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Is the 'Rejection Reaction' Inducing Ability in Sporophytic Self-incompatible Systems Restricted Only to Pollen and Tapetum?

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Summary. The present investigation describes the stigmatic rejection response, induced by anther or pollen at developmental stages ranging from anther primordia to mature pollen, as well as in other floral and vegetative tissues. This has been studied in a sporophytic self-incompatible system, *Brassica campestris* (Brassicaceae). The implications of these observations have been discussed.

Key words: Sporophytic self-incompatibility – Rejection reaction – Vegetative and floral tissues – *Brassica campestris*

Introduction

The breeding experiments conducted by Correns (1916) and East and Mangelsdorf (1925) during the first quarter of this century led to the recognition of two types of control mechanisms of self-incompatibility among angiosperms. In gametophytic self-incompatible (GSI) systems, the incompatibility behaviour is governed by the genotype of individual microspores. Contrary to this, in sporophytic self-incompatible (SSI) systems, the parental genotype determines the expression of incompatibility. The SSI systems are characterized either by (i) total inhibition of pollen germination or (ii) failure of the pollen tubes to penetrate the stigmatic papillae (Bateman 1955). In both situations, the stigmatic papillae adjacent to the point of contact with self (incompatible) pollen are occluded with a polysaccharide, β, 1–3 glucan (callose), within a few hours of pollination (Dickinson and Lewis 1973). This deposition has been termed 'rejection reaction'. Subsequently, it has also been shown that callose is deposited in response to an interaction between the pollen-wall and stigma-surface (Dickinson and Lewis 1975). Such an interaction involves the specific binding of pollen-wall proteins to receptors on the surface of stigmatic papillae (Knox et al. 1976).

The biochemical and physiological bases of this distinction between GSI and SSI systems have so far remained inconclusive. Although two main views have been expressed to explain the two types of self-incompatibility controls, neither of them is fully satisfactory. The proponent of the first hypothesis (Pandey 1960, 1970) explained the differential genetical behaviour of the two types by assuming that in SSI systems, the incompatibility gene (S-gene) is activated prior to cytokinesis in microspore mother cells. Thus, all the microspores acquire the products of the incompatibility gene(s) of the parent. In contrast, the time of gene action in GSI systems is post-meiotic (post-cytokinesis), so that two microspores of a tetrad possess similar products of incompatibility gene(s). On the contrary, the supporters of the second concept – Heslop-Harrison and his co-workers – have indicated that in SSI systems, the incompatibility factor(s) is/are synthesized in tapetum and is/are subsequently deposited in exine repositories during pollen development (Heslop-Harrison et al. 1973, 1974; Knox et al. 1975).

The present investigation was undertaken to test the validity of the above-mentioned explanations for SSI systems. *Brassica campestris* cv. 'Pusa Kalyani', belonging to the family Brassicaceae and having an SSI system (Bateman 1955) was selected. We originally intended to pin-point the exact stage of microsporogenesis at which the microspores acquire the ability to invoke 'rejection reaction'. The second step would then have been to cultivate, under in vitro conditions, microspores isolated prior to this developmental stage, to test if they still develop this ability when grown free from the influence of tapetum. However, the results of experiments conducted to achieve the first aim completely modified the second. These experiments showed that anthers and microspores at all developmental stages possess callose-inducing ability. Therefore, the in vitro culture of microspores was not attempted, instead various other sporophytic tissues (vegetative and floral) were tested for their rejection-reaction-causing ability.

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Materials and Methods

The Plant Material

The experiments included in this paper were performed on garden-grown plants of *Brassica campestris* Linn. cv. 'Pusa Kalyani' during January 1980 and 1981. Vigorously growing plants, showing bright callose rejection reaction and no pollen germination upon selfing, were selected for experimentation. The experimental unit was a single plant, with extracts from a plant, tissues applied to the stigmas on that same plant. However, the same experiments were repeated with other plants individually.

Application of Anther Primordia to Mature Pollen on Emasculated Pistils

The buds, one day prior to anthesis, were emasculated and bagged in order to avoid any contamination. The following day the pistils were excised from the emasculated flowers and implanted in agar plates (2% agar + 15% sucrose + 100 ppm boric acid). These pistils were free of contaminating pollen. For treating these pistils, floral buds containing stamens at different stages of development (anther primordium to mature pollen) were brought to the laboratory. The buds were dissected on a clean slide under a stereoscopic binocular microscope. Four of the six anthers were cut near the filament with a sharp blade and pressed at the apical end, thus squeezing out the microspore mother cells (MMCs) to mature pollen from the cut end onto a clean glass slide. The stigma of emasculated pistil was then placed in contact with the squeezed-out contents. The other two anthers from each bud were fixed in Carnoy's fluid (ethanol : acetic acid, 3 : 1) for 24 h. They were squashed in 2% propionocarmine in order to determine the cytological stage of the anther/pollen at the time of application.

For anther stages younger than those containing MMCs, the four anthers were crushed and applied to the stigmas. Controls comprised (i) emasculated unpollinated pistils and (ii) emasculated pistils rubbed against a clean slide. All such pistils were implanted in agar plates and kept for 20 h at room temperature.

Application of Extracts of Other Sporophytic Tissues

Different vegetative as well as such floral tissues as leaf, stem, sepal, petal, anther filament, dehisced anther, unfertilized and fertilized ovule, ovary wall and fruit wall (100 mg each) were ground separately in 0.2 ml of 0.02 M barbiturate buffer (pH 8.8). The floral parts were removed from emasculated flowers to avoid any pollen contamination. A small piece of filter paper dipped in each extract was applied to stigmas of an emasculated pistil. Here, the two controls were (i) emasculated unpollinated pistils and (ii) emasculated pistils touched with buffer-soaked filter paper.

Observations of the Treated Pistils

The pistils were softened in 8 M NaOH for 3–4 hours, washed with distilled water and stained with 0.1% aniline blue in 0.1N K_3PO_4. These were observed under a fluorescence microscope (Kho and Baër 1968). The relative scores for the intensity of callose deposition were recorded on a visual basis with respect to the selfed control which depicted a maximum intensity of callose deposition (Score 6). The intensity of callose deposition was assessed by considering the percentage of papillae showing callose deposition, as well as by the extent of callose deposits within each papilla.

Observations

The data presented here are the mean of experiments conducted during January 1980, are comprise of three replicates for each stage of development and are repeated at least thrice. The experiments were repeated again in January 1981, with the same results. Anthers at different stages of development were capable of inducing rejection reactions in self-stigmas. The extent and the intensity of the deposition of callose in stigmatic papillae varied with the developmental stages of the anthers. They are described in the following paragraphs in five categories, i.e. (i) anther primordia, (ii) pre-meiotic, (iii) tetrad, (iv) pre-mitotic 1 and (v) pre-mitotic 2 to post-mitotic 2.

Anther Primordia

Buds of a length less than 0.5 mm contained anther primordia which had a mass of undifferentiated parenchymatous cells surrounded by an epidermis (stage 1, Fig. 1). Even at such an early stage, anthers induced rejection reactions, though only in a few papillae (Fig. 2).

Pre-Meiotic

The pre-meiotic anthers (stage 2, Fig. 3), carrying archesporium and sporogenous tissue, did not show very well defined anther wall layers. Anthers at the archesporium stage were composed of a homogeneous mass of cytoplasm-rich cells with densely staining nuclei. The rejection reaction induced by such anthers was more than that invoked by anther primordia (Figs. 4 and 17).

Tetrad

Buds (1.0–1.5 mm long) showed green anthers containing spore tetrads (stage 3, Fig. 5). These microspore tetrads induced brighter callose deposition in stigmatic papillae than did the pre-meiotic anthers (Figs. 6 and 17).

Pre-Mitotic 1

This phase begins with the release of microspores by the dissolution of the callose wall of the microspore mother cells. The released microspores possess a

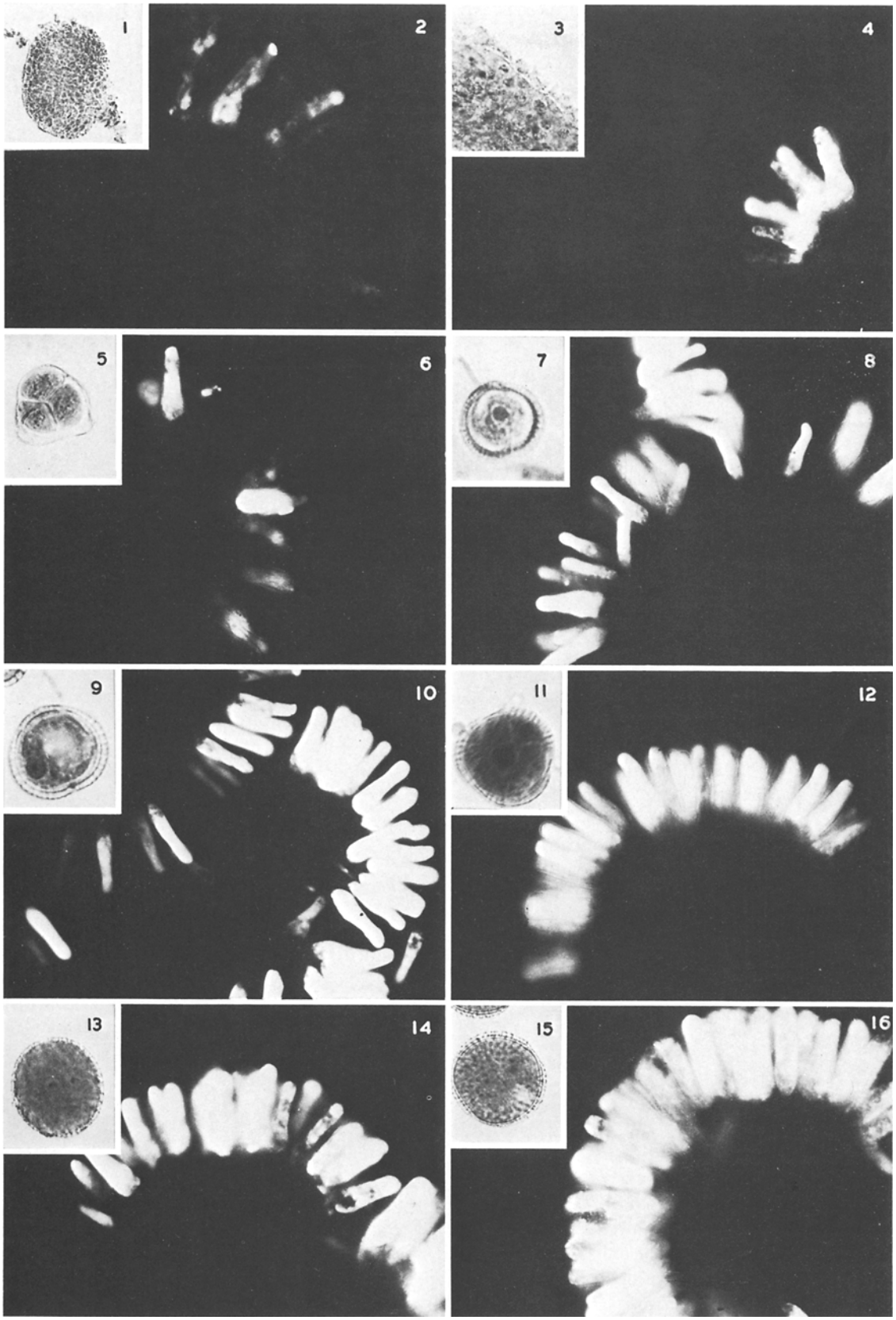

Fig. 1 – 16. 1, 2: Anther primordium and the rejection induced by it in the stigmatic papillae, respectively (1 – 14 X, 2 – 168 X). **3, 4:** Sporogenous tissue and a stronger callose deposition invoked by it as compared to that depicted in Fig. 2 (3 – 505 X, 4 – 135 X). **5, 7, 9, 11, 13 and 15:** The spore tetrad and microspore at stages 5, 6, 8, 9 and 10, respectively (5 – 505 X, 7 – 450 X, 9 – 337 X, 11 – 374 X, 13 – 505 X and 15 – 505 X). **6, 8, 10, 12, 14 and 16:** Callose rejection reaction induced by tetrad and microspores at stages 5, 6, 8, 9 and 10, respectively (6 – 117 X, 8 – 108 X, 10 – 108 X, 12 – 126 X, 14 – 117 X and 16 – 128 X)

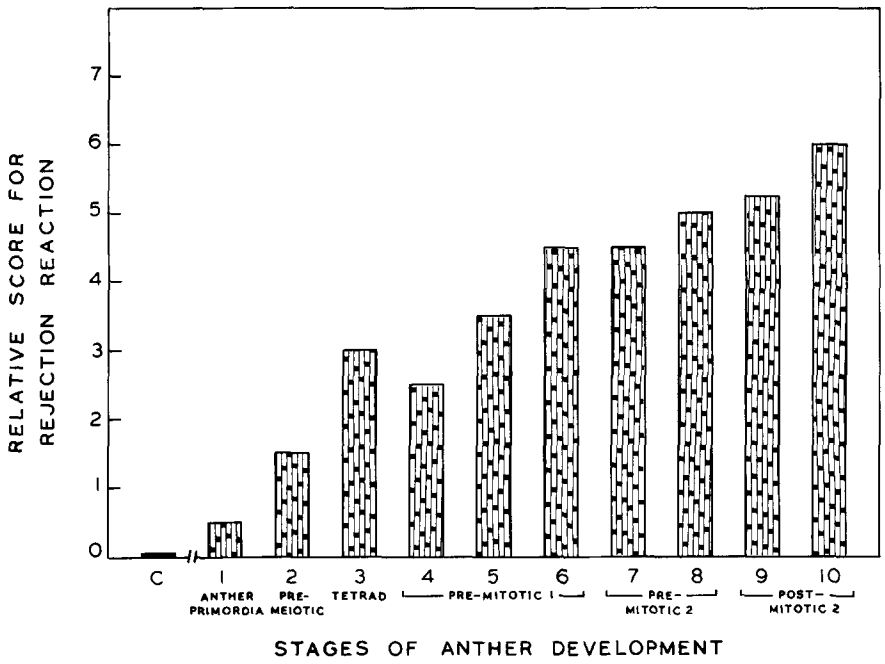

Fig. 17. Histogram illustrating the relative intensities of callose deposition in the stigmatic papillae after the application of tissue contents taken at different stages from the same plant (anther primordium to mature pollen; c = control)

centrally located nucleus (stage 4). The subsequent development of a vacuole begins to push the nucleus towards the periphery of the pollen cell (stage 5, Fig. 7). Simultaneously, the microspore nucleus undergoes S-phase, in preparation for the first pollen mitosis and comes to lie at the periphery (stage 6, Fig. 9). Buds (1.5–2.0 mm in length) with green anthers contained microspores at stage 4 and although the intensity of the rejection reaction showed a decline, it was more than that induced at stages 1 and 2 (Fig. 17). Microspores at stages 5 and 6 were found in buds varying in length from 2.0–2.5 and 2.5–3.0 mm, respectively; anthers were yellow-green. The intensity of the rejection reaction increased gradually at stages 5 and 6 (Figs. 8, 10, 17).

Pre-mitotic 2 to Post-mitotic 2

This period starts with the completion of the first pollen mitosis when the pollen becomes 2-celled and when the generative cell is situated peripherally (stage 7). The end of this stage is marked by the disappearance of the central vacuole. At the next stage, 8, the microspore becomes full of homogeneous cytoplasm, with the generative cell still attached to the wall (Fig. 11). Subsequently, the generative cell detaches from the peripheral wall and undergoes mitosis to form two male cells. Simultaneously, the cytoplasm becomes granular owing to the accumulation of starch (Fig. 13, stage 9). The microspore at anthesis has been recognised to represent mature pollen (stage 10, Fig. 15). The entire post-mitotic events occur in 3.5–4.7 mm long buds during which the microspores show a gradual increase in their potentiality to induce callose in stigmatic papillae (Figs. 12, 14, 16, 17).

Vegetative and Floral Tissues

The various sporophytic tissues tested for their rejection reaction inducing ability were vegetative (stem and leaf) as well as floral parts (sepal, petal, anther filament, dehisced anther, unfertilized ovule, fertilized ovule, ovary wall and fruit wall). All these tissues induced callose deposition in self-stigmatic papillae but the intensity of callose deposition was variable.

Discussion

In SSI systems, the incompatibility phenotype of the male gametophyte is controlled by the parental (sporophytic) genotype (Riley 1936). This has been ascribed to early S-gene action, pre-meiotic or meiotic, by Pandey (1960). However, Heslop-Harrison et al. (1973) consider that the synthesis of the incompatibility factor does not take place in meiocytes themselves; instead it is synthesized in tapetum and is subsequently transferred to pollen exine during development. This con-

clusion is essentially based on their two observations, namely (i) during the later stages of anther development, several tapetally synthesized substances are transferred to pollen in *Lilium* (Heslop-Harrison 1968 b) and (ii) pollen leachates as well as tapetum fragments are capable of inducing rejection reactions in self-stigmatic papillae (Heslop-Harrison et al. 1974; Heslop-Harrison 1975). In the meantime, Dickinson and Lewis (1973, 1975) have also demonstrated that tryphine, synthesized in tapetum, can invoke-stigma rejection reaction response in the absence of pollen in yet another crucifer, *Raphanus sativus*.

However, the present investigations have shown that not only pollen at different developmental stages, but even anther primordium without any trace of wall differentiation, is capable of inducing a rejection reaction in self-stigma. This indicates that the capacity to induce rejection reactions is not *limited* to tapetum or pollen grains exclusively; rather it *is* present in all sporophytic tissues (vegetative and floral). Even earlier, antigenic similarity has been shown between various sporophytic tissues (petal, corm and stigma) on one hand and pollen on the other (Clarke et al. 1977).

Another observation made during our study is the increasing potential of pollen for inducing the rejection reaction subsequent to the release of microspores from the callosic microspore mother cell (MMC) walls. This indicates that with the development of pollen, the concentration of the leachable active fraction responsible for inducing the rejection reaction also increases. The enhancement in its concentration may be augmented by tapetum during anther development, as already suggested by Heslop-Harrison (1968 a). Although there are many studies indicating the transfer of substances (proteins, enzymes, lipids, etc.) from tapetum to exine (Heslop-Harrison et al. 1973; Knox et al. 1975), in none of them has the transfer of specifically identified factor(s) responsible for the rejection reaction ever been shown.

Alternatively, the increased capacity of mature pollen to incite callose deposition may be due to the gradual transfer of factor(s) from the pollen cytoplasm to the exine carried over from the diplophase, thus making them easily leachable in higher amounts during the pollen-pistil interaction. The earlier investigators (Heslop-Harrison et al. 1973, 1974) based their conclusion about the role of tapetum in contributing incompatibility factor(s), on the assumption that isolated tapetal cells, or even their fragments, were free of any physical or chemical contamination. However, it is well known that contact of even a short duration (1 minute) is sufficient for the elution of the rejection reaction inducing fraction(s) from pollen exine. Moreover, it is very difficult to isolate tapetal cells without any contamination from either pollen or other anther wall layers. Further, Heslop-Harrison (1978) has himself remarked that "Certainly it seems inconceivable that the kinds of communication between cells seen in pollen-pistil interactions should be restricted wholly to the reproductive phase and indeed there is evidence that the S-gene specificities themselves are present in somatic cells". Therefore, the views expressed by Heslop-Harrison et al. (1973, 1974) and Dickinson and Lewis (1973, 1975) need some more careful rethinking.

To conclude, the observations presented here prove that rejection reaction inducing factor(s) is/are present in all the sporophytic tissues of a sporophytic self-incompatible plant. As a consequence of the present study, for isolation and characterization of this/these factor(s), one need not restrict the explorations to pollen and/or tapetum. Rather, it should now be easy to carry out such investigations with vegetative tissues (leaf or stem) which are available in ample amounts in comparison to pollen and/or tapetum.

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