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EXTRACTS FROM STYLES, DEVELOPED AT DIFFERENT TEMPERATURES, AND THEIR EFFECT ON COMPATIBILITY OF PETUNIA HYBRIDA IN EXCISED-STYLE CULTURE

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SUMMARY

The growth of *Petunia* clone W166K (S_1S_2; self-incompatible) pollen tubes in Brewbaker & Kwack's medium is inhibited by extracts from clone T_2U (S_3S_3; also self-incompatible) styles. The styles were collected one day before anthesis from plants grown either at a day/night temperature regime of 25.5/18°C (extracts A) or 19.5°/18°C (extracts B). High molecular (protein) extracts B had a much stronger effect than high molecular extracts A. After enzymatic breakdown of the proteins the inhibition by extract A was as strong as that by extract B. From these results it is concluded that prior to anthesis pollen tube growth stimulating proteins are being synthesized in the style when the day temperature is sufficiently high.

The inhibitory effects of the low molecular weight carbohydrate extracts A and B were the same.

1. INTRODUCTION

Temperature during style development has an effect on the length of pollen tubes after a compatible pollination: the length of those tubes is greater when the styles developed under the 25.5/18°C temperature regime instead of under the lower temperature regime of 19.5/18°C (VAN HERPEN & LINSKENS 1981).

There is no difference in total protein content between unpollinated styles either developed under the high or low temperature regime, but the immunologically determined protein patterns of those styles differ significantly (VAN HERPEN 1981).

So the question arises whether or not polypeptides, synthesized before pollination could have an impact on the length of pollen tubes after compatible pollinations. According to the model of VAN DER DONK (1975) the question seems rather irrelevant, because the stylar polypeptides do have an influence on the tube length in a compatible situation, but their synthesis is only restricted to the progamic phase.

Labelling experiments (LINSKENS & ESSER 1959), E.M. studies (VAN DER PLUIJM & LINSKENS 1966) and biochemical analysis (LINSKENS 1955) have indicated that pollen tubes take up material, such as free sugars from the style during

their growth through the intercellular substance (I.S.). The length of the pollen tubes resulting from a compatible pollination may be determined by the mass of intercellular substance (I.S.) or by the amount of available free carbohydrates in the style; unless of course the I.S. is completely broken down to free carbohydrates by the growing pollen tube, the total amount of free carbohydrates could not have an influence on the pollen tube length, because the measured free carbohydrate content in unpollinated styles developed at the low temperature regime is the highest and the pollen tube length in those styles the least compared to the styles developed at the high temperature regime (VAN HERPEN & LINSKENS 1981; VAN HERPEN, 1983).

With the help of the excised style technique it should be possible to determine whether or not proteins and free carbohydrates have an influence on the length of the compatible pollen tubes.

Two methods were available: the 'improved excised style culture" from NIIMI (1982) and the "semi-vitro culture" (SCHOCH-BODMER 1932; STRAUB 1946; BREWBAKER & MAJUMDER 1961).

2. MATERIALS AND METHODS

2.1. Plant Material

Two clones of self-incompatible *Petunia hybrida* were used: T_2U (S_3S_3) as style, and W166K (S_1S_2) as pollen provider. The T_2U summer-cuttings were grown in two plant growth chambers with day/night regimes at 19.5/18°C and 25.5/18°C respectively, as described previously (VAN HERPEN & LINSKENS 1981). W166K was grown in the greenhouse, because temperature during pollen development had no effect on the tube length of that pollen (VAN HERPEN & LINSKENS 1981). Only cross-pollinations, W166K (S_1S_2) pollen on T_2U (S_3S_3) styles, were performed.

2.2 Collection and storage of pollen, and pollination

Anthers were collected from W166K flower buds just before anthesis, and dried at 23°C for 24 h in the dark. The pollen was separated from the anther tissue by sieving and afterwards stored at −70°C. To avoid self-pollination the T_2U flowers were emasculated, and then pollinated with the stored W166K pollen. Only T_2U styles, developed at the temperature regime of 19.5/18°C, were used for these pollination experiments.

2.3. Pollen tube growth in vivo and vitro

Two methods were available: "the improved excised style culture" (NIIMI 1982) and the "semi-vitro culture" (SCHOCH-BODMER 1932; STRAUB 1946; BREWBAKER & MAJUMDER 1961). Both were tested and the last one was improved in such a way that it became a good tool for studying the effect of style extracts on pollen tube growth. The pollen germination and subsequent pollen tube growth were allowed for exactly 4 hours on the plant at a temperature of 19.5 $\pm$ 0.2°C.

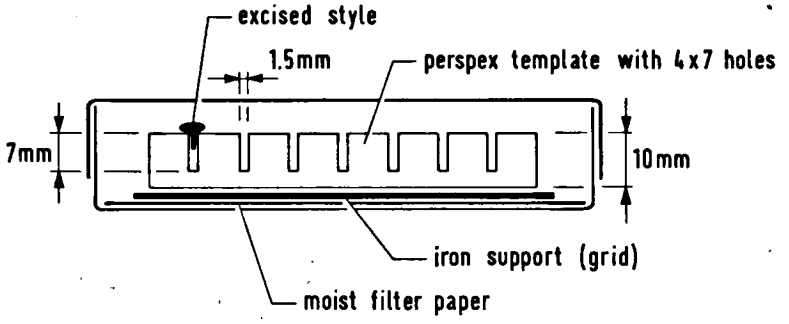

Fig. 1. Apparatus for excised style culture.

Seven flowers (pollinated) were collected at random and the styles were cut with a sharp, scoured, razor blade 5 mm from the stigma tip. These excised-styles were put into holes filled with 10 µl of medium (pollen culture medium according to SHARMA & SHIVANNA 1982 or BREWBAKER & KWACK 1963) with or without style extracts. The perspex template with 4 × 7 holes was placed on a grid into a water saturated petri-dish (*fig. 1*). The pollen tubes were allowed to grow into the medium for 20 hours at 25°C in the dark.

2.4. Determination of pollen tube length

The excised styles and protruding pollen tubes were straightened on a microscope slide and placed under a light microscope with stage micrometer. The length of the protruding pollen tubes was measured from the cut end of the excised style to the point reached by 90% of the pollen tubes.

2.5. Medium

Two media were tested: BREWBAKER & KWACK medium (1963), adjusted to pH 5.6–5.7 with 0.1 N NaOH or 0.1 N HCl before addition of 10% sucrose, was much better suited for our plant material than the pollen culture medium of SHARMA & SHIVANNA (1982) which had a strong inhibitory effect on the length of the protruding pollen tubes, reducing the length almost to nil. The Brewbaker & Kwack medium was used as "the standard medium" for all experiments.

Table 1. Length in mm of W166K pollen tubes, protruding from cut-ends of excised styles and grown in low molecular carbohydrate extracts of styles developed either at the low or high temperature regime. Low molecular carbohydrate extracts are dissolved in Brewbaker & Kwack medium. Average of 28 observations ± standard error of the mean.

conc. of extract (number of styles per 10 µl medium)	0	0.01	0.03	0.05	0.1
19.5/18°C	3.15 ± 0.15	2.80 ± 0.16	2.70 ± 0.15	2.20 ± 0.12	1.25 ± 0.09
25.5/18°C	3.15 ± 0.15	2.90 ± 0.13	2.50 ± 0.10	2.30 ± 0.20	1.20 ± 0.11

Table 2. Length in mm of W166 pollen tubes, protruding from cut-ends of excised styles and grown in differentially treated extracts of styles developed either at the low or high temperature regime. Average of 28 observations ± standard error of the mean.

	Extract 5′ at 25°C	Extract 5′ at 70°C	Extract 5′ at 100°C
25.5/18°C extract	2.17 ± 0.10	3.04 ± 0.14	3.08 ± 0.15
19.5/18°C extract	1.90 ± 0.12	2.06 ± 0.13	3.07 ± 0.15
	Extract 5′ at 70°C	(Extract + Protease K) 30′ at 25°C, inactivated for 5′ at 70°C	(Extract + Protease K) immediately inactivated for 5′ at 70°C
25.5/18°C extract	3.04 ± 0.14	1.71 ± 0.10	2.60 ± 0.12
19.5/18°C extract	2.06 ± 0.13	1.91 ± 0.10	2.04 ± 0.10
	Extract 5′ at 70°C	(Extract + DNA'se) 30′ at 25°C, inactivated for 5′ at 70°C	(Extract + DNA'se) immediately inactivated for 5′ at 70°C
25.5/18°C extrat	3.04 ± 0.14	2.61 ± 0.09	2.48 ± 0.11
19.5/18°C extract	2.06 ± 0.13	2.63 ± 0.10	2.44 ± 0.08
	Extract 5′ at 100°C	(Extract + RNA'se) 30′ at 25°C, inactivated for 5′ at 100°C	(Extract + RNA'se) immediately inactivated for 5′ at 70°C
25.5/18°C extract	3.08 ± 0.15	2.78 ± 0.11	2.64 ± 0.12
19.5/18°C extract	3.07 ± 0.15	2.71 ± 0.10	2.74 ± 0.10

2.6. Preparation of extracts

High molecular substances: Unpollinated T_2U styles developed under the low or the high temperature regime were collected one day before anthesis, immediately frozen in liquid nitrogen, pulverized and homogenized in 0.1 M Tris-HCl pH 7.2 using a Braun Potter homogenizer (30 strokes at 700 r.p.m.); after centrifugation (15,000 × g) the layer of lipids was removed, and the supernatant was passed through a G-25 column, equilibrated with BREWBAKER & KWACK medium (1963); only the high molecular fractions with more than 25 µg protein per 100 µl (protein determination according to LOWRY et al. 1951) were collected and used for further experiments. The concentration of the extract is presented as the number of styles per 10 µl of the "standard medium".

Low molecular carbohydrates: As described above, with the exception that the styles were homogenized in 80% ethanol and the supernatant was passed through a Dowex 50 × 8 (H^+) column. The concentration of the free sugars in the effluent of the column was measured by the method of DUBOIS et al. (1956). After vacuum-evaporation of the ethanol the free carbohydrate-residue is dissolved in "standard medium" and ready to be used for further experiments. The concentration of the extract was expressed as the number of styles per 10 µl of "standard medium".

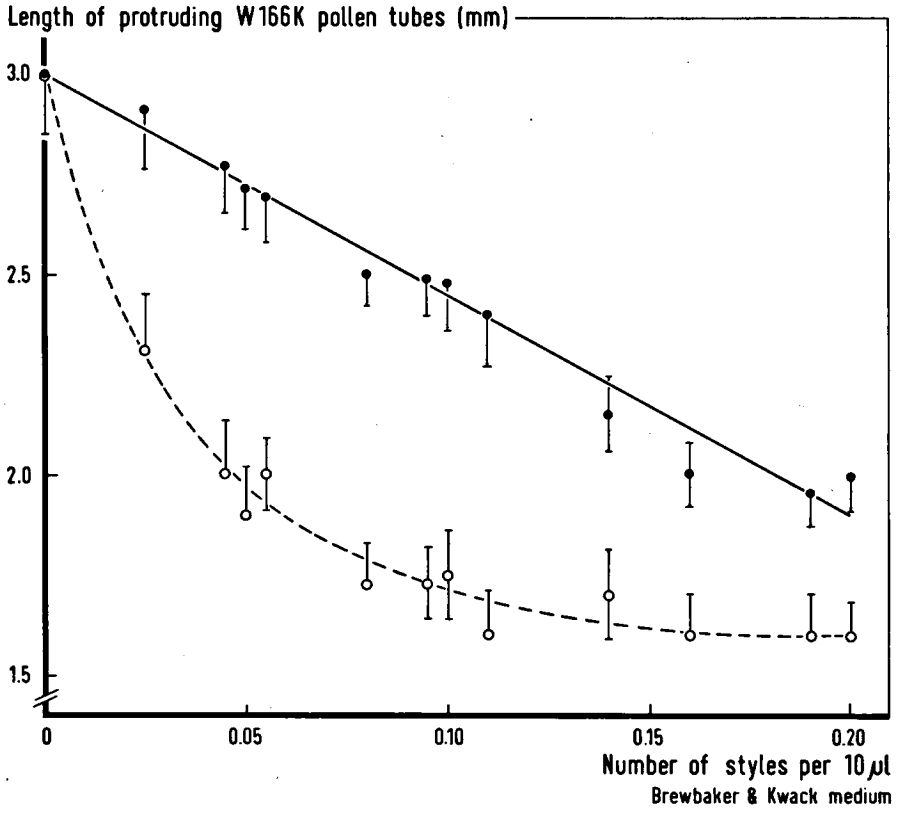

Fig. 2. Length of W166K (S_1S_2) pollen tubes grown on the plant for 4 hours at 19.5°C, then 20 hours at 25.0°C in excised style culture with T_2U (S_3S_3) extracts of styles developed at 25.5/18°C (•) or 19.5/18°C (○). Average of 28 observations ± standard error of the mean.

2.7. Enzymes

Protease K, DNA'se and RNA'se are used in final concentrations of 100 µg/ml. Protease K is added to the high molecular extract for 30′ at 25°C and inactivated by a temperature of 70°C for 5′. RNA'se (KUNITZ 1940) and DNA'se are applied for 30′ at 25°C and inactivated respectively at 100°C and 70°C for 5′.

3. RESULTS

Methods: The disadvantage of the "improved excised style culture" from NIIMI (1982) was that the pollen tubes growing in and on the agar were thin and did not make uniform contact with the extracts dissolved in it. The negative point of the "semi-vitro culture" (SCHOCK-BODMER 1932; STRAUB 1946; BREWBAKER & MAJUMDER 1961) was its difficulty to prepare and incubate a large number of excised styles respectively in a short time over a long period of time. Improve-

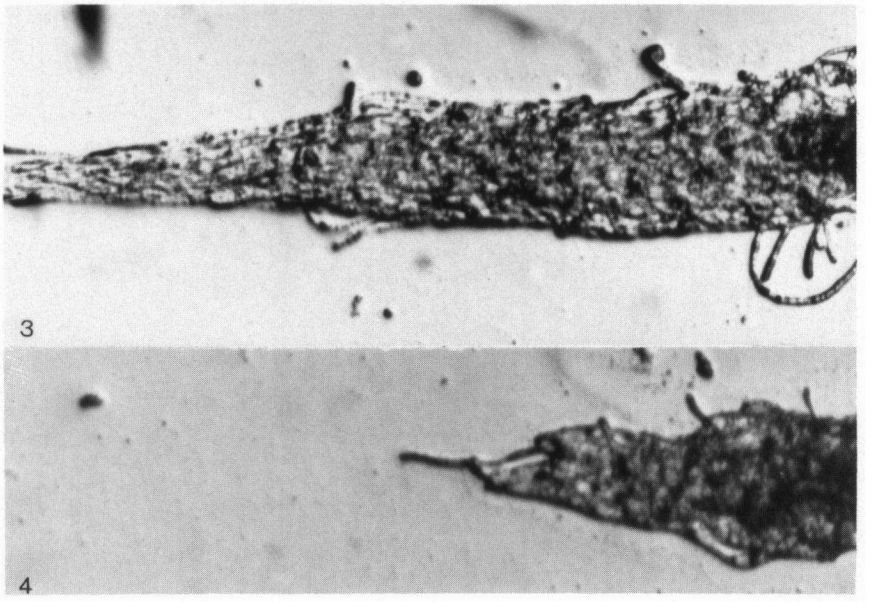

Fig. 3. Protruding pollen tubes growing in extracts of styles developed at the high temperatue regime of 25.5/18°C.
Fig. 4. Protruding pollen tubes growing either in extracts of styles developed at the low temperature regime of 19.5/18°C or in extracts of styles developed at 25.5/18°C and treated with protease K.

ment of the last method (see Materials and Methods 2.3) finally made it a good technique for our purpose.

Low molecular carbohydrate extracts: No differences in pollen tube length could be detected between pollen tubes growing in the low molecular carbohydrate extracts of unpollinated styles developed either at the low (19.5/18°C) or the high temperature (25.5/18°C) regime (*table 1*).

High molecular extracts: The length of the protruding pollen tubes depends on whether the extract is made of styles developed at the high or low temperature regime, and on the concentration of the extract (*fig. 2*). The difference between the style 25.5/18°C and the 19.5/18°C extract is the most when the concentration of the extracts equals 0.05 style per 10 µl standard medium of BREWBAKER & KWACK (*figs. 2, 3* and *4*). This particular concentration will be used in further experiments to determine which compound, in the high molecular extract, is responsible for the differential effect.

Heating the style 25.5/18°C extract up to 70°C, increases the length of the pollen tubes compared to the untreated 25.5/18°C extract. The same is true for the style 19.5/18°C extract if heated up to 100°C (*table* 2).

Application of protease K to the style 25.5/18°C extract decreases the length of the pollen tubes growing in that extract and there is no longer any difference

in pollen tube length between the enzyme treated 25.5/18°C extract and the untreated 19.5/18°C extract (*fig. 4* and *table 2*). No change in pollen tube length could be observed when the style extracts were treated with RNA'se or DNA'se (*table 2*).

4. DISCUSSION

The low temperature regime (19.5/18°C) during style development and progamic phase does not only prolong the recognition process in *Petunia,* but also alters it in such a way that acceptance of the compatible pollen tubes (tubes resulting after a cross-pollination) does not occur (VAN HERPEN & LINSKENS 1981). According to the model of VAN DER DONK (1975), the pollen genome is not activated because the style specific polypeptides are probably not synthesized. If this "incompatible-like" situation exists it can be used to determine, with the help of the excised-style technique, whether or not proteins, synthesized in the style before pollination, could change that "incompatible-like" situation into a compatible one. Our experiments (*table 2*) prove the existence of S_3S_3 stylar proteins, only synthesized in the style before pollination under a temperature regime of 25.5/18°C, which have an effect on the length of the S_1S_2 cross-pollen tubes in semi-vitro culture. So the "incompatible-like" situation, after a compatible pollination, as first postulated by VAN HERPEN & LINSKENS (1981) is confirmed. However, it is not impossible that stylar polypeptides are formed during pollination, but according to the experiments of VAN HERPEN (1981), the gene activity and subsequent polypeptide synthesis before the progamic phase is independent of the kind of pollination. The translation of masked messenger RNA to stylar polypeptides found by VAN DER DONK (1975) during the progamic phase is completely evaded.

The conclusion of SHARMA & SHIVANNA (1982) that "unpollinated pistil contains self-incompatibility factors which affect *in vitro* germination and tube growth of self- and cross-pollen differently" gives more than reasonably can be concluded from those few experiments performed, and so the remark on the stylar polypeptides of VAN DER DONK (1975) is rather preliminary.

The use of DNA'se and RNA'se in combination with the unpollinated T_2U extracts had no effect on the length of the W166K pollen tubes. It is possible, unless the excised style technique is invalid, that the pollen genome, activated by the stylar polypeptides, does not activate the Stylar S-gene but results in a more and better use of the compounds available for the pollen tubes in the unpollinated style extracts. This would be in contrast with the findings of VAN DER DONK (1975) who postulated the onset of the stylar S-gene by the activated pollen genome.

The proteins in the style extract of styles developed at 25.5/18°C promote more or inhibit less than the proteins in the 19.5/18°C style extract. The experiments with protease K indicate that the 19.5/18°C proteins have no effect, and because of that the 25.5/18°C proteins must have a promoting effect on the

length of the protruding pollen tubes. However, it is still possible that there are inhibitory proteins in the 25.5/18°C extract. Heating up the 25.5/18°C extract to 70°C has an effect on the pollen tube length, so it is not unlikely that an inhibiting compound in the style extract is inactivated.

The length of the pollen tubes resulting from a compatible pollination is certainly not determined by the amount of available free carbohydrates, because no differences could be detected between pollen tubes growing in the free carbohydrate extract of unpollinated styles developed either at the low or high temperature regime (*table 1*). Our excised style technique gives a completion on the previously made assumption that free carbohydrate content in the style could not be correlated with the length of the cross-pollen tube (VAN HERPEN, 1983). Determination of the free carbohydrate content in styles developed at the low temperature regime and supplemented with 24 hours of the high temperature regime (25.5/18°C) revealed a sudden drop in the free carbohydrate content of the style in the first day after the high temperature treatment, immediately followed by a rise in free carbohydrate content in the next two days, and again in a drop and subsequent rise in free carbohydrate content (VAN HERPEN, in preparation). These results could also not be correlated with the greater length of the pollen tubes growing in those styles as described previously (VAN HERPEN & LINSKENS 1981).

Our excised style technique is a good and reliable method for studying pollen-style interactions through pollen tube growth.

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