POLLEN SELECTION IN BRASSICA SPECIES

A.B. Wills and T. Hodgkin

Scottish Crop Research Institute, Invergowrie, Dundee, DD2 5DA, U.K.

INTRODUCTION

The possibility that plant breeders might bring about desirable changes in the frequencies of genes controlling crop economic characters by artificial selection of pollen has recently become a topic of considerable interest (e.g. Ottaviano, Sari Gorla and Pe, 1982; Pfahler, 1983; Hodgkin, 1985). If this possibility is to be realised a precondition is either that the genes controlling the target characters in the sporophyte should also be active in the male gametophyte, or that selection can be made for closely linked genes. Tanksley, Zamir and Rick (1981) estimated from isozyme studies of tomato (Lycopersicon esculentum Mill.) that 60% of the genes expressed in the sporophyte were also expressed in the male gametophyte. Willing and Mascarenhas (1984) found by heterologous hybridisation of pollen cDNA and shoot poly(A)RNA of Tradescantia paludosa L. that at least 64% of pollen sequences were expressed in shoots and 60% of shoot mRNAs were expressed in pollen. They estimated that 20,000 genes were expressed in pollen compared to 30,000 in vegetative shoots. From these and other studies (see review by Ottaviano and Mulcahy, 1986) it is apparent that there is an adequate genetic basis for pollen selection in plant breeding.

There are many examples of functional genetic correlations between sporophyte and male gametophyte. In corn (Zea mays L.), pollen resistance to the pathotoxin from Helminthosporium maydis, Nisik and Miyake, race T, was correlated with responses from corn cytotypes (Laughnan and Gabay, 1973). Similarly, Smith and Moser (1985) found that the success of selection for tolerance of sugarbeet (Beta vulgaris L) to the herbicide ethofumesate could be verified by pollen germination in the presence of the herbicide. Further examples will be found in the comprehensive review by Ottaviano and Mulcahy (1986).

To date, only a few pollen selection experiments have been described and these have all involved in vivo selection. Thus, Ottaviano, Sari Gorla and Pe (1982) selected for pollen of high competitive ability in Zea mays and reported that, after 3 generations of selection, seedling dry weight and root growth were significantly improved in the resulting progeny. Similarly, Zamir, Tanksley and Jones (1982) showed that pollinations carried out at low temperatures favoured the transmission of Lycopersicon hirsutum Humb. & Bonpl. genes in the F2 and backcross from a cross between the cold tolerant L. hirsutum and the less tolerant L. esculentum.

Pollen selection is an attractive tool for the improvement of Brassica crops by plant breeding, particularly if it can be performed

on pollen grains in vitro. It is a theoretically efficient procedure (Pfahler, 1983) and pollen grains are available in large numbers (ca. 50,000 per flower in Brassica napus L., Hodgkin, unpub.). Following the development at the Scottish Crop Research Institute of a liquid medium which will reliably promote high germination rates of pollen of Brassica species (Hodgkin, 1983) the other necessary components for carrying out in vitro pollen selection in the genus have been established. These have been used to select for resistance to a phytotoxic extract from the fungus Alternaria brassicicola (Schw.) Wilts. pathogenic to oilseed and other brassicas.

MATERIALS AND METHODS

Germination of fresh and incubated Brassica pollen was assessed in hanging drop cultures using the medium of Hodgkin and Lyon (1986). Germination tests comprised at least two replicate hanging drops. Counts were made after 4 h at 20C of 300 grains per hanging drop from random fields at 60x magnification.

Incubation treatments were in shake cultures and after appropriate time periods the samples were centrifuged at 11,500 g for 2 mins, resuspended in fresh medium and recentrifuged (Hodgkin, 1987). Pollen viability after treatment was assessed by fluorescein diacetate staining. Pollinations were done by applying treated pollen with a brush to stigmas or the cut surface of decapitated pistils.

Phytotoxic extracts of A. brassicicola culture filtrate were prepared as described by MacDonald and Ingram (1986). Methods for thin layer chromatographic bioassay and use of the culture filtrate were described in Hodgkin and Lyon (1986) and Hodgkin and MacDonald (1986). For pollen selection the culture filtrate extract was partitioned three times with ethyl acetate; water soluble components were dried and redissolved in water to give the desired concentration.

For the selection experiment pollen from glasshouse grown plants of B. napus L. cv. Herkules (oilseed rape) and cv. Arran (forage rape) was incubated, as described above, for 1 h either in germination medium or in medium containing 20 mg ml^{-1} culture filtrate (water soluble fraction). It was then centrifuged, rinsed twice, recentrifuged and applied to decapitated pistils of flowers of B. napus L. cv. Primor (oilseed rape) which had been emasculated prior to anther dehiscence. Mature seed from pollinated flowers was collected and sown in batches on different dates.

Cultivars Herkules and Arran differed genotypically from cv. Primor in respect of the polyallelic acid phosphatase locus Acp-3 (Wills, Fyfe and Wiseman, 1979). The sexual origin of seeds was checked by comparing acid phosphatase isozyme profiles following polyacrylamide gel electrophoresis of seedling cotyledon extracts.

RESULTS AND DISCUSSION

1. Germination of incubated pollen and use in seed production

Fresh Brassica spp. pollen germinated readily in hanging drop culture but germination was somewhat depressed by prior incubation treatments. For example, after 10 min incubation, germination of B. alboglabra Bailey pollen decreased from 89% (control mean) to 73% although longer periods of incubation (up to 2 h) did not result in

further reduction in germination. Incubated pollen also germinated after transfer to stigmas, but there was no visual evidence of stigma penetration even with the minimum incubation period used (10 min). Pollen tubes grew over the stigma surface; those from the 1 hr incubation reached 50-100 µm length.

By contrast, incubated pollen applied to the cut surface of the style after removal of the stigma produced tubes which grew successfully through the style to the ovary. Similar numbers of pollen tubes in the decapitated style were found after application of treated (22.7) as untreated (25.1) pollen, although these were much lower than those from compatible pollinations of whole pistils (100-200). Interestingly, treated pollen could grow only through decapitated mature styles, failing to penetrate either entire or decapitated pistils in flower buds.

It is likely that failure of tube penetration on the intact stigma results from diffusion into the incubation medium of essential exine-held components, and enzymes such as acid phosphatase and esterase have been detected in brassica pollen exudates. Such components could perhaps be supplied by mentor pollen and, in earlier experiments, Wills (unpublished) investigated the use of mentor pollen to promote germination on stigmas of pollen which was air-dried at room temperature following incubation in high osmoticum medium. Small numbers (<10) of pollen tubes were found in styles when cross-compatible pollen was incubated for periods up to ca. 30 min, air-dried, and applied to stigmas 5 min after untreated incompatible pollen. No tubes were found when the pollens were applied together, or when the treated pollen was applied 30 min after incompatible pollen. Pollinations were also made using treated pollen from a plant with a dominant marker on a self-incompatible plant whose pollen was used as mentor. Following incubation periods of 0.5-30 min all pollinations set seed (av. 9.0/flower). Twenty-one progeny plants from the 0.5 min treatment showed the dominant phenotype while a further 11 plants were recessive. In the longer treatments only two of 129 plants had the dominant character. The observed pollen tube growth and production of plants carrying the dominant character might both be attributable to incomplete immersion of pollen during incubation. As the seed parent appeared to be fully incompatible when tested with untreated self pollen it seems that the combination of pollinations promoted self-pollination, but the possibility of parthenocarpic development cannot be excluded.

Seed production from pollen incubated in standard medium and applied to decapitated pistils was determined in two inbred lines of B. oleracea L. var. acephala DC. Seed set per flower by incubated pollen (1.7-2.1) and by unincubated pollen (2.5-2.9) did not differ markedly. As with pollen tube number these rates were much lower than those in conventional compatible pollinations (19.8-22.5). Despite the failure to detect pollen tubes in intact pistils after application of incubated pollen, such pollination treatments sometimes yielded a low rate of seed set (0.2-0.3 seeds/flower). Possibly, some tubes from incubated pollen did penetrate the stigma surface but were undetected in tests 20-24 hr after pollination because penetration was delayed.

2. Response of pollen to A. brassicicola culture filtrates

MacDonald and Ingram (1986) partially purified culture filtrates of A. brassicicola and prepared extracts lethal to secondary embryoids

of B. napus which they used to select secondary embryoids for resistance to these toxins.

Germination and tube growth of B. napus pollen were inhibited by the toxic culture filtrate. At 10 mg ml^{-1} of the culture filtrate in germination medium germination was reduced to 17% and tube lengths to 40 µm. At 20 mg ml^{-1} only occasional deformed tubes were detected. Hodgkin and MacDonald (1986) provided evidence that the same toxic compounds affect both secondary embryoids (sporophyte) and pollen (gametophyte). Using a thin layer bioassay technique they also showed that there were at least three toxic compounds in the culture filtrate with Rf values of 0.03, 0.30 and 0.53 on chromatography with acetonitrile.

3. Use of culture filtrate extracts in pollen selection

At 20 mg ml^{-1} equivalent concentration the Rf 0.03 component of the culture filtrate extract completely inhibited germination of pollen of cultivars Herkules and Arran. Pollen, removed from this medium, centrifuged, rinsed and transferred to fresh medium lacking the toxin also failed to germinate and was considered to be inviable.

In view of these results it was surprising that seed production on cv. Primor from pollen incubated with the water soluble fraction of the partitioned extract was 0.5 seeds/flower for cv. Herkules and 0.96 for cv. Arran (Table 2). These were only slightly lower rates than those obtained from pollen incubated in the absence of the extract (0.67 and 1.13), but were significantly lower than those from unincubated pollen. As care was taken to ensure that pollen was fully immersed during treatment it seems that toxin-treated pollen may have recovered on the decapitated style.

A significant proportion (20%) of the progeny obtained did not possess the paternal Acp3 isozyme bands. These seeds may have arisen from accidental self contamination but, as they were almost entirely present in progeny from pollinations with toxin-incubated pollen, it is more likely that they were apomictic maternals (Mackay, 1972)

Table 2. Seed production in Brassica napus cv. Primor following pollination of decapitated pistils with pollen of cvs Herkules and Appin

Pollen parent	Treatment	No. flowers pollinated	Seeds/flower
cv. Appin	incubated + toxin	163	0.50
	incubated - toxin	142	0.67
	control	129	4.29
cv. Herkules	incubated + toxin	251	0.96
	incubated - toxin	209	1.13
	control	92	2.72

CONCLUSIONS

In 1987 experiments will be completed to demonstrate whether the

procedures used in these experiments have resulted in any increased resistance of either pollen or plants to the A. brassicicola toxin. As the progeny flower their pollen will be tested for ability to germinate in the presence of the water soluble fraction of the toxic culture filtrate. Individual plant reaction to the toxin will also be investigated using a leaf disc test (MacDonald and Ingram 1986). In addition an F2 generation will be produced and tested.

Although the outcome of the selection experiment is not yet known, the results presented demonstrate the successful development of procedures which permit the potential of in vitro pollen selection to be adequately tested in brassicas. The techniques are simple and employ no expensive or sophisticated equipment other than a small bench centrifuge and an electrophoresis kit. Seed production, using the methods described above, is adequate for the type of selection envisaged; in these experiments over 300 seeds were produced from pollinations on 400 flowers by 5 h labour. It is probable that further investigations would result in improvements in seed production, either through the use of mentor pollen or through alternative stigma treatments. The failure of bud pollination and the possible production of maternals are potential disadvantages to the current procedures.

Any in vitro selection procedure involves problems with respect to the activity of normally expressed sporophytic genes in the culture system employed. Indeed, there are numerous examples of in vitro selection in which expression of the desired character in culture was not followed by expression in the regenerated progeny or was not inherited (see Chaleff,. 1981 for further discussion). In some respects pollen selection may offer advantages over other forms of in vitro selection. The genes expressed are likely to be those normally expressed in the gametophyte, significant overlap of gene expression has been demonstrated, parallel phenotypic responses can be established for particular characters and culture does not involve the development of abnormal plant states such as protoplasts or callus. Obvious candidates in Brassicas might include cold tolerance where this depends on membrane integrity, resistance to the fungus Phoma lingam which has been reported to involve the production of a phytotoxin (Sacristan, 1982), resistance to certain herbicides (see e.g. LaRossa and Falco, 1984) and resistance to antibiotics (Chaleff, 1981).

More generally the techniques described in this paper offer opportunities for the development of a number of other procedures with potential value in crop improvement. Of particular interest at present is the possibility of introducing DNA into pollen (Hess, Dressler and Konle, 1985) and the development of in vitro fertilisation techniques (Tilton and Russell, 1984).

REFERENCES

Chaleff, R.S. 1981. Genetics of Higher Plants: applications of cell culture. C.U.P., Cambridge, pp. 184 + xiii.

Hess, D., Dressler, K. and Konle, S., 1985. Gene transfer in higher plants using pollen as vectors: Bacterial transferase activity expressed in Petunia progenies. In The Experimental Manipulation of Ovule Tissues, Ed. G.P. Chapman, S.H. Mantell and R.W. Daniels, Longman, New York and London, pp. 224-239.

Hodgkin, T., 1983. A medium for germinating brassica pollen in vitro.

Cruciferae Newsletter, 8, 63.

Hodgkin, T., 1985. The potential for pollen selection in brassicas. In Proceedings of Better Brassicas '84 Conference, Ed. W.H. MacFarlane Smith and T. Hodgkin, Scottish Crop Research Institute, Dundee, pp. 51-56.

Hodgkin, T., 1987. A procedure suitable for in vitro pollen selection in Brassica oleracea. Euphytica, 36 (in press).

Hodgkin, T. and Lyon, G.D., 1986. The effect of Brassica oleracea stigma extracts on the germination of B. oleracea pollen in a thin layer chromatographic bioassay. Journal of Experimental Botany, 37, 406-411.

Hodgkin, T. and MacDonald, M.V., 1986. The effect of a phytotoxin from Alternaria brassicicola on brassica pollen. New Phytologist, 104, 631-636.

LaRossa, R.A. and Falco, S.C., 1984. Amino acid biosynthetic enzymes as targets of herbicide action. Trends in Biotechnology, 2, 158-161.

Laughnan, J.R. and Gabay, S.J., 1973. Reaction of germinating maize pollen to Helminthosporium maydis pathotoxins. Crop Science, 13, 681-684.

Macdonald, M.V. and Ingram, D.S., 1986. Towards the selection in vitro for resistance to Alternaria brassicicola in Brassica napus ssp. oleifera, (Metzg.) Sinsk., winter oilseed rape. New Phytologist, 104, 621-629.

Mackay, G.R., 1972. On the genetic status of maternals induced by pollination of B. oleracea L. with B. campestris L. Euphytica, 21, 71-77.

Ottaviano, E. and Mulcahy, D.L., 1986. Gametophytic selection as a factor of crop plant evolution. In The Origin and Domestication of Cultivated Plants. Ed., C. Barigozzi, Elsevier, Amsterdam, Oxford, New York, Tokyo, pp. 101-120.

Ottaviano, E., Sari Gorla, M. and Pe, E., 1982. Male gametophyte selection in maize. Theoretical and Applied Genetics, 63, 249-254.

Pfahler, P.L., 1983. Comparative effectiveness of pollen genotype selection in higher plants. In Pollen: Biology and Implications for Breeding. Ed., D.L. Mulcahy and E. Ottaviano, Elsevier, New York, Amsterdam, Oxford, pp. 361-366.

Sacristan, M.D., 1982. Resistance responses to Phoma lingam of plants regenerated from selected cell and embryogenic cultures of haploid Brassica napus. Theoretical and Applied Genetics, 61, 193-200.

Smith, G.A. and Moser, H.S., 1985. Sporophytic-gametophytic herbicide tolerance in sugarbeet. Theoretical and Applied Genetics, 71, 231-237.

Tanksley, S.D., Zamir, D. and Rick, C.M., 1981. Evidence for extensive overlap of sporophytic and gametophytic gene expression in tomato. Science, 213, 453-455.

Tilton, V.R. and Russell, S.H., 1984. Applications of in vitro pollination fertilization technology. BioScience, 34, 239-242.

Willing, R.P. and Mascarenhas, J.P., 1984. Analysis of the complexity and diversity of mRNAs from pollen and shoots of Tradescantia. Plant Physiology, 75, 865-868.

Wills, A.B., Fyfe, S.K. and Wiseman, E.M., 1979. Testing F1 hybrids of Brassica oleracea for sibs by seed isoenzyme analysis. Annals of Applied Biology, 91, 263-270.

Zamir, D., Tanksley, S.D. and Jones, R.A., 1982. Haploid section for low temperature tolerance of tomato pollen. Genetics, 101, 129-137.