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# Flavanoids in Pollen and Stigma of *Brassica oleracea* and Their Effects on Pollen Germination *In Vitro*

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## ABSTRACT

*Brassica oleracea* pollen was applied to a basic medium of 1·5 per cent agar and 15 per cent sucrose to which flavanoids were added at three concentrations. Two types of agar were used; with agar 1, quercetin at a concentration of $0{\cdot}5 \times 10^{-3}$ per cent gave an increase in percentage germinating grains. With agar 2, an increase in germination occurred with kaempferol and naringin at concentrations of $0{\cdot}5 \times 10^{-3}$ and $0{\cdot}5 \times 10^{-4}$ per cent respectively. Increase in pollen tube length occurred with agar 2 and quercetin at a concentration of $0{\cdot}5 \times 10^{-3}$ per cent.

The stigma tissue of *B. oleracea* contains at least three and the pollen at least one glycoside of quercetin. The sugars in the glycosides were not identified. Pollen germination and pollen tube extension were not stimulated exclusively by the flavanoids present in the stigma. The flavanoid composition of the stigma did not vary amongst five different S-allele genotypes, indicating that flavanoids are probably not directly involved in the incompatibility reaction of *B. oleracea*.

## INTRODUCTION

Sisa (1930, 1933) obtained germination of *Brassica* pollen on a sucrose/gelatin medium adjusted to pH 7·5 with sodium acetate, and Schwanitz (1942) found that boron added to the medium improved the germination of *Brassica* pollen as for many other genera. Using a 5 per cent gelatin medium containing 0·6 M sucrose, Loo and Hwang (1944) obtained 23–29 per cent germination of *Brassica juncea* pollen, with the production of pollen tubes 12–14 $\mu$m long. Addition of manganese sulphate, colchicine and indol-3yl-acetic acid to this medium each increased germination and tube extension, although the last two treatments produced abnormal pollen tubes. Minaeva and Gorbaleva (1968) found that some flavanoids had a stimulatory effect on *in vitro* germination and tube extension of pollen of some Umbelliferae when added to the basic medium of 20 per cent sucrose. Quercetin, isorhamnetin, rutin and isoquercetin each increased germination and tube length.

Martin (1969, 1970*a*, *b*, *c*) and Martin and Telek (1971) have found that the principal components of stigmatic exudates and secretions are phenolic compounds and lipids. Martin (1969) tested ten species from different families by paper chromatography and noted that the phenolic compounds occurred as glycosides or esters and that the greater part of the sugar in the exudates was tied up in phenolic compounds. He suggested that these compounds in the stigma may 'interact with growth substances to control pollen germination and growth', and 'account for the specificity of stigmas which permit only a few types of pollen to germinate on their surfaces'.

Work described in this paper extends Martin's findings and reports the effect of some

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flavanoids on the germination of *B. oleracea* pollen and compares this with the flavanoid composition of the stigma and pollen.

## MATERIALS AND METHODS

All the pollen germination tests and most of the paper chromatography involved a marrow stem kale plant homozygous for the $S_{16}$-incompatibility allele (Thompson and Taylor, 1966) and the selfed progeny of this plant. Other chromatography tests involved the following genotypes: an $S_{23}S_{23}$ kale, an $S_{45}S_2$ Brussels sprout, an $S_{16}S_2$ kale/Brussels sprout hybrid and an $S_{16}S_{23}$ kale.

Two sorts of agar were used: Oxoid Ionagar No. 2 (agar 1) and Oxoid agar No. 3 (agar 2). The 15 per cent sucrose/1·5 per cent agar medium was poured on to 3 × 1·5 in (7·6 × 3·8 cm) glass microscope slides to a depth of about 3 mm. The medium incorporating agar 1 was called medium 1, that with agar 2 was medium 2. Pollen, collected in a watch glass by tapping the anthers of a flower, settled in aggregates which were broken up with a paint brush, and the pollen was applied to the agar by light strokes with the brush. Each slide was placed in a separate Petri dish lined with filter paper moistened with 0·5 ml distilled water and left in the dark at a constant temperature of 24 °C. After 16 h, photographs were taken through a stereomicroscope of three fields of view of each slide. The number of germinated grains and the lengths of the tubes were measured from the photographs. Regression analysis and one-way analysis of variance of percentage germination and pollen tube length were calculated.

The effect of pH was assessed by adjusting with dilute NaOH or HCl. The basic sucrose/agar medium was compared with one including boric acid and salts as described by Brewbaker and Kwack (1964): 0·1 per cent boric acid, 0·03 per cent calcium nitrate, 0·02 per cent magnesium sulphate and 0·01 per cent potassium nitrate.

The flavanoids incorporated into the basic medium at concentration of $0{\cdot}5 \times 10^{-1}$, $0{\cdot}5 \times 10^{-2}$ and $0{\cdot}5 \times 10^{-3}$ per cent were quercetin, kaempferol, naringenin, rutin and naringin. Quercetin and kaempferol are flavonols and rutin is a flavonol glycoside (quercetin-3-rutinoside); naringenin is a flavanone and naringin a flavanone glycoside (naringenin-7-neohesperidoside). The aglycones were added directly to the basic medium as they will withstand boiling, the less stable glycosides were dissolved in water at 50–55 °C and incorporated into the medium after the agar had been dissolved and then cooled to 55 °C. The pH of all media was measured.

Ascending paper chromatography of stigma and pollen extracts was carried out according to the methods of Martin (1969). The extracts were prepared from 100 excised stigmas or pollen from 100 flowers by extracting for 20 min with 0·4 ml of 95 per cent ethanol with 1 per cent concentrated HCl. Control solutions of the same flavanoids as used in the pollen germination tests were prepared by dissolving 1 mg in 0·5 ml of the above solvent. Whatman 3MM paper was used with *n*-butanol-acetic acid-water (B.A.W.) in the proportion of 4:1:5 as a solvent. Volumes of 0·03 ml of extract or control were applied to the paper in both hydrolysed and unhydrolysed form. Acid hydrolysis, which permits identification of the aglycone present in a glycoside, was achieved by boiling for 30 min with an equal volume of 2 N HCl. The hydrolysed flavanoids were extracted with 0·1 ml of amyl alcohol and this was spotted onto the chromatogram. The extracts were concentrated and the spots and paper dried in a stream of warm air. After the solvent front had travelled 15 cm, the chromatogram was examined under visible and ultraviolet (u.v.) light of 350 nm, fumed with ammonia vapour and re-examined in a similar manner. Other treatments applied to the chromatogram were dipping in 5 per cent ethanolic aluminium chloride, in 1 per cent aqueous ferric chloride and 1 per cent aqueous potassium ferricyanide and in 1 per cent sodium borohydride dissolved in isopropanol, followed by fuming with concentrated HCl vapour.

## RESULTS

### *Pollen germination*

Medium 1 had a pH of 7·6 and no significant difference in percentage germination or pollen tube length occurred over the pH range 6–9. With boric acid and salts added to the medium 16–19 per cent germination occurred with pollen tube length of 10–13·5 times the diameter of the pollen grain. Quercetin at $0{\cdot}5 \times 10^{-3}$ per cent stimulated germination but not pollen tube extension (Table 1); naringenin gave no stimulation at any concentration used.

Medium 2 also had a pH of 7·6, and when quercetin was incorporated at $0{\cdot}5 \times 10^{-3}$ per cent, there was a significant increase in pollen tube length, but no increase in percentage germination. Kaempferol at $0{\cdot}5 \times 10^{-3}$ per cent and naringin at $0{\cdot}5 \times 10^{-1}$ per cent both increased germination, but naringenin and rutin gave no stimulation at any concentration used (Table 2). All media incorporating flavanoids had a pH of 7·2–7·6 except those with the highest concentration of flavanoid which had a pH of 6·7–7·1.

TABLE 1. *Effects on pollen germination and tube extension of supplementing medium 1 with flavanoids*

| Flavanoid | Average germination (%) | | | Average pollen tube length (× diameter of pollen grain) | | |
|---|---|---|---|---|---|---|
| | $0{\cdot}5 \times 10^{-3}$ | $0{\cdot}5 \times 10^{-2}$ | $0{\cdot}5 \times 10^{-1}$ | $0{\cdot}5 \times 10^{-3}$ | $0{\cdot}5 \times 10^{-2}$ | $0{\cdot}5 \times 10^{-1}$ |
| None | | 12·5 | | | 6·0 | |
| Quercetin | 21<br>Stimulation<br>($P = 0{\cdot}001$) | 11<br>n.s. | 0 | 5·8<br>n.s. | 5·2<br>Inhibition<br>($P = 0{\cdot}05$) | 0 |
| Naringenin | 13·4<br>n.s. | 0 | 0 | 5·8<br>n.s. | 0 | 0 |

n.s. = Not significantly different from the control; $P$ = probability.

TABLE 2. *Effects on pollen germination and tube extension of supplementing medium 2 with flavanoids*

| Flavanoid | Average germination (%) | | | Average pollen tube length (× the diameter of the pollen grain) | | |
|---|---|---|---|---|---|---|
| | $0{\cdot}5 \times 10^{-3}$ | $0{\cdot}5 \times 10^{-2}$ | $0{\cdot}5 \times 10^{-1}$ | $0{\cdot}5 \times 10^{-3}$ | $0{\cdot}5 \times 10^{-2}$ | $0{\cdot}5 \times 10^{-1}$ |
| None | | 14·5 | | | 4·3 | |
| Quercetin | 15·2<br>n.s. | 8·0<br>Inhibition<br>($P = 0{\cdot}001$) | 0·9<br>Inhibition<br>($P = 0{\cdot}001$) | 5·7<br>Stimulation<br>($P = 0{\cdot}001$) | 4·0<br>n.s. | 1·9<br>Inhibition<br>($P = 0{\cdot}01$) |
| Naringenin | 15·4<br>n.s. | 0·5<br>Inhibition<br>($P = 0{\cdot}001$) | 0·6<br>Inhibition<br>($P = 0{\cdot}001$) | 4·3<br>n.s. | 2·2<br>Inhibition<br>($P = 0{\cdot}05$) | 1·6<br>Inhibition<br>($P = 0{\cdot}001$) |
| Kaempferol | 19·2<br>Stimulation<br>($P = 0{\cdot}05$) | 0 | 0 | 4·6<br>n.s. | 0 | 0 |
| Rutin | 14·2<br>n.s. | 13·0<br>Inhibition<br>($P = 0{\cdot}05$) | 0 | 4·1<br>n.s. | 3·6<br>Inhibition<br>($P = 0{\cdot}01$) | 0 |
| Naringin | 15·0<br>n.s. | 12·0<br>Inhibition<br>($P = 0{\cdot}05$) | 19·6<br>Stimulation<br>($P = 0{\cdot}05$) | 4·5<br>n.s. | 4·5<br>n.s. | 4·4<br>n.s. |

n.s. = Not significantly different from the control; $P$ = probability.

Medium 1 with $0{\cdot}5 \times 10^{-3}$ per cent quercetin gave the highest germination of 21 per cent medium 1 with salts and boric acid gave the longest pollen tubes of 10–13·5 times the diameter of the pollen grain.

### *Chromatography*

Seven spots were detected on the chromatogram of the unhydrolysed stigma extracts, five of which stained blue with ferric chloride and potassium ferricyanide indicating that they were probably phenolic substances. Ethanolic aluminium chloride and fuming with ammonia showed that the three phenolic spots of lowest $R_F$ value 0·111, 0·240 and 0·404, were flavonols as they fluoresced yellow or yellow-green under u.v. light. They migrated more slowly than the aglycone controls and were probably flavonol glycosides. Only the naringenin control stained magenta in the sodium borohydride test which is specific for flavanones (Eigen, Blitz, and Gunsberg, 1957). After acid hydrolysis of the stigma extracts, the $R_F$ and appearance of all spots on the chromatogram were unchanged except that the two flavonol glycosides of lowest $R_F$ value were no longer visible, and a new spot appeared with an average $R_F$ value of 0·78. This showed all the characteristics of the quercetin control.

The chromatogram of unhydrolysed pollen extracts showed five spots, only one of which, with an $R_F$ value of 0·5 was identified as a flavonol glycoside. On acid hydrolysis, this spot disappeared to be replaced by a spot with quercetin-like characteristics at an average $R_F$ of 0·79.

No differences in flavanoid composition which could be attributed to genotype were detected amongst the five stigma extracts, involving four different incompatibility alleles, tested in either hydrolysed or unhydrolysed condition.

## CONCLUSIONS AND DISCUSSION

Media 1 and 2 gave different pollen germination results; longer pollen tubes and higher germination respectively. Agar 1 was supplied as highly refined and had a lower salt content than agar 2. It is possible therefore, that the higher concentration of certain salts stimulated pollen germination but not pollen tube extension. However, the addition of salts and boric acid to agar 1 increased both germination and tube extension, although this was probably largely attributable to the boric acid (Schwanitz (1942)). When $0{\cdot}5 \times 10^{-3}$ per cent quercetin was incorporated into medium 1, germination but not pollen tube extension was stimulated; the reverse was true with medium 2. This result may have been affected by the reactivity of flavanoids with many metal salts, which is the basis of most of the detection methods for flavanoids after chromatography. Kaempferol and naringin with medium 2 stimulated germination at $0{\cdot}5 \times 10^{-3}$ and $0{\cdot}5 \times 10^{-1}$ per cent respectively.

Three glycosides of quercetin were detected in the stigma, and one in the pollen of *B. oleracea*. The identity of the sugars in the glycosides was not established. However, since the control had a higher $R_F$ value than any of the glycosides, it is unlikely that rutin was present. One glycoside was not hydrolysed after 30 min, suggesting that the sugar may have been attached in the 7-position, as this confers greater resistance to acid hydrolysis (Harborne, 1967). The glycoside of $R_F$ 0·111 probably had more than one sugar attached to the quercetin, as increasing glycosylation results in lower $R_F$ values in BAW.

Quercetin stimulated the growth of *B. oleracea* pollen and was detected as glycosides in the pollen and stigma. Kaempferol, which also encouraged germination, has been detected as glycosides in other crucifers (Maksyntina, 1965; Harborne, 1965; Paris and Charles, 1962; Bate-Smith, 1962). However, whereas naringin stimulated pollen germination it has not been reported, nor have any other flavanones, as occurring in crucifers.

Thus, some flavanoids stimulate pollen germination or pollen tube extension of *B. oleracea in vitro*, and may have a similar effect *in vivo*, but this effect is not specific to the quercetin which is present in the stigma.

Since no difference was detected in the flavanoid composition of the stigmas of five different genotypes involving four different incompatibility alleles, it is unlikely that these substances are directly associated with intraspecific incompatibility reactions in *B. oleracea*.

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