequency of resistant mutants, the experimental design (principally very large M_2 population size) is inadvertently selected which will result in isolation of only those mutants which occur at a very low frequency. The screening of small M_2 populations, on the other hand, permits the isolation of the much more frequent micro-mutants, exhibiting increased partial resistance.

This hypothesis may also go some way to explaining the high frequencies of mutants isolated following adventitious regeneration (somaclonal variation), compared to mutagenesis (Daub, 1986; Jones, 1990). In these programmes, relatively few adventitious regenerants (R_0 plants) were usually obtained, resulting in rather small R_1 populations (the equivalent of the M_2 generation). This permitted intensive screening and subsequently the isolation of micro-mutants present at high frequencies. When similar-sized R_1 and M_2 populations were compared, similar frequencies of mutants were generally obtained (Gavazzi et al., 1987). It should be noted, however, that the mechanisms causing somaclonal variation frequently differ from those involved in mutagenesis (Karp, 1991), so that intrinsic mutation frequencies may vary somewhat between R_1 and M_2 populations.

Whereas oilseed rape mutants with higher (e.g. HH-1) or lower (e.g. HH-13) *Sclerotinia* resistance

could be isolated, the trend of the increased variation in the M_2 population was towards increased resistance. This is in general agreement with other intensive screening programmes, following induced mutagenesis or adventitious regeneration. The explanation for this phenomenon is, given that the 'Linetta' inbred was moderately susceptible to *Sclerotinia*, the minor genes (exhibiting additive effects) which controlled *Sclerotinia* response would be present predominantly as the susceptible alleles; consequently, most mutations would be expected to exhibit a change in phenotype from susceptible to resistant, resulting in an M_2 population with a greater average resistance than 'Linetta'.

The resistance exhibited by HH-1 proved to be greater than that of the only commercial cultivar with reported *Sclerotinia* resistance, 'Briol'. Partial disease resistance can be ineffective under high disease levels (Sweet and Pope, 1992), as appeared to happen with 'Briol' in the 1994 field trial, but not with HH-1 in the same trial. Independent field trials in France and Canada have confirmed the significant *Sclerotinia* resistance of HH-1 compared to 'Linetta' and standard rapeseed varieties (data not shown). Expression of this character by HH-1 in different environments and over more than five years (data not shown) indicated that the resistance is a stable genetic trait.

The neglect of mutation breeding in European crop improvement programmes is based, in part, on the widespread occurrence of negative phenotypic effects in otherwise desirable mutants, resulting from multiple mutations and/or pleiotropy (Dhillon et al., 1993). These effects are usually expressed as reduced yield. In the 1994 trial, however, mutant HH-1 out-yielded (under high inoculum pressure), not only the inbred parental line (derived from 'Linetta', one of the first-released double-low spring oilseed rape varieties in the 1980s) but also 'Starlite', which was the most widely-grown cultivar in Ireland in 1993; in the absence of *Sclerotinia*, the yields of 'Linetta' (906 g m^{-2}) and HH-1 (1010 g m^{-2}) were not significantly different ($p > 0.05$).

A spring oilseed rape population was used in this study because of the shorter period from sowing to growth stage 2.5, the stage at which inoculation was conducted, than with winter oilseed rape varieties, which require vernalisation, despite the fact that *Sclerotinia* stem rot is more important in winter oilseed rape than in spring varieties. For small-population mutation breeding to be of value in oilseed rape breeding programmes, it should be applicable to oilseed rape populations other than 'Linetta', including winter varieties. Inoculation studies on 100-strong M_2 and M_0 populations of winter variety 'Cobra' (M_2 mean ($\pm$SD) lesion size 1.61 ± 0.88 cm, M_0 1.90 ± 0.68 cm) and spring variety 'Mars' (M_2 2.31 ± 1.35 cm, M_0 2.69 ± 1.08 cm) produced similar results (reduced lesion size, increased variation in the M_2 generation, compared to the M_0 generation) to those obtained for 'Linetta'. These results confirm the potential value of mutation breeding employing small-population intensive screening for the introduction of novel partial disease resistance genes into crops, particularly those (like *B. napus*) with narrow gene pools. The particular problem of isolating mutants with a quantitatively altered phenotype from a genetically heterogenous parent population, such as oilseed rape, was partly overcome by several generations of inbreeding and single-seed descent to develop the parental population; a more rapid and complete method would have been to isolate a homogenous population from a single doubled haploid of 'Linetta'.

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