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Resistance Responses to *Phoma lingam* of Plants Regenerated from Selected Cell and Embryogenic Cultures of Haploid *Brassica napus* *

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Summary. Resistant plants and plants with reduced susceptibility against the pathogen *Phoma lingam* could be regenerated from selected callus and embryogenic cultures of haploid rape (*Brassica napus*) previously treated with mutagens. In the two in vitro selection systems used – absence of fungus growth on the cultures after incubation with parasite spores and resistance to the toxic filtrate – the resistance to the toxin was effective. In addition, some regenerants with increased tolerance were obtained from unselected cultures. Resistance tests on regenerated plants were carried out by inoculation of whole plants in the greenhouse, reproducing as much as possible the infection mechanisms which take place under natural conditions. Preliminary results on resistance of the progeny of single susceptible and tolerant regenerants seem to indicate that the acquired resistances are of a genetic nature.

Key words: *Phoma lingam* – Resistance in callus and embryogenic cultures – Haploid *Brassica napus*

Introduction

In addition to the efforts made in resistance breeding of introducing resistance genes from wild species into crop plants, attempts have been made in the last years to produce novel resistant plants through induced mutations. These attempts, using intact plants, are well documented in the Proceedings of two Symposia organized by FAO/IAEA in 1973 and 1977 with the topic "Induced mutations against plant diseases" (IAEA 1974, 1977). Theoretically, the use of cell culture systems instead of intact plants should drastically reduce the time required for obtaining novel resistant plants, since they permit work with large populations of haploid cells (Melchers and Bergmann 1959). In fact, since the first reported application of selective techniques in vitro for the production of resistant plants (Carlson 1973), several reports on this subject have been published (for reviews see Thomas et al. 1979; Brettel and Ingram 1979; Siegemund 1981; Chaleff 1981).

The prerequisite for an effective selection for resistance at the cell level is the expression of the resistant character of the intact plant in its cultured cells, a condition which is not satisfied by all plant-parasite systems. Moreover, the biochemical basis of the resistant reaction is mostly unknown. Knowledge of this would be very helpful in the development of a selection system in vitro. For this reason, nearly all work done on this subject refers to host-parasite systems in which toxins produced by the pathogen play a role in the disease. The criterium for resistance is then the resistance of the cultured cells against the toxin.

In this paper, results are presented of experiments, carried out with cell and embryogenic cultures of haploid rape (*Brassica napus*), with the aim to induce and select resistant plants against the pathogen *Phoma lingam*. *P. lingam*, the imperfect form of the ascomycete *Leptosphaeria maculans*, is the causal agent of the blackleg disease of Crucifers, a disease which in the last years has become an important cause for low yields in Central Europe. Although adult-stage resistance has been identified in some French breeding lines of rape, at present little is known about the heritability and specificity of the resistance responses. This resistance is considered in general as being of a quantitative type. However, the studies of McGee and Petrie (1978), Williams and Delwiche (1979) and Cargeeg and Thurling (1980) supply some evidence for host specialization in the pathogen.

* Part of these results was reported in a scientific meeting („Die höhere Pflanzenzelle: Möglichkeiten und Grenzen der Forschung") dedicated to Professor G. Melchers in the Max-Planck-Institut für Zellbiologie (Ladenburg) in January 1981

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Materials and Methods

Cell and Embryogenic Culture Lines

The cell cultures were established from vegetatively propagated plants derived from a spontaneously originated haploid plant of summer rape "Early Cauba", Zero Erucic (line H1). The callus cultures were initiated from mid-rib explants of leaves and from segments of flower stems on Murashige and Skoog (1962) agar medium (MS) containing 0.1–0.2 mg/l 2,4-dichlorophenoxyacetic acid and 3.0 mg/l kinetin, in the dark at 24 °C. Suspension cultures were started from callus which had been placed in liquid medium (same composition but without agar) on a giratory shaker (120 rpm). The cell suspensions were filtered weekly through 500–800 µm sieves and diluted 1:1 with fresh medium. The method used for regeneration of plants from callus cultures has been already described (Sacristán 1981).

In addition to cell cultures, two lines of stem embryo cultures (SE) were used for the experiments: line P10/17 originated from regerating plantlets of protoplast cultures of androgenic *B. napus,* spring line 24/72 (Thomas et al. 1976). This line was used only in the first experiments because it lost its original haploid character (it became hypodiploid). Line 79 ha was derived from androgenic embryos of anther cultures of rape (line 307, Rosenhof) and was haploid (Thomas and Wenzel 1975). The embryogenic cultures were maintained on MS-agar medium with 1% sucrose without hormones. For plant regeneration, developing shoots from the embryos were isolated and transferred to the medium of Gamborg et al. (1968) containing α-naphthylacetic acid (0.2 mg/l) for development of roots.

All plant material was originally developed by the "Projektgruppen Haploide in der Pflanzenzüchtung" in the Max-Planck-Institut für Pflanzengenetik (Ladenburg) and was kindly supplied to us by Dr. F. Hoffmann.

Chromosome Numbers

In regenerated plants, the chromosome numbers were determined in root tips after treating them with 2 mM 8-hydroxyquinoline, fixation in ethanol/acetic acid 3/1 and staining with orcein-HCl.

Mutagen Treatment

This treatment was applied to callus cell suspensions and to stem embryo cultures, which were cut into inocula of 1–2 mm and transferred to liquid medium. Either ethyl methanesulfonate (EMS) or N-methyl-N-nitro-N-nitrosoguanidine, in concentrations of 1–2% and 20–100 µg/ml, respectively, were utilized as mutagenic agents. Time of treatment (2.5 to 5 h) and mutagen concentration were adjusted to give an inactivation of at least 40%.

Phoma lingam Cultures

Dr. Schneider (Biologische Bundesanstalt Berlin-Dahlem) kindly supplied us with the pathogen (Nr. 63698) which had been isolated in 1975 form *B. napus* "Lesira" in Saarbrücken. In order to obtain pycnidia and spore production, the fungus was inoculated on oat-meal agar plates, precultured for 5–6 days in the dark at 24 °C and then transferred to near-ultraviolet illumination (Philips TL 40W/08/RS, 400 nm). Spore suspensions were obtained by flooding the surface of the culture plates with sterile water. The spore suspensions were filtered through 5 µm sieves under sterile conditions and, if necessary, adjusted to the desired concentration by centrifugation (5,000 rpm) and re-suspension of the spore sediments. These sterile spore suspensions remained active for at least two months when kept in the refrigerator.

Toxic sterile culture filtrates were obtained from fungus cultures in Czapek liquid medium (composition as given by Nienhaus 1969). Cultures three to four weeks old (stationary phase) were filtered through filter paper, then through 0.1 or 0.2 µm Millipore filters, and tested for sterility.

Tests for Pathogenicity and for Filtrate Toxicity

In order to detect a possible loss of virulence of the parasite by culturing on artificial media, a rapid test for pathogenicity was carried out periodically: seeds of the susceptible rape variety "Lesira" were surface-sterilized (0.1% $HgCl_2$ + 1 drop Tween 80, 5 min), thoroughly washed with sterile water, soaked overnight in a spore suspension containing 10^7–10^8 spores/ml, and germinated for 5 days on sterile filter paper moistened with the same suspension in sterile petri dishes. The germinated seedlings, planted into soil in the greenhouse, always showed symptoms of the disease after 5–7 days, and all plantlets died within 3 weeks.

The same method was applied to test the toxicity of the culture filtrate by using sterile filtrate instead of spore suspensions. The toxic effect became apparent immediately after germination by a drastic inhibition of root growth and damage of the root tips.

Selection Systems in vitro

Two selection systems were applied. In one series of experiments the selection criterium was the absence of visible fungus growth on cultures which had been treated with spores of the pathogen. For this, two infection procedures were used (Sacristán 1979): in the first one, small (2–3 mm) callus colonies or stem embryo inocula were shaken for 16 to 20 h in liquid nutrient medium containing pycnidiospores at concentrations of 2×10^3 to 2×10^6/ml, then washed with nutrient medium and plated on agar. The second procedure consisted of plating the cultures directly onto agar already containing a top layer with spores (at concentrations of 10^2 to 10^4/cm^2). In both cases the developing mycelium covered all or most of the cultures and extended over the agar after an incubation period of 6 days. Cultures which did not show signs of attack were selected.

The second selection system was based on the resistance or tolerance of the cultures against the toxic culture filtrate of the fungus. The selection medium was a mixture 1:1 of toxic filtrate and the normal nutrient medium for the respective plant tissue culture (for controls the tissue culture medium was mixed with pure fungus culture medium). Cultures which survived and grew further after at least two passages on medium containing toxin were selected as resistant.

Resistance Tests on Regenerated Plants and their Progeny

Regenerated plants of selected and control cultures and cuttings of them were grown in a greenhouse at 18–20 °C with additional illumination during the months October–April. The

plants were inoculated with the pathogen when they showed good growth and when the basal part of their stems had a diameter of at least 4 mm (depending on the lines, from one to three months after they had been planted into soil). For infection, each plant was superficially wounded with a lancet or needle at the basis of its stem and a cellulose tampon soaked with a fixed volumen of a spore suspension was placed over the wound and attached with a parafilm strip. The spore concentration was adjusted to give an inoculum of 2×10^6–10^7 spores/plant. The tampons were removed 7–10 days after inoculation. The wounding is not necessary for infection, but it facilitates the diagnosis of the local symptoms in the first phases. The first blackleg symptoms, blackening and/or corking of the crown region, appeared 10 to 14 days after infection, and susceptible plants collapsed 3 to 8 weeks later. Using this method an accidental escape of susceptible plants from the infection was never observed.

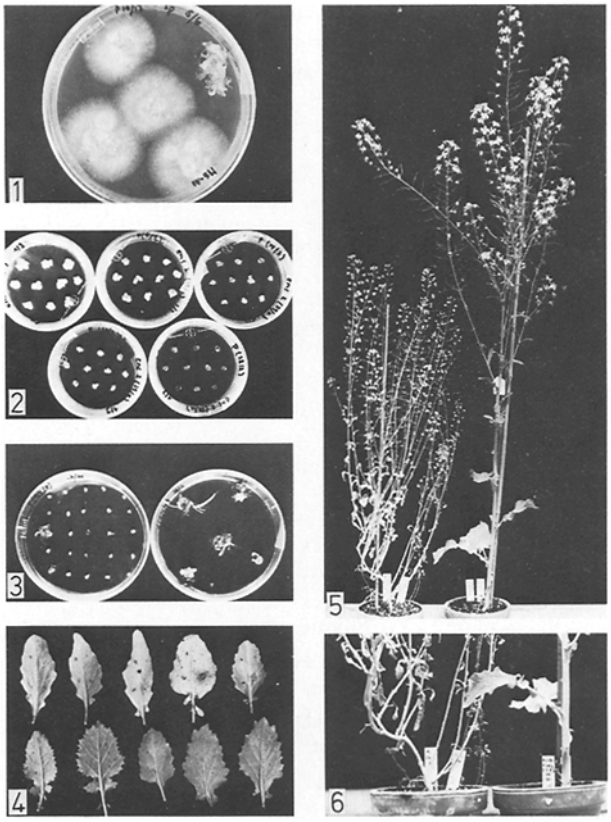
In a few cases, especially when plants showed a certain degree of resistance, an additional test on cut leaves was carried out. Excised, surface-sterilized leaves were placed on sterile moistened filter paper in petri dishes and infected by depositing 1 µl-drops of a spore suspension (10^5 spores/µl) on their surface. The severity of the lesions could be evaluated 7 days after inoculation. Moreover, this test permits the comparison in the same leaf of the effect of the infection with that of the toxic filtrate, which can be applied in the same manner to the leaf.

For the few regenerated plants which were diploid or became diploid through colchicine treatment and set seed, it was possible to test their progeny for resistance. In this case the inoculation was carried out in seedlings, in order to reproduce the infection mechanism as it takes place under natural conditions. Seven days after germination (fully expanded cotyledons, first leaf just appearing) the seedlings were inoculated in a growth chamber (30,000 lux, 16-h photoperiod, 20/16 °C, 80% rel. humidity) as follows: each cotyledon was punctured and a 2 µl-drop of inoculum containing 4×10^4 spores was placed over the wound. The lesions of the cotyledons were evaluated 10 days after inoculation, and the development of the disease was followed by rating the severity of the lesions on the crown region and the general condition of the plants up to three or four months after infection (plants at maturity).

Results

1 Selection of Cell and Embryogenic Cultures and Regeneration of Plants

If infection efficiency and growth of the parasite are factors which mainly depend on the resistant or susceptible character of the plant, one could expect that in vitro resistant cells can also be selected from susceptible ones on the basis of a substantially inferior attack by the fungus. In fact, Helgeson et al. (1976) found differences in the growth of *Phytophthora parasitica* var. 'nicotianae' between tobacco callus of resistant and susceptible plants, and we could also observe quantitative differences in the growth rate of *P. lingam* on callus of the tolerant rape variety "Jet Neuf" and that of the susceptible "Lesira" (unpublished results). The experiments in which the selection criterium was the absence of attack after treatment of the cultures with



Figs. 1–6. 1. Embryogenic cultures 8 days after they had been treated for 16 h with a *Ph. lingam* spore suspension (2×10^5 spores/ml) and then plated (exp. 5/6). One of the 5 cultures in the plate is not covered by mycelium. **2** Toxic effect of *Phoma lingam* culture filtrate from fungus cultures of different age on rape callus after 9 days culture on the respective medium. Upper row, left: control. The other plates contain filtrates from 11 day (upper, middle), 19 day (upper, right), 25 day (bottom, left) and 33 day (bottom, right) – old fungus cultures. The filtrate in the last plate with necrotizing calli corresponds to a fungus culture in stationary phase. **3** Stem embryo colonies after 3 weeks culture on toxin-medium (left) compared with controls (right). One colony grows on toxin-medium. **4** Test on excised, surface-sterilized leaves of susceptible plants regenerated from SE-cultures (upper row) and from toxin-resistant callus cultures (lower row), 7 days after inoculation. Onto each leaf two 1 µl-drops of toxin medium (left of the mid-rib) and two 1 µl-drops of spore suspension containing 10^5 spores/µl (right) were placed. Leaves of susceptible plants are already yellowed. **5** Healthy plant regenerated from a toxin-resistant cell culture of exp. 1/3-F (right) and collapsed control plant of the same line (H1), two months after inoculation with the same spore suspension. **6** Lower parts of the plants represented in Fig. 5: "corking" at base of stem and faded leaves in the control plant (left), no disease symptoms in the regenerated plant from a selected culture (right)

pathogen spores, and the plants regenerated from them, are summarized in Table 1 (also Fig. 1).

Before starting experiments on selection against the toxic filtrate of *Phoma,* it was investigated to what extent the phytotoxin produced by the fungus plays a

Table 1. Selection of cell and stem embryo colonies after treatment with *Ph. lingam* spores and regeneration of plants

Exp. no	Material	Number of cells (approx.)	Spore conc.	Selected colonies	Regenerated [a] plants	Dead in soil
15/2	callus (H1)	10^9	2×10^5/ml	3	1	1
1/3-S	callus (H1)	3×10^8	4×10^2/ml	3	–	
2/6	callus (H1)	2×10^9	2×10^5/ml	8	6	2
5/6	SE (P10/17)	not det.	2×10^5/ml	4	20	18
27/2	SE (79 ha)	10^8	1.3×10^3/ml	5	28	7
					(18)	(3)

[a] Number in brackets: plants from non-selected cultures (controls) of the respective experiment material

Table 2. Effect of *Ph. lingam* culture filtrates on seedlings of rye, wheat and red clover compared in each case with rape seedlings

Exp. no.		Medium	Number of seeds	Germination %	Root length [a] (mm)	Hypocotyl length [a] (mm)
1	Rye	Czapek	75	74.7	14.5±0.5	22.3±1.7
		Filtrate	75	69.3	11.7±0.6	19.7±2.2
	Rape	Czapek	100	93.0	28.8±1.9	18.1±0.9
		Filtrate	100	93.0	1.6±0.1	5.1±0.4
2	Wheat	Czapek	100	88.0	17.3±0.6	13.5±0.6
		Filtrate	100	84.0	14.2±0.6	12.4±0.6
	Rape	Czapek	100	87.0	12.5±0.8	5.2±0.4
		Filtrate	100	80.0	1.4±0.2	3.4±0.5
3	Clover	Czapek	100	42.0	9.7±1.3	5.6±0.8
		Filtrate	100	16.0	2.1±0.2	2.6±0.2
	Rape	Czapek	100	86.0	31.8±1.5	21.2±0.9
		Filtrate	100	89.0	2.1±0.1	6.7±0.4

[a] Measured 4 days (exp. 1 and 3) or 3 days (exp. 2) after germination

role in the disease. It could be established, (1) that the sterile filtrate induced necrotic spots on cotyledons and hypocotyls of seedlings and, also in the seedling stage, induced blackleg symptoms which were similar to those induced by the pathogen, and (2) that the tissues necrotized by the filtrate were colonized more quickly by the pathogen than intact tissues. With respect to host specificity of the toxin, the response of rape seedlings to it (see Materials and Methods) was compared with the response of non-host plants. As it can be seen in Table 2, the inhibition produced by the filtrate in rape seedlings is of a higher magnitude than that produced in rye or wheat seedlings, but comparable to the effect on red clover. Although the response of red clover could be related to the fact that this species is a host for a related species of *P. lingam* (*P. medicaginis*), we cannot ascribe a strict host-specific character to this toxin but only a relative specificity (see also Bousquet et al. 1977; Boudard 1978). However, from the drastic reaction of rape seedlings to the toxic filtrate and the type of symptoms that it produces, it could be assumed that the toxin is a co-determinant of pathogenicity, probably by conditioning the host to the invasion by the parasite. Resistance to the toxin should then also result in some resistance to the disease.

The inhibitory effect of the *Phoma* culture filtrate on callus and embryogenic cultures of rape was apparent a few days after culture on medium containing filtrate (Fig. 2). Figure 2 also visualizes the increasing accumulation of the toxic principle in the course of the fungus culture to a maximal value in the stationary phase.

The experiments in which colonies resistant against the toxic filtrate could be selected are summarized in Table 3. Although resistant colonies did not grow as well on selective as on control medium, they could be easily distinguished from sensitive cultures by the production of chlorophyll in the light and the development of roots (Fig. 3). In the experiment with unorganized callus cultures (the first one in Table 3) selected colonies survived 7 passages on selective medium. In the experiments with stem embryo cultures, their spontaneous regeneration allowed not more than two passages on selective medium.

The relative high mortality of regenerated plantlets from callus and SE-cultures of line P10/17 when

Table 3. Selection of cell and stem embryo colonies resistant to *Ph. lingam* culture filtrate, and regeneration of plants

Exp. no.	Material	Number of cells (approx.)	Selection[a] medium	Selected colonies	Regenerated[b] plants	Dead in soil
1/3-F	callus (H1)	10^8	ag	20	77 (8)	42 (4)
26/5	SE (P10/17)	10^6	ag	9	2	2
22/2	SE (79 ha)	3×10^6	ag	11	13 (33)	4 (4)
22/10	SE (79 ha)	2×10^6	l	9	19 (6)	8 (5)
7/12	SE (79 ha)	2×10^6	l	1	21 (33)	2 (2)
18/2	SE (79 ha)	2×10^6	l	2	–	–

[a] Filtrate mixed 1 : 1 with agar (ag) or liquid (l) medium
[b] Numbers in brackets: see Table 1

transferred into soil was due to a deficient development of the root system, probably caused by unbalanced chromosome complements (hypodiploid).

2 Tests for Resistance on Regenerated Plants

The inoculations were carried out in the greenhouse as described in Materials and Methods. Resistance tests were repeated at least once on cuttings of the original regenerants. The disease symptoms were observed 10–15 days after infection and then, periodically, up to four months later. They were classified according to the following rating system:
0: no visible expression of disease; 1: light, limited corking at base of stem; 2: corking and/or blackening encircling the stem basis, but general condition of the plant not yet affected; 3: profound lesion, stem basis narrowed; plant beginns to fade or/and falls down; 4: collapse of the plant.
Control plants of line H1 showed local lesions 7–10 days after infection and died 4–6 weeks thereafter. Plants which did not show visible symptoms (rated "0") three months after infection were classified as "resistant". These plants showed a strictly localized necrotic spot under the epidermis of the stem basis, probably an expression of a hypersensitive reaction. Plants considered "tolerant" were those which showed essentially lighter lesions (severity classes 1–2) in comparison with others simultaneously tested, and which survived for 3–4 months after infection. In such plants disease symptoms appeared clearly later than in susceptible plants, in virtue of a slower progress of the disease. As a comparison model for a resistant reaction that of *Brassica juncea* was taken, for a tolerant reaction that of rape "Jet Neuf". The results of the resistance tests on regenerated plants are presented in Table 4.

In addition to individual differences in susceptibility between plants of a single experiment, global differences between plants from different experiments could be observed. Thus plants from experiments 1/3-F and 22/10 showed in total a higher tolerance in comparison with regenerants of other experiments. This increased tolerance was also reflected in the leaf test (Fig. 4). From the only experiment started with cell cultures (1/3-F) two plants were regenerated which did not show any disease symptoms several months after infection – a reaction which is comparable only with that of the resistant species *B. juncea* (Figs. 5, 6). Although the proportion of tolerant plants was in general higher within the regenerants from toxin-selected cultures than within those from non-selected ones (Table 4), quantitative differences in susceptibility could also be ob-

Table 4. Responses of regenerated plants to infection with *Ph. lingam*

Exp. no.	Selection method in vitro	Number[a] inoculated plants	Susceptible	Tolerant	Resistant
2/6	spores	1	1	–	–
5/6	spores	2	2	–	–
27/2	spores	21 (15)	18 (12)	3 (3)	– –
1/3-F	filtrate	29 (4)	20 (4)	7	2
22/2	filtrate	8 (29)	6 (27)	2 (2)	–
22/10	filtrate	7 (1)	5 (1)	2	–
7/12	filtrate	19 (30)	18 (29)	1 (1)	–

[a] Numbers in brackets: see Table 1

Table 5. Chromosome numbers of plants regenerated from callus and stem embryo cultures

Exp. no.	Number of plants with the cromosome number:								
	19	19+38	34	35	36	37	38	72	74–76
15/2 (callus)							1		
1/3-F (callus)			2	1	15	7	9	1	4
2/6 (callus)		1					2		
27/2 (SE)	4	1							
22/10 (SE)	4	2							
7/12 (SE)	10	3							

served in this last group. This is probably an expression of the genetic variability of the cultures.

3 Chromosome Numbers of Regenerated Plants

Whereas the majority of plants derived from embryogenic cultures were haploid, no plant regenerated from callus cultures had retained the haploid character of the original cells, most of them being diploid (Table 5, also Sacristán 1981). The frequent hypodiploidy of these plants caused an important decrease in fertility, or even sterility. However, it was possible to obtain seed from some plants. Concerning the regenerants of stem embryo cultures, it would be necessary to diploidize them for obtaining seed. In a preliminary assay with colchicine treatment it has been possible to diploidize 3 plants and to obtain seed from them.

4 Tests for Resistance on the Progeny of Single Diploid Regenerants (preliminary results)

In order to ascertain to what extent the observed differences in susceptibility among the regenerants have a genetic basis, the progeny of a few plants of known reaction type against *Phoma* was tested for resistance. Unfortunately the two mentioned plants which gave a resistant response (Table 4) did not set seed. Since the pollen of these plants was sterile, attempts are in progress to cross them with other plants.

As described in Materials and Methods the infection was performed with seedlings. The lesions of the cotyledons were classified according to the following rating system (based on that described by Williams and Delwiche 1979):

0: no visible lesion (not included in the analysis, probable escape of infection); 1: punctiform necrosis; 2: chlorotic lesion $\leqq 2$ mm around the wound; 3: chlorotic lesion 2–5 mm; 4: chlorotic lesion > 5 mm, often irregular shape; 5: chlorotic lesion + few pycnidia; 6: chlorotic lesion + abundant pycnidia; 7: collapse of the cotyledon.

For each progeny an average disease severity value in the seedling state was calculated according to the formula:

$$\frac{(n\times 1)+(n\times 2)+\ldots+(n\times 7)}{n},$$

where n = number of lesions in the corresponding class.

The further development of the disease was controlled weekly up to 3–4 months after inoculation (plants at maturity). Plants were classified as susceptible if they died or presented clear blackleg symptoms within the first 8 weeks after infection (disease severity "2", see Results, 2.). Plants which showed only light local symptoms at maturity (class "1") were considered to be tolerant. The third group did not present visible disease symptoms at maturity; in these plants a small and sharp localized necrotic spot in the interior part of their stem basis was often visible. As material for comparison seedlings of "Lesira", "Jet Neuf" and *B. juncea* were simultaneously infected.

The results of these experiments are summarized in Table 6. They should be considered as preliminary since the number of progeny plants was small and the infection tests have not yet been repeated. But it was of interest to present them because they show a positive correlation between the susceptible or tolerant character of the parent plant and the proportion of susceptible or resistant/tolerant plants in the respective progeny. This indicates that the observed differences in susceptibility among the regenerated plants are probably of genetic nature. In the case of diploid parent plants regenerated from callus cultures (Table 6, the last four), the segregation in their progeny could be explained by assuming that the diploidization in the cell culture took place before the mutagen treatment and/or selection. But an explanation for the segregation of plants from SE-cultures (Table 6, the first two), which had been haploid as regenerants and were experimentally diploidized, is not possible with the present data.

With respect to the relation between the mean disease rating in the seedling stage and the symptoms at maturity, the results indicate that, except for an absolute resistance reaction at the seedling stage (class "1"), the seedling test does not allow the prediction of susceptibility or resistance of the mature plant.

Table 6. Resistance tests against *Phoma lingam* on the progeny of single regenerated plants

Parent plant [a]	Rection [b] to Ph. l.	Number inoculated progeny plants	Disease value in seedling stadium	Disease evaluation at maturity: number of plants			% plants tolerant and/or without symptoms
				susceptible	tolerant	without symptoms	
EMS-4 (27/2)	t	43	1.82	22	4	17	48.8
F-E1-6 (7/12)	s	38	1.81	34	–	4	10.5
F-1a (1/3-F)	s	29	3.77	28	–	1	3.4
F-1b (1/3-F)	s	39	4.05	37	2	–	5.1
F-2 (1/3-F)	t	8	3.75	2	–	6	75.0
F-19/10 (1/3-F)	t	16	4.62	9	–	7	43.8
Reference plants							
'Lesira'	s	10	3.97	10	–	–	0
'Jet Neuf'	t	10	2.94	2	7	1	80.0
Br. juncea	r	8	1.0	–	–	8	100

[a] In brackets: Exp. no. EMS-4: plant from mutagen-treated but non-selected culture. F: plants from filtrate-selected cultures
[b] s = susceptible, t = tolerant, r = absolutely resistant

Discussion

Despite the expected advantages for the selection of resistant mutants, cell culture methods have so far provided few resistant plants which could be introduced in breeding programs (for review s. Brettel and Ingram 1979). Most work on this subject has more the character of a model than of direct application. On the one hand, the resistance of the regenerated plants is often tested only on the basis of the toxin effect or, by using the pathogen, in leaf tests (Carlson 1973; Behnke 1979, 1980 a, b). On the other hand, studies of resistance in the progeny of resistant regenerants are frequently lacking. Extensive experience in producing resistant plants through selection in cell cultures was gained in the system cms-T-maize – *Helminthosporium* (= *Drechslera*) *maydis*, in which true resistances were found in regenerants and their progeny (Gegenbach et al. 1977, 1981). However, a practical application for these plants has so far not been found since they are not cms-T (see also Brettel et al. 1979; Brettel and Thomas 1980).

Each plant disease affects the plant as a whole and has a particular time course. Therefore, in our opinion, tests for resistance should implicate the whole plant by reproducing as much as possible the conditions of natural infection. This has been done in the present study concerning *Phoma* resistance in plants derived from cultured cells.

From the two in vitro selection systems, absence of fungus growth on the cultures and resistance to the toxic filtrate, only the second was effective, as evidenced from the proportion of tolerant/resistant plants among the regenerants from selected cultures as compared to those derived from non-selected ones. By using the pathogen principal difficulties arose from the growth of the fungus on the plant nutrient media and in the estimation of spore densities which permitted to detect true, not casual, differences in attack. But it is conceivable that such a system could be useful for other pathogens which cannot develop on plant culture media (Sacristán and Hoffmann 1979).

Apart from plants derived from toxin-selected cultures, a few plants regenerated from control cultures which also showed an increased tolerance to *Phoma lingam*. This means that, irrespective of the in vitro selection system, the cell culture as such can provide a genetic variation with potential applications, as has been reported for sugar cane clones with increased resistance to eyespot and Fiji disease derived from tissue cultures (Heinz et al. 1977) and for potato clones resistant to *Alternaria solani* derived from a protoplast population (Matern et al. 1978).

Embryogenic cultures have the advantage over callus cultures that they regenerate easily. However, because of their rapid growth and of the fact that in general toxic metabolites are poorly transported through the tissue, the possibility of a misjudgement as "resistant" is higher than by using callus cultures. Perhaps this is the cause for the better results obtained with callus cultures in the present study, although the possibility of selecting cell colonies composed of a mixture of resistant and sensitive cells is still at hand. Singel-cell cultures would be the ideal system for such studies.

The preliminary studies on the progeny of a few single regenerated plants which set seed seem to indicate that the resistances detected in some regenerants are of genetic nature.

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