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Pollen-stigma interactions in *Brassica oleracea*; a new pollen germination medium and its use in elucidating the mechanism of self incompatibility

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Summary. A simple medium is described which supports the germination of *Brassica* pollen in vitro. The method is completely reliable and the percentage germination achieved is comparable to that found on a compatible stigma. The pollen tubes produced attain lengths equal to those growing in the style and exhibit similar growth kinetics. The major difference between this medium and other less-reliable media is the addition of 1 mM Tris. The efficacy of Tris is shown to be due in part to the establishment of an optimum pH but an "amine effect" is also identified. The optimum pH for growth in vitro may be different from that encountered on the stigma. Atmospheric concentration of CO_2 and pollen population density are also shown to have pronounced effects on germination in vitro.

Germination in vitro is not significantly affected by prior incubation on the stigma surface. Neither cross- nor self-stigmas exert any apparent influence on pollen viability in this respect. Similarly the numbers of tube initials produced in atmospheres of 98% relative humidity (RH) are not altered by such treatments. Conversely, incubation in germination medium and in 98% RH prior to pollination appears to modify pollen-stigma interactions to some extent.

Stigma extracts added to the germination medium cause an increase in the rate of hydration of both cross- and self-pollen, but this effect is greater in the case of cross-pollen. Evidence for specific inhibition of self-pollen germination by stigma extracts added to the medium was not obtained. The relevance of these phenomena to current concepts of the mechanism of self incompatibility (SI) are discussed and a potential use of the medium in overcoming the SI response is suggested.

Key words: *Brassica* – Pollen – Pollen germination – Germination medium – Self incompatibility

Introduction

A major factor hindering investigations into pollen-stigma interactions in species with sporophytic self-incompatibility (SI) systems has been the difficulty in achieving satisfactory germination of the pollen of these species in vitro. Since the majority of species with sporophytic SI systems produce pollen that is trinucleate at anthesis (Brewbaker 1957) and possess stigmas of the "dry" type (Heslop-Harrison and Shivanna 1977), it has been suggested that the trinucleate condition may be associated with a requirement for a slow, controlled re-hydration on the stigma surface. Indeed, many trinucleate pollens have been shown to germinate successfully on a partially dried agar medium which, in its fully hydrated state, would not support germination (Kroh 1956; Bar Shalom and Mattsson 1977). The latter authors also present evidence that a "membrane", potentially capable of slowing down the flow of water to the grain, forms on the surface of the agar medium during drying. Further, Heslop-Harrison (1979, 1982) has shown that water potential relationships between the pollen and stigma play a crucial role in the reformation of the pollen membranes during grain re-hydration and thus significantly affect germination. In *Brassica* some germination has been achieved in a humid environment (Roberts et al. 1979) and Ferrari et al. (1981) have reported that tube initials are consistently produced at 98% RH. These authors interpret this as demonstrating that a definite, critical turgor pressure is required for grain germination.

Although such phenomena are of interest when considering the mechanism of SI in *Brassica oleracea* (where evidence exists that denial of stigmatic water to self-pollen is central to the operation of the SI system

[Roberts et al. 1980; Dickinson and Roberts 1983]) many other factors have to be taken into account when formulating a medium for pollen germination. For example the osmotic potential of the medium (Johri and Vasil 1961; Stanley and Linskens 1974), its pH (Kwack and Kim 1967) and the inclusion of various mineral salts, notably of calcium and boron (Brewbaker and Kwack 1963; Dickinson 1978) have all been shown to markedly affect the germination of pollen of many species. Equally, increasing the number of pollen grains per unit volume of medium (the so-called "population effect") and increasing the atmospheric concentration of CO_2, have both frequently been reported to be effective in improving germination and tube growth in vitro.

In *Brassica* reports of media supporting germination in vitro have been limited to the raffinose based media of Chiang (1974) and Dhaliwal et al. (1981), and the polyethylene glycol (PEG)-supplemented medium of Ferrari and Wallace (1975). All these have been found to have severe drawbacks, whether due to low percentage germination, poor reproducibility, or unsuitability for more than a few different genotypes. In this paper we report the discovery of a medium which reliably supports the germination of pollen of *Brassica* at levels comparable with those achieved on a compatible stigma. Using this medium we have attempted to define further the requirements of *Brassica* pollen for satisfactory germination in vitro. In addition we have tried to mimic in vitro some aspects of previous studies carried out in vivo to determine whether the rejection of self-pollen is reversible (Kroh 1966) and whether stigmatic molecules added to the medium affect the rate of hydration (Roberts et al. 1980) and prevent germination (Ferrari et al. 1981) of self- and cross-pollen. In an attempt to extend the practical applications of this work, we have also applied pollen suspended in droplets of the medium to cross and self stigmas.

Materials and methods

Seeds of *Brassica oleracea* of known S-genotype and of a variety not requiring vernalisation for flowering were kindly supplied by Dr. D. J. Ockendon, (N.V.R.S., Wellesbourne, Warwicks, UK). Plants were grown in a heated greenhouse until the first flower buds were produced whereupon they were transferred to a growth chamber and maintained at 15 °C and 70% RH for the duration of the flowering period.

Freshly dehisced anthers were collected each morning and pollen germination experiments were carried out at 20 °C either in 3.5 mls of medium in a 5 cm Sterilin Petri dish or in 250 µl aliquots of medium in the wells of a Dynatech Microelisa (R) Immunoprecipitation plate. Pollen was applied to the medium either by brushing (5 cm Petri dish) or dabbing (Microelisa plate) pollen from the anthers onto the liquid surface. Initially clumps of pollen formed but these spread out under the influence of surface tension to produce an even distribution of pollen. After varying time intervals the percentage germination was scored using a microscope fitted with an eyepiece grid. A grain was scored as germinated if it produced an entire tube greater than the grain diameter. Burst grains and tubes were scored as ungerminated. For each treatment 400–1,000 pollen grains were scored and each treatment was replicated 2 to 5 times.

Media were made up fresh each day by the addition of 3 g sucrose to a 15 ml aliquot of sterilised stock solution containing KNO_3 (0.1 g/l), $CaCl_2 \cdot 6H_2O$(0.362 g/l) and H_3BO_3 (0.01 g/l). This was followed by the addition of various amounts of sodium hydroxide' Tris, or methylamine (all supplied by Sigma, UK) and the pH of each solution was recorded. The addition of 3 g of sucrose and 1–2 mg of Tris to 15 ml aliquots of the stock solution was found to give the best germination and was used in all subsequent experiments.

CO_2 experiments

A saturated solution of sodium bicarbonate was pipetted into a small plastic container which was then placed in a 5 cm Petri dish containing germination medium such that the two solutions were kept separate. The lid was replaced and the Petri dish was left to equilibrate overnight, thus generating a high CO_2 concentration. Pellets of KOH were added in a similar fashion to another Petri dish and left to equilibrate, thus scavenging CO_2 and producing a low CO_2 concentration.

Rate of hydration experiments

Forty stigmas were homogenised in 0.1 ml of germination medium and the resultant homogenate was centrifuged for 2 min in a Beckman Airfuge. Grains were applied singly to 25 µl of the supernatant on a microscope slide and a stopwatch was started at the moment of contact. Each grain was observed under the microscope and when full rehydration was achieved (marked by a change in shape from elliptic to spheroidal and in colour from gloss to matt yellow) the watch was stopped and the time taken to rehydrate recorded. Fifty grains were thus observed for each treatment. "Cross" and "self" pollen were scored in the presence and absence of stigma extracts.

Effect of stigma extracts on % germination experiments

These experiments were carried out largely as described in Ferrari and Wallace (1975). Stigmas were immersed in germination medium (2 stigmas per 10 µl) for 30 min with occasional shaking. The resulting eluate was then added to fresh germinating medium (1 : 1, v : v) on which pollen had been left for 1–2 min. "Cross" and "self" eluates were thus applied. Controls were carried out in which no eluate was added and in which eluate was present from the beginning.

Pollen transfer experiments

Fresh pollen was applied to stigmas on the plant which were then brushed such that a monolayer of pollen remained on the stigma surface. Cross- and self-pollinations were carried out and left for either 1 to 2 h. After these times the stigma was excised and applied to the surface of germination medium in the well of a Microelisa plate, thus transferring pollen grains from the stigma to the medium. Percentage germination for each treatment was scored after 4 h and 24 h.

Similarly pollen grains were transferred from cross- and self-stigmas to microscope slides in a Petri dish equilibrated at 98% RH with $K_2Cr_2O_7$ (Ferrari et al. 1981) and left overnight, after which time the number of the initials produced for each treatment was scored. In the converse experiments pollen was incubated for varying time intervals in germination medium and in 98% RH and then transferred to cross- and self-stigmas and left overnight. Stigmas were squashed in aniline blue and observed as described previously (Roberts et al. 1979).

pH of pollen and stigma eluates

Excised stigmata were immersed in distilled water and in 20% sucrose solutions adjusted to varying pH with 1 mM citrate buffer (approx. 20 stigmas/0.2 ml). The pH was monitored for 2 h at 20 °C and any change was recorded. Control solutions at identical pH's were also monitored over this period. Similarly pollen was added to the above solutions (approx. 2 mg/0.2 ml) and any pH change recorded. The change in the pH of germination medium caused by the addition of pollen was also measured.

Results

The nature of pollen germination and tube growth in vitro

The percentage germination achieved in vitro using the Tris medium was comparable to that found on a compatible stigma which, although very variable, is usually in the range 70%–100%. Perfectly formed tubes were produced and normal cytoplasmic streaming could be observed. The rate of growth was also similar to that found during the first stages of growth in vivo (i.e. some 30–50 μm h^{-1}).

The effect of pH on pollen germination in vitro

Adjusting the pH of germination medium with sodium hydroxide was found to affect the percentage germination to some extent but Tris was found to be far more effective in stimulating germination at all pH's (Fig. 1). Indeed at pH 8–9 a dramatic increase from 20% to 80% germination was achieved. Hepes and phosphate buffer systems were also tested in the medium and found ineffective; Hepes buffer supported around 20% germination but no germination was achieved in the phosphate buffered medium. Occasionally media adjusted with NaOH to pH 8 supported up to 50% germination but this was always surpassed by at least 40% by Tris-supplemented medium at the same pH.

The effects of Tris, methylamine, CO_2 and pollen population on germination in vitro

The effects of Tris and methylamine, grain population, and atmospheric CO_2 are presented in Figs. 2, 3, and 4 respectively. Figure 2 shows that the stimulating effect of Tris when added to the standard medium is greatest

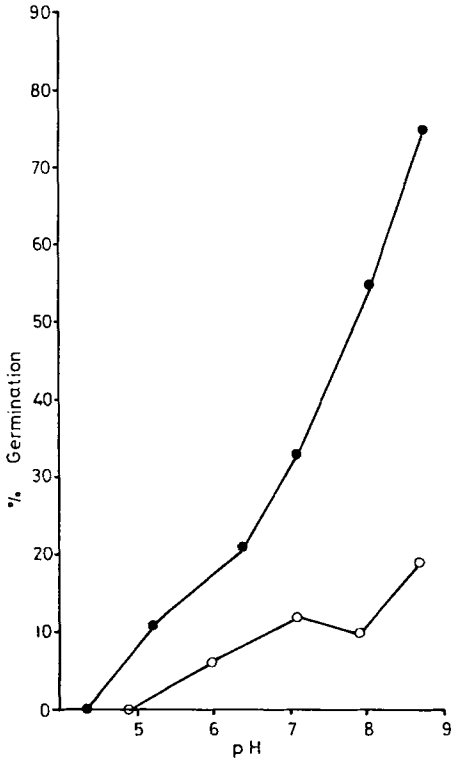

Fig. 1. The effect on percentage germination of modifying the pH of the medium using Tris (● —●) and NaOH (○ —○). Between 400 and 600 grains were scored for each determination

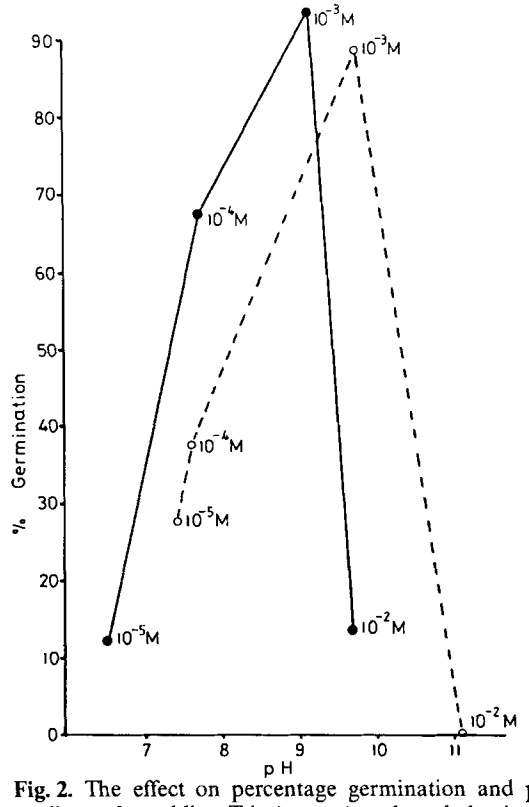

Fig. 2. The effect on percentage germination and pH of the medium after adding Tris (● —●) and methylamine (○ ---○) at different molarities. Between 400 and 600 grains were scored for each treatment

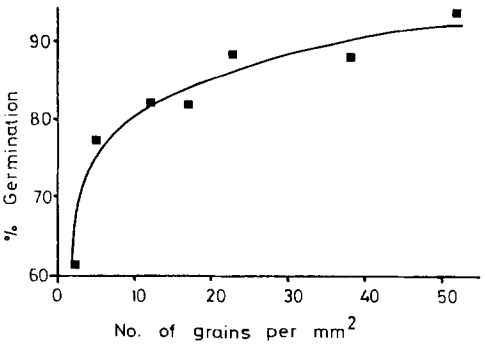

Fig. 3. The effect on percentage germination of adding increasingly larger populations of grains to the same amount of germination medium. Between 400 and 600 grains were scored for each determination

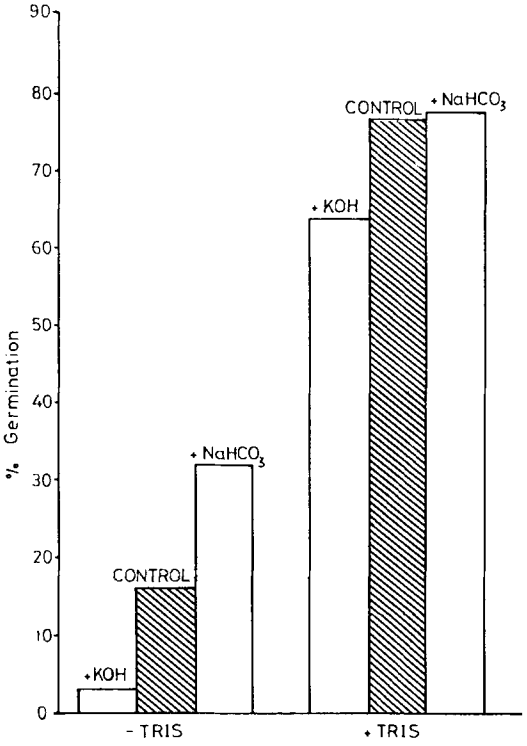

Fig. 4. The effect on percentage germination of increasing (+$NaHCO_3$) and decreasing (+KOH) the atmospheric concentration of CO_2 over germination medium with (+Tris) or without (– Tris) the addition of Tris. (Control = pollen grown in the normal way with no atmospheric modification.) Between 400 and 600 grains were scored for each determination

at 10^{-4} to 10^{-3} M and that the addition of methylamine to the medium produces a similar pattern of response. The number of grains per unit area also has significant effects on the percentage germination, an increase from 60% to 90% germination is achieved by increasing grain density from 2 to 50 grains/mm² (Fig. 3). Although elevated atmospheric concentration of CO_2 causes a two-fold increase in percentage germination in the absence of Tris, no stimulatory effect was obtained in the presence of Tris. Reducing the atmospheric concentration of CO_2 effects a reduction in percentage germination of some 15% both in the presence and absence of Tris (Fig. 4).

The effects of stigma extracts on hydration and germination in vitro

The effects of stigma extracts on the rate of hydration of pollen in vitro are presented in Fig. 5. The addition of stigma extracts was found to result in very variable percentage germination. No specific inhibition of self pollen was detected in such experiments independent of whether stigma extracts were added after 1–2 min or present from the beginning of the experiment. When the concentration of stigma extracts reached that used in the rate of hydration experiments both cross- and self-pollen were prevented from germinating.

Pollen transfer experiments

Incubation on the stigma surface for 1 or 2 h was found to have no statistically significant effect on subsequent germination in vitro, independent of the compatibility of the stigma on which incubation was carried out. Similarly when pollen was transferred from cross- and self-stigmas to atmospheres of 98% RH the numbers of tube initials produced (40% ± 5%) were not different from those produced by control pollen incubated on cover slips instead of stigmas prior to transfer.

Incubation of pollen in germination medium and in 98% RH prior to transfer to cross- and self-stigmas gave

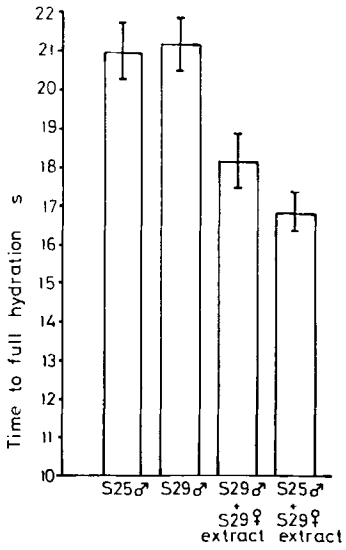

Fig. 5. The effect of addition of S29 stigma molecules to the germination medium on the time taken by cross (S25) and self (S29) pollen grains to rehydrate on the medium. Fifty grains were scored for each determination. Bars represent standard errors

Table 1. The effect on pH of adding stigmata and pollen grains to lightly buffered solutions

Solution	Initial pH	pH after 2h		Change in pH relative to control
		+ Stigmata	Control	
1 mM Citrate Buffer	5.5	5.68	5.57	+0.11
	6.0	5.96	6.02	−0.06
	6.5	6.17	6.54	−0.37
1 mM Citrate Buffer + 20% Sucrose	5.5	5.54	5.49	+0.05
	6.0	5.81	5.93	−0.12
	6.5	6.06	6.55	−0.49
		+ Pollen	Control	
1 mM Citrate Buffer	5.5	6.01	5.57	+0.44
	6.0	6.31	6.02	+0.29
	6.5	6.43	6.54	−0.11
1 mM Citrate Buffer + 20% Sucrose	5.5	5.91	5.49	+0.42
	6.0	6.12	5.93	+0.19
	6.5	6.30	6.55	−0.25

very variable results. The germination of compatible pollen was considerably reduced in most instances whereas in some cases the germination of self-pollen increased. Occasionally self-pollen tubes were observed to penetrate the stigma indicating that the SI response may have been by-passed.

The pH of pollen and stigma eluates

The results from these experiments (Table 1) indicate that the pH of solutions into which substances from the stigma have diffused is between pH 5.5 and pH 6.0. The pH of the pollen eluates appears to be in the region pH 6.0–pH 6.5. Significantly pollen applied in large quantities to germination medium caused a change in pH from pH 7.78 to pH 6.48 thus confirming the acidic nature of pollen eluates.

Discussion

The nature of germination and tube growth on the medium

Although good percentage germination and pollen tube growth were consistently achieved, the artificial nature of the germination medium must be stressed and great caution must be exercised in interpreting these results in terms of events occurring in vivo. Certainly the kinetics of germination, tube growth and the observed cytoplasmic streaming suggest that the mechanism of growth is comparable to that found on the stigma. Equally the "population effect" and the effect of raised atmospheric CO_2 indicate that the grain metabolism is also similar to that of grains germinating on the stigma. However many additional and different factors must be operating in vitro. Most significant perhaps is the fact that the pH optimum in vitro does not appear to correspond to the pH that is likely to be encountered on the stigma surface. Unfortunately it proved impossible directly to measure the surface pH on the "dry" stigma of *Brassica* and it remains possible that the indirect method employed using excised stigmata may be inaccurate. Nevertheless, since similar pH changes occur in 20% sucrose it appears that this is a true measure of the pH of surface eluates and is not due to cells bursting, although leaching of solutes normally retained in vivo cannot be discounted. It is also possible that pH only becomes significant when the grain is germinating on a liquid medium, pH optima of around pH 8 have also been found in studies of other pollens germinating in vitro (Kwack and Kim 1967). The other major difference detected between germination in vitro and germination in vivo was in the rate of hydration, as measured by the time taken for the grain to take up sufficient water to become spherical. This lasted only some 20 s on the liquid germination medium but in excess of 1 h on the stigma surface (Roberts et al. 1980). Solidification of the medium with agar followed by partial drying may shed some light on this problem (Bar Shalom and Mattsson 1977).

The amine effect

The addition of various sugars and salts is common practice in studies of pollen germination in vitro, and although some sugars may be taken up and utilised in tube wall synthesis (Hrabětova and Tupý 1961; Kroh and Van Bakel 1973), these

substances are not likely to seriously disrupt normal grain metabolism. Indeed the provision of calcium and boron and certain other nutrients is frequently essential for germination both in vitro and in vivo (Johri and Vasil 1961; Brewbaker and Kwack 1963; Stanley and Linskens 1974). On the other hand Tris cannot be regarded as a substance occurring naturally on stigma surfaces and its efficacy in increasing percent germination, and its apparent similarity to the effects of methylamine, indicate a fairly potent effect on grain metabolism which has yet to be fully investigated. It should be noted here that polyamines have recently stimulated much interest as potential growth regulators (e.g. Dai et al. 1982) and have been shown to be synthesised by pollen tubes growing in vitro (Speranza and Calzoni 1980). Exogenous polyamines have significant effects on the germination of pollen in vitro (Speranza et al. 1982). However, it must be remembered that the initial phase of tube growth in an atmosphere of 98% RH consistently takes place and very occasionally longer tubes may form without any provision of more complex media (Ferrari et al. 1981). Our present view is thus that exogenous amines and polyamines are unlikely to play significant roles in recognition reactions in vivo, at least during the early stages of the SI response which are undoubtedly the most important in terms of self-recognition and rejection events (Dickinson and Roberts 1983). We would prefer to interpret the observed "amine effect" as an artificial stimulation of grain metabolism in a liquid environment, not required in the natural situation and certainly not as simulating an in vivo recognition or "trigger" event inducing a second phase of tube growth not normally achieved in vitro.

The CO_2 and population effects

The stimulatory effect of CO_2 in the absence of Tris is interesting as this treatment is also effective in overcoming SI (Nakanishii and Hinata 1973). The fact that CO_2 is not stimulatory in the presence of Tris may indicate that both Tris and CO_2 affect a similar aspect of grain metabolism. However, the maximum percent germination may have already been achieved in the presence of Tris and thus any positive effect of CO_2 was not revealed. The stimultory effect of CO_2 was also found in another study of *Brassica* pollen germination in vitro (Dhaliwal et al. 1981) and these authors suggest that enhanced malate synthesis in the presence of CO_2 balances the uptake of K^+ resulting in increased turgor pressure. Since elevated relative humidity may also overcome SI in vivo (Carter and McNeilly 1975, 1976) these results provide further evidence that the denial of stigmatic water to the self-pollen grain, and consequent failure to achieve "correct" turgor, is central to the mode of operation of SI.

The "population effect" or "mutual growth stimulation" (Stanley and Linskens 1974) has been found in virtually all studies but has yet to be satisfactorily explained.

Expression of SI in vitro

Much progress has recently been made in attempts to examine the SI response in vitro. Extracts of pistils when added to the medium have been shown to specifically inhibit the growth of incompatible pollen in a number of studies (Golynskaya et al. 1976; Shivanna et al. 1981; Dickinson et al. 1982). In all cases where the factors responsible have been investigated glycoprotein molecules have been implicated in the response. In *Brassica* such molecules have been isolated and shown to specifically inhibit self-pollen (Ferrari et al. 1981). Whereas S-specific stigmatic glycoprotein molecules have been isolated and shown to specifically inhibit self-pollen (Ferrari et al. 1981). Whereas S-specific stigmatic glycoprotein molecules are now definitely established as stigmatic receptors involved in SI reactions (Nishio and Hinata 1982, Hinata et al. 1982; Dickinson and Roberts 1983) their mode of action remains unclear, largely because studies such as are described above have failed to identify the recognition molecule in the pollen. The results presented here indicate that stigma molecules may have an effect on grain hydration, as crude extracts appear to be capable of affecting the rate of hydration in vitro. These results must be treated with some caution as the time scales for rehydration in the medium are vastly different from those on the stigma (20 s as compared to 1h+). Also, continued germination is not found in such concentrated stigma extracts. It is interesting however that small differences between cross- and self-pollen over a 20 s rehydration period have been detected on the stigma of *Secale cereale* (Heslop-Harrison 1978). How such differences might be related to continued germination is unclear; we have been unable to show any consistent effects of stigma extracts on the germination and the growth of cross- and self- pollen in vitro. Thus it appears that expression of SI does not occur in our medium in the way that it does in the, admittedly, very different PEG supplemented medium of Ferrari and Wallace (1975). The inability of stigma extracts to effect SI in a medium providing a plentiful supply of water and optimal growth conditions would be predicted by our model (Dickinson and Roberts 1983).

Pollen transfer experiments

Transferring pollen from stigmas, whether cross or self, to germination medium or 98% RH after varying periods of incubation on the stigma did not significantly affect the percent germination or number of tube initials produced relative to control pollen. In similar experiments Kroh (1966) found that transfer of pollen from a self-stigma to a cross-stigma did not prevent normal germination. Thus we must conclude that inhibition of self-pollen on the stigma is readily reversible and is not effected by toxic molecules. Alternatively, it may be argued that the inhibiting factors are readily metabolised and the grain recovers rapidly

when removed from their influence. However evidence of stigmatic factors entering the grain has yet to be obtained in the first critical hours of pollination (Roberts, Harrod and Dickinson, in prep.) and the simplest hypothesis remains that self-pollen is rejected initially by a simple denial of stigmatic water, although the presence of "back-up systems" cannot be discounted (Dickinson and Roberts 1983). A more complex model might involve self-recognition by the stigmatic glycoprotein resulting in the release of low MW complex carbohydrates or amines at very low concentration from the papillar wall which, in turn, could regulate pollen metabolism and prevent turgor being achieved.

Curiously the experiments involving transfer of pollen from germination medium and 98% RH to stigmas of differing compatibility gave very variable results. In many cases pollen simply failed to continue germinating while, on other occasions, continued growth of cross- and self-pollen was observed and rarely self-tubes were seen to penetrate the papillae. It is therefore possible that modifications of these techniques may provide a simple, inexpensive technique for the plant breeder interested in overcoming the SI response.

Conclusion

Despite the many difficulties associated with relating in vitro germination responses to events occurring in vivo, it is clear that many of the results presented above do have a direct bearing on the mechanism of SI in *Brassica,* not least in that they suggest an inexpensive was in which the plant breeder may overcome the response. Although purified stigmatic glycoproteins would be required for a convincing demonstration of whether SI may be expressed in vitro our hypothesis that regulation of water flow to the grain is central to the mechanism of SI remains valid. It is also apparent from the transfer experiments that the grain is not irreversibly inhibited by self-recognition. Thus we may conclude that successful germination in vitro and expression of SI in vivo both hinge on regulating the supply of water to the grain whether by metabolic regulators or by molecular rearrangements acting as barriers to water flow. A major step in testing such an hypothesis would be the identification of the pollen receptor.

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Note added in proof

While this work was in press Hong-Qi Z and Croes AF (Acta Bot Neerl 31:113–119, 1982) demonstrated that (PEG)-400 is superior to sucrose as the major osmoticum in media for the germination of *Petunia hybrida* pollen. We have found this also to be true for the *Brassica* medium. However, we have still been unable to show any S-specific effects of stigma extracts in media in which (PEG)-400 has been substituted for sucrose.