Acta Bot. Neerl. 27 (3/4), August 1978, p. 205–212.

FUNCTION OF THE POLLEN COAT IN DIFFERENT STAGES OF THE FERTILIZATION PROCESS

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key words: germination, incompatibility, *Petunia*, pollen coat, pollen tube growth, pollen washing, seed setting

SUMMARY

The weight of released proteins after four subsequent washings of pollen of *Petunia hybrida* L. in germination medium (containing 10% saccharose and 0.01% boric acid) is calculated to be c. 18% of the original dry pollen weight. Although washed-and-dried pollen did germinate very badly after replacement in the germination medium, it produced normal pollen tube growth in the style and normal seed setting in the ovary. It is concluded that released pollen coat substances are not functional in the *in vivo* fertilization process. Another conclusion is that these eluted pollen coat substances have no role as recognition factors in the gametophytically-determined incompatibility system of *Petunia*.

1. INTRODUCTION

The pollen coat is defined as the total of loosely bound substances, laid down between and upon the fibrils and strands of the pollen grain wall during maturation in the anther. The pollen grains almost immediately release amino acids, proteins and carbohydrates from the pollen coat, when brought into a liquid environment. STANLEY & LINSKENS (1965) and LINSKENS & SCHRAUWEN (1969) observed rapid release of proteins and amino acids during the first minutes after dispersion of the pollen grains in the germination medium. GILBERT (1972) found that *Lepidoptera* of the genus *Heliconius* utilized released amino acids: the moths collected pollen grains on the proboscis, washed them actively with nectar and ingested the released amino acids.

These investigations left unanswered some questions about the function of these substances on the one hand, and about the viability after washing treatments of the pollen grains on the other.

KIRBY & SMITH (1974) found significant differences of released pollen coat substances between pollen of the binucleate and trinucleate type. They suggested qualitative studies to elucidate control-processes of intraspecific incompatibility systems in which these substances might be involved. This suggestion was also based upon an assertion of TSINGER & PETROVSKAYA-BARANOVA (1961) about the role of wall proteins and other compounds in interaction processes between the pollen grain and its substrate. HESLOP-HARRISON et al. (1973) distinguished between gametophytic and sporophytic fractions in the pollen walls of the Malvaceae. They proposed that in intraspecific incompatibility systems of the gametophytic type control is mediated through

the gametophytic fraction, whereas in sporophytic systems the sporophytic fraction will be responsible for supply of the "recognition substances". The proteins held in the wall of poplar and Cruciferae pollen grains were directly concerned in the control of pollen germination and the sporophytically-determined incompatibility system (KNOX et al. 1972; HESLOP-HARRISON et al. 1974). ROGGEN (1974) tried to overcome the sporophytically-determined incompatibility barrier in *Brassica* by washing the pollen grains for a short time with water, acetone or chloroform: pollination with pollen washed in water or in acetone-water-mixtures produced seed setting in a pseudo-compatible clone, but incompatibility was hardly influenced.

Till now there is only speculation about the function of the substances, which are released from the pollen coat, in the gametophytically-determined incompatibility reaction. Moreover, the influence of pollen washing on *in vitro* germination is not well known either.

Consequences of washing-out of pollen exudates for *in vitro* germination and *in vivo* pollen tube growth and seed setting in *Petunia hybrida* after (gametophytically-determined) compatible and incompatible pollinations are described and discussed in this paper.

2. MATERIALS AND METHODS

2.1. Pollen collecting

Ripe anthers of *Petunia hybrida* L. clones W166K and T2U, with incompatibility alleles S1S2 and S3S3 respectively, were collected and dried at RH = c. 40% and at room temperature (20–24°C) for one day. The liberated pollen grains were sifted out from the opened anthers.

2.2. Pollen washing and drying

Five ml of germination medium (10% saccharose and 0.01% boric acid) containing 50 mg of pollen was swirled in a centrifuge tube on a *Vortex*. After 5 min the suspension was centrifugated at 2000 × g for 5 min. The supernatant was used for protein measurement (LOWRY et al. 1951). The pellet was dried on filter paper, or rewashed maximally 3 times before drying. This drying lasted 5–10 hrs at room temperature and RH = c. 40%

2.3. Pollen germination in vitro

Seven mg portions of washed-and-dried pollen were applied to 25-ml Erlenmeyer flasks containing 1 ml of germination medium (modified according to SCHRAUWEN & LINSKENS 1967). The flasks were shaken on a reciprocating shaker (120 strokes/min). Percent germination was calculated from at least 800 pollen grains. Pollen grains were considered to be germinated when they had produced a pollen tube, which was at least equal or longer than half the grain diameter. Pollen diameter was measured (blind scoring), using a Reichert projection microscope, in units relative to the pollen grain image diameter projected on the microscope screen (1 image unit = 1.67 μm). Diameter measure-

ments were carried out after the washed-and-dried pollen grains were brought in a drop of germination medium on a glass-slide.

2.4. Pollination

Washed-and-dried pollen was used for pollination. One day before anthesis flowers of clone T2U were emasculated to avoid self-pollination. Pollinations were carried out on plants in the greenhouse.

2.5. Pollen tube length measurements

The length of the pollen tubes was determined with a U.V.-light microscope (LINSKENS & ESSER 1957): eight styles were picked at random and the length of the bundle of the ten fastest-growing pollen tubes in each style was measured.

2.6. Seed setting

The number of seeds formed in seed capsules after cross- or self-pollination with pollen, washed 0, 2 or 4 times in germination medium and dried after the washing procedure, was determined. Per pollination 15 seed capsules were used.

3. RESULTS

3.1. Protein release

After four washings the total amount of protein released from pollen of both clones into the germination medium appeared to be almost 18% of the original dry pollen weight (*fig. 1*).

3.2. Germination

Germination *in vitro* of washed-and-dried pollen was very poor (*table 1*). The germination percentage of pollen of clone T2U appeared to be lower than that of clone W166K. The main reason for this low germination percentage was the high number of aborted pollen grains (c. 25%). These pollen grains have diameters of less than 15 units (*fig. 2, 1*).

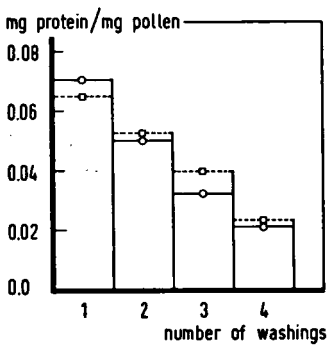

Fig. 1. The effect of washing in germination medium on the release of protein from pollen of *Petunia hybrida* clones T2U and W166K. ——O—— clone T2U, – – –□– – – clone W166K.

Table 1. Germination percentages of washed-and-dried pollen of *Petunia hybrida* clones T2U and W166K after replacement in germination medium.

number of washings	germination percentage	
	clone T2U	clone W166K
0	31	62
1	1	3
2	5	6
3	3	9
4	2	11

The diameter of washed-and-dried pollen grains after replacing in the germination medium had much decreased in comparison to the diameter of the control. This decrease must be ascribed to rupture of a number of pollen grains after replacing the pollen in the germination medium, as was observed under the microscope. The diameter of both washed-and-dried and control pollen grains decreased during the first three hrs after germination (*fig. 2*). This decrease demonstrated in *fig. 2, III* must be ascribed mainly to germination, whereas the decrease shown in *fig. 2, IV* was caused by pollen rupture.

3.3. Pollen tube growth and seed setting

Pollen tube growth – after compatible or incompatible pollinations with washed-and-dried pollen – and the subsequent seed setting equalled their controls (*fig. 3, table 2*). Differences in length between pollen tubes of washed-and-dried and control pollen grains were not significant ($P < 0.05$), as were the differences in seed setting between washed-and-dried and control pollen grains after compatible or incompatible pollinations.

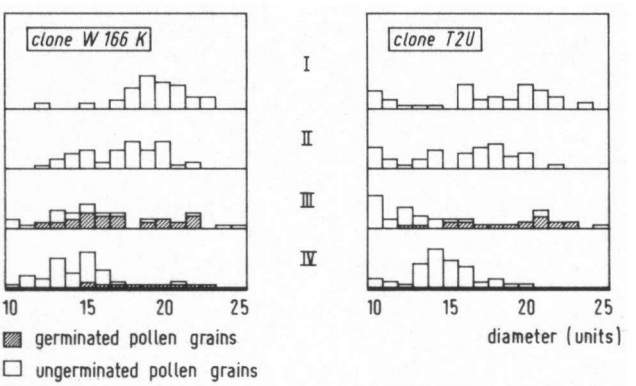

Fig. 2. Diameters of pollen grains of *Petunia hybrida* clones T2U and W166K after four washings in germination medium.
I and II: after 0 hr of germination (I: control pollen, II: washed-and-dried pollen); III and IV: after 3 hr of germination (III: control pollen, IV: washed-and-dried pollen). Each histogram consists of 50 pollen grains.

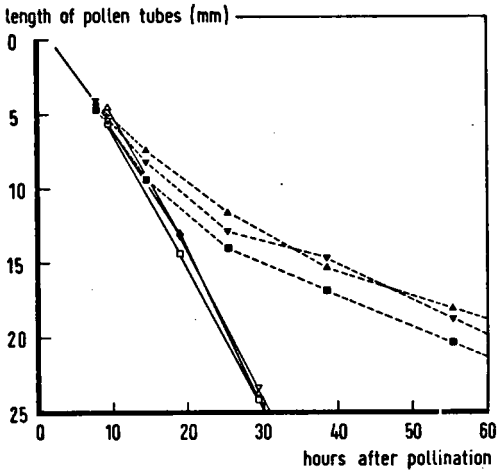

Fig. 3. Compatible and incompatible pollen tube growth in styles of *Petunia hybrida* clone T2U of washed-and-dried pollen. □——□ compatible growth (W166K) control, ▽——▽ and △——△ compatible growth after 2 resp. 4 subsequent washings in germination medium and drying thereafter; ■—— ■ incompatible growth (T2U) control, ▲——▲ and ▲——▲ incompatible growth after 2 resp. 4 subsequent washings in germination medium and drying thereafter.

Table 2. Seed setting produced by washed-and-dried pollen of *Petunia hybrida* clones W166K and T2U (after compatible or incompatible matings respectively).

pollination			number of washings	number of seeds per capsule		
♀		♂		$\bar{X} \pm 2\sigma_x$	lowest	highest
T2U (S3S3)	×	W166K (S1 + S2)	0	184 ± 35	61	295
			2	226 ± 27	99	328
			4	181 ± 37	94	313
T2U	×	T2U	0	14 ± 8	0	46
			2	1 ± 1	0	9
			4	1 ± 2	0	14

4. DISCUSSION

After transfer into germination medium the pollen grains released many substances and took up a large amount of water very rapidly. When the pollen grains were then dried, this water was yielded to the surrounding air within a few minutes, as could be followed under the microscope (Gilissen, unpublished results): the original oval shape of the dry pollen grain before swelling had returned. Consequently, water molecules taken up during swelling in germination medium were able to leave the pollen wall and the pollen protoplasm very easily. It is known that water molecules from the air are able to penetrate and then leave the pollen wall and the pollen protoplasm very rapidly, without changing the oval shape of the pollen grain (GILISSEN, 1977b). It appeared that in the *in vivo* system water molecules, which entered or left the pollen grain, did influence its metabolism in a reversible way.

In nature pollen often becomes wetted during the pollination event by water or nectar (a sugar solution, which is much alike the germination medium).

EISIKOWITCH & WOODELL (1975a) found that pollen grains in *Primula vulgaris* are protected against adverse effects of rainwater: the anthers produce a substance, which inhibits premature pollen germination when rainwater has entered the flower. These authors do not mention a possible effect of rainwater on the self-incompatibility system in the heterostylic *Primula*. EISIKOWITCH & WOODELL (1975b) found that pollen of *Armeria maritima* was less resistent to rain water than to sea water. Their results might be due to the fact that the osmotic value of sea water is much higher than that of rainwater and thus can prevent pollen rupture. HAGERUP (1950) described pollination via rainwater (rain pollination), but did not suggest any physiological effects of this kind of pollination on the pollen grains.

In many plant species water acts as a vector in pollen transport. Dispersal can take place under water (hyphydrogamy) or on the water surface (enhydrogamy) (SCULTHORPE 1967; FAEGRI & VAN DER PIJL 1971). Little is known about the self-incompatibility in these species, in which pollen always comes in contact with water. ERNST-SCHWARZENBACH (1953) investigated morphologically inter-generic and inter-specific incompatibility phenomena in Hydrocharitaceae, but did not pay attention to pollen physiology and self-incompatibility.

Petunia hybrida is pollinated by hawk moths (Sphingidae, Lepidoptera). During transfer, the pollen sticks to the proboscis (BRANTJES, 1973), which is often wetted by nectar. As shown above washing and subsequent drying did not destruct the *in vivo* pollen viability and the incompatibility reaction. It can now be concluded that wetting of pollen grains and subsequent release of pollen coat substances on the moth's proboscis during the pollination event in nature also does not influence the pollen function. However, germination *in vitro* of washed and dried pollen was strongly decreased. In a previous paper (Gilissen, in press) different radiosensitivities of pollen of *Petunia* in germination medium (*in vitro*) and in stigmatic exudate (*in vivo*) were demonstrated. It was suggested there, that these differences in radiosensitivity were dependent on differences in chemical composition of germination medium and stigmatic exudate. It is likely that here also the chemical composition of germination medium and stigmatic exudate influenced washed-and-dried pollen similarly as it influenced irradiated pollen.

The calculated amount of protein released into the germination medium after four subsequent washings was 18% of the total pollen weight. The total loss by release of protein and other organic and inorganic substances must therefore be considerably higher. During each distinct washing about 2–3% of the pollen grains ruptured, as was observed by pollen-diameter measurements. These ruptured pollen grains had pressed part of their protoplasm out. So it must be noticed here that a little portion of the protein measured (*fig. 1*), did come from plasma of ruptured pollen grains.

The fact, that compatible and incompatible pollen tube growth and seed

setting were not affected by the loss of the released substances, indicates that these substances are not functional in the fertilization process. This statement is in agreement with the results of studies in gametophytically-determined self-incompatible *Lilium longiflorum* (FETT et al. 1976). It is concluded that these loosely bound, water soluble substances have no role as recognition factors in the incompatibility system either. So it can be hypothesized now that:

1. recognition substances in the gametophytically-determined incompatibility reaction are not water soluble. They might be soluble in oil or other fatty substances, for the stigmatic exudate contains about 80% oil and other fatty substances (KONAR & LINSKENS 1966).

2. the recognition reaction does not take place at the stigmatic surface at all, but just starts after penetration of the pollen tubes into the stigmatic tissue. An indication for this second explanation lies in the observation that pollination alone was not enough to activate the stylar tissue as far as wilting of the corolla is concerned: to initiate the wilting-regulating function of the style (which was different in cross- and self-pollinated styles) penetration of the pollen tubes into the stigma was indispensible (GILISSEN, 1976, 1977a). This indication is strengthened by observations by DEURENBERG (1977) who found differentiated protein synthesis with polysomes from *Petunia* ovaries during progressing tube growth in the style after pollination with compatible or incompatible pollen.

ACKNOWLEDGEMENT

The authors are much indebted to prof. H. F. Linskens for his stimulating interest, to J. van Straten (Dept. of Microbiology, Agricultural University, Wageningen) for correcting the manuscript and to Miss J. Verhoeven for typing it. Thanks are also due to Mr. W. van den Brink and Mr. A. Glaap for growing excellent plant material. This research was made possible by financial support of the Euratom-ITAL-association, contract 002-094-71-1 BIA N.

REFERENCES

BRANTJES, N. B. M. (1973): Sphingophilous flowers, function of their scent. In: *Pollination and dispersal*, pp. 27–46. Eds.: N. B. M. BRANTJES & H. F. LINSKENS, Nijmegen: University, Department of Botany.

DEURENBERG, J. J. M. (1977): Differentiated protein synthesis with polysomes from Petunia ovaries before fertilization. *Planta (Berl.)* **133**: 201–206.

EISIKOWITCH, D. & S. R. J. WOODELL (1975a): Some aspects of pollination ecology of Armeria maritima (Mill.) Willd. in Britain. *New Phytol.* **74**: 307–322.

—, & —, (1975b): The effect of water on pollen germination in two species of Primula. *Evolution* **28**: 692–694.

ERNST-SCHWARZENBACH, M. (1953): Zur Kompatibilität von Art- und Gattungsbastardierungen bei Hydrocharitaceen. *Öst. bot. Z.* **100**: 403–423.

FAEGRI, K. & L. VAN DER PIJL (1971): *The principles of pollination ecology*. Pergamon Press, Oxford, New York, second revised edition.

FETT, W. F., J. D. PAXTON & D. B. DICKINSON (1976): Studies on the self-incompatibility response of Lilium longiflorum. *Amer. J. Bot.* **63**: 1104–1108.

GILBERT, L. E. (1972): Pollen feeding and reproduction biology of Heliconius butterflies. *Proc. Nat. Acad. Sci. U.S.A.* **69**: 1403–1407.

GILISSEN, L. J. W. (1976): The role of the style as a sense-organ in relation to wilting of the flower. *Planta (Berl.)* **131**: 201–202.

—, (1977a): Style-controlled wilting of the flower. *Planta (Berl.)* **133**: 275–280.

—, (1977b): The influence of relative humidity on the swelling of pollen grains in vitro. *Planta (Berl.)* **137**: 299–301.

—, (1978): Post-X-irradiation effects on Petunia pollen germinating in vitro and in vivo. *Env. Ex. Bot.* (in press).

HAGERUP, O. (1950): Rain-pollination. *D. Kgl. danske Vidensk. Selsk. Biol. Medd.* **18**: 5.

HESLOP-HARRISON, J., Y. HESLOP-HARRISON, R. B. KNOX & B. HOWLETT (1973): Pollen-wall proteins: "gametophytic" and "sporophytic" fractions in the pollen walls of the Malvaceae. *Ann. Bot.* **37**: 403–412.

—, R. B. KNOX & Y. HESLOP-HARRISON (1974): Pollen-wall proteins: exine-held fractions associated with the incompatibility response in Cruciferae. *Theoret. Appl. Genetics* **44**: 133–137.

KIRBY, E. G. & J. E. SMITH (1974): Elutable substances of pollen grain walls. In: *Fertilization in higher plants*, pp. 127–130. Ed.: H. F. LINSKENS, Amsterdam: North-Holland Publishing Company.

KNOX, R. B., R. R. WILLING & A. E. ASHFORD (1972): Role of pollen-wall proteins as recognition substances in interspecific incompatibility in poplars. *Nature* **237**: 381–383.

KONAR, R. N. & H. F. LINSKENS (1966): Physiology and biochemistry of the stigmatic fluid of Petunia hybrida. *Planta (Berl.)* **71**: 372–387.

LINSKENS, H. F. & K. ESSER (1957): Über eine spezifische Anfärbung der Pollenschläuche im Griffel und die Zahl der Kallosepfropfen nach Selbstung und Fremdung. *Naturwissenschaften* **44**: 16.

—, & J. SCHRAUWEN (1969): The release of free amino acids from germinating pollen. *Acta Bot. Neerl.* **18**: 605–614.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL (1951): Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265–275.

ROGGEN, H. (1974): Pollen washing influences (in)compatibility in Brassica oleracea varieties. In: *Fertilization in higher plants*, pp. 273–278. Ed.: H. F. LINSKENS, Amsterdam: North-Holland Publishing Company.

SCHRAUWEN, J. & H. F. LINSKENS (1967): Mass culture of pollen tubes. *Acta Bot. Neerl.* **16**: 177–179.

SCULTHORPE, C. D. (1967): *The biology of aquatic vascular plants*. Edward Arnold Ltd., London.

STANLEY, R. G. & H. F. LINSKENS (1965): Protein diffusion from germinating pollen. *Physiol. Plant.* **18**: 47–53.

TSINGER, N. V. & T. P. PETROVSKAYA-BARANOVA (1961): The pollen grain wall – a living physiologically active structure. *Dokl. Akad. Nauk. SSSR* **138**: 466–469.