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Pollen grain on the compatible and incompatible stigma of *Secale cereale* L.

Ziarno pyłku na zgodnych i niezgodnych znamionach *Secale cereale* L.

ABSTRACT

The course of pollination processes, pollen morphology and the degree of stigma pollination both in self-compatible (four lines) and in self-incompatible (two cultivars) plants of *Secale cereale* were examined. It was ascertained that either the self-compatible or the self-incompatible plants produced in anthers large amounts of the vital pollen, which during pollination was deposited in different manner on the stigma surface. It is worth noting that the presence of the leftovers of tapetum between mature pollen grains only in the anther of two self-incompatible cultivars was observed.

Keywords: pollination, compatibility, stigma, pollen grain, rye

STRESZCZENIE

Tematem badań było prześledzenie budowy morfologicznej ziaren pyłku oraz procesu zapylenia u kilku linii *Secale cereale*. Badano cztery linie, dwie z nich były samozgodne, a dwie samoniezgodne. Obserwowano różnice w reakcji ziaren pyłku w zależności od tego, czy zostały zdeponowane na zgodnym lub niezgodnym znamieniu. Ponadto w pylnikach samoniezgodnych linii odnotowano obecność resztek tapetum pomiędzy dojrzałymi ziarnami pyłku.

Słowa kluczowe: zapylenie, zgodność, znamię, ziarna pyłku, żyto

INTRODUCTION

Self-incompatibility has been known in flowering plants for over a century since Darwin's description in 1876 (5). The physiological mechanisms involved in self- or cross-fertilization

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have significant effects on population structure and have potential diversification and evolutionary consequences (32). From the moment of the release from the anther, the mature pollen grain functions as an independent organism, the main task of which is to deliver the sperm cells to the embryo sac. However, recognition processes take place before this happens – the processes of acceptance or rejection of incompatible pollen. These mechanisms take place on the surface of the stigma, inside the stigmatoid layer and in the tissues of the pistil. If the pollen is incompatible, then processes preventing possible fertilization take place, on the surface of the stigma or in the tissues of the pistil part – style and ovary. Whereas, when the pollen is compatible, all the processes preceding fertilization occur correctly. On the stigma of the pistil the accumulation of many matured pollen grains takes place. These pollen grains interact and compete with one another. The rivalry between the pollen grains depends on their natural feature combination, genetic unity within the population and the degree of homozygocity (19, 22). This phenomenon influences the flow of alleles to the genome of the future embryo and next sporophyte and plays a vital part in the evolutionary development of higher plants (21, 32).

The phenomenon of self-compatibility is defined by Nettancourt (26) as the unfitness of fertile hermaphroditic seed-bearing plants to create zygotes after self-pollination. According to Nasrallah (25), self-incompatible is pollen rejection at some point in the pollination process, depending on the species. It is estimated that self-incompatibility takes place in more than the half of all angiospermous plants (17).

Self-incompatibility is a genetically controlled mechanism, functioning in female sporophyte tissue, encompassing the reactions of recognition and rejection of a pollen of a specific genotype. In heterostylous flowers, self-incompatibility is controlled by a singular multiallelic S locus, by many researchers considered to be the most polymorphic plant locus (11). It is commonly assumed that the expression of the same S alleles in the pistil and in the pollen placed on its stigma leads to the recognition of the pollen as incompatible and as a consequence causes the rejection of the pollen (17).

Two different mechanisms of genetic self-incompatibility among angiospermous plants are described: gametophytic and sporophytic (8, 13). Gametophytic self-incompatibility is most common among plants and thoroughly examined at the representatives of the following families: Papaveraceae, Poaceae, Rosaceae, Solanaceae, Scrophulariaceae (27). In most of the listed plants, the process of self-pollination is controlled by a single locus, but it also may be controlled by 2 loci (some of Poaceae), or even by 3 or 4 loci (some of Ranunculaceae and Chenopodiaceae) (10). Inhibition of the growth of the pollen tube takes place as a result of interaction between the products of the same alleles of pollen and pistil, and the system of incompatibility has to be achieved by the proteinous products of S gene, which are synthesized and located in the cytoplasm or exine of the pollen grain (15). For example, in the anther of *Brassica oleracea* two cooperating proteins of molecular mass 7.5–10 kDa were identified, existing in the pollen and tapetum and taking part in the recognition reaction (1, 9).

Rye (*Secale cereale* L.) is an anemophilous plant and produces huge amounts of light pollen. Released pollen grains are moved by the wind to the pistil's stigmata. The rye's pistil did not have a style, and branched stigma was directly joined to the ovary. The proximal part of both forks was covered with tiny hairs directed upwards. The distal part of stigma contains feathery, multicellular, extensive papillae (multicellular and multiseriate papillae). The stigma of rye was dry, forked into two lobes; it had feathery structures, which was conductive to the retention of large amounts of pollen grains. The whole stigma formed the receptive area (15).

In the present work, four lines of rye (*S. cereale*) with the self-compatible gene and two cultivars (Amilo and Kier) characterized by self-incompatibility were examined. The aims of this work were examination of morphology of anther and matured pollen grains, the course of the pollination

process, the degree of the stigma pollination both in self-compatible and in self-incompatible plants, respectively.

MATERIAL AND METHODS

The material of the research consisted of plants (seedlings) received from DANKO Institution of Plans Breeding (Plant Breeding Department DANKO) (located in Laski, Poland): four self-compatible lines: Ls190, Ls193, Ls225, Ls250 and two self-incompatible: Amilo and Kier. The self-compatible lines were the inbreeding lines originating from the crossing of self-incompatible varieties with the self-compatible line S 176_{86} from Stuttgart.

The observations concerning the course of the pollination processes were carried out at the same time for all tested rye lines and cultivars. At the beginning of anthesis phase, appropriate spikes of the examined plants were covered with isolators. During anthesis phase, spikes with isolators were shaken from time to time. To estimate the degree of stigmata pollination, cutting the basal, mature florets from spikelets and next stigmata were taken from pistils which under a stereoscopic microscope were observed. The morphology of anthers and pollen grains of particular tested lines and cultivars under scanning electron microscope was observed. The material (anthers and pollination stigmata) was previously fixed in a mixture of 2.5% glutaraldehyde and 2.5% paraformaldehyde in phosphate buffer of pH = 6.8. Next, the material was dehydrated in an acetone series to dry acetone, and saturated with CO_2 at the temperature of 40^0C under the pressure of 70 standard atmospheres. The examination of the ultrastructure of the pollen wall was conducted using the transmission electron microscope Tesla BS 500 after standard coating with epoxide resin LR White.

To identify the presence of callose, specific staining with Aniline Blue and fluorescence microscopy was used. The florets from central part of spikes were fixed in 3:1 mixture of ethanol: glacial acetic acid. Fixed anthers were stained with 50 µg/ml Aniline Blue in 50 mM KH_2PO_4, pH 8.2 (18). The paraffin sections were observed under a Nikon Optiphot-2 microscope with DM 400 nm filter set for callose detection. To check, if pollen tubes grew into stigmatoid tissue, macerated preparations, dyed with aniline blue were prepared and analyzed under a fluorescence microscope.

In *in vitro* conditions, 2% sucrose solution, the ability of mature pollen grains, taken from cracking anthers, to germinate into pollen tube was checked (2).

RESULTS

Stigma surface of *Secale cereale* has feathery structure. It is adaptation to the retention of the large amounts of pollen grains.The whole stigma forms the receptive surface (Fig. 1a).

The observation of the degree of pollination of the stigma of all the tested lines and cultivars were conducted on the spikes covered with isolators. On the stigma, relatively large amounts of pollen were situated, but one could observe a kind of diversity in the degree of stigmata pollination depending on the examined line. It was arbitrarily assessed as large or medium. The greatest amount of pollen was placed on the stigma of self-incompatible Amilo and Kier cultivars (Fig. 1b) and self-compatible Ls193, Ls225 lines. Whereas, in the remaining self-compatible Ls190 and Ls250 lines medium pollination of stigmata was observed. Moreover, significant differences in the manner of placement of pollen on the stigmata among the examined lines were observed. In most of the examined lines

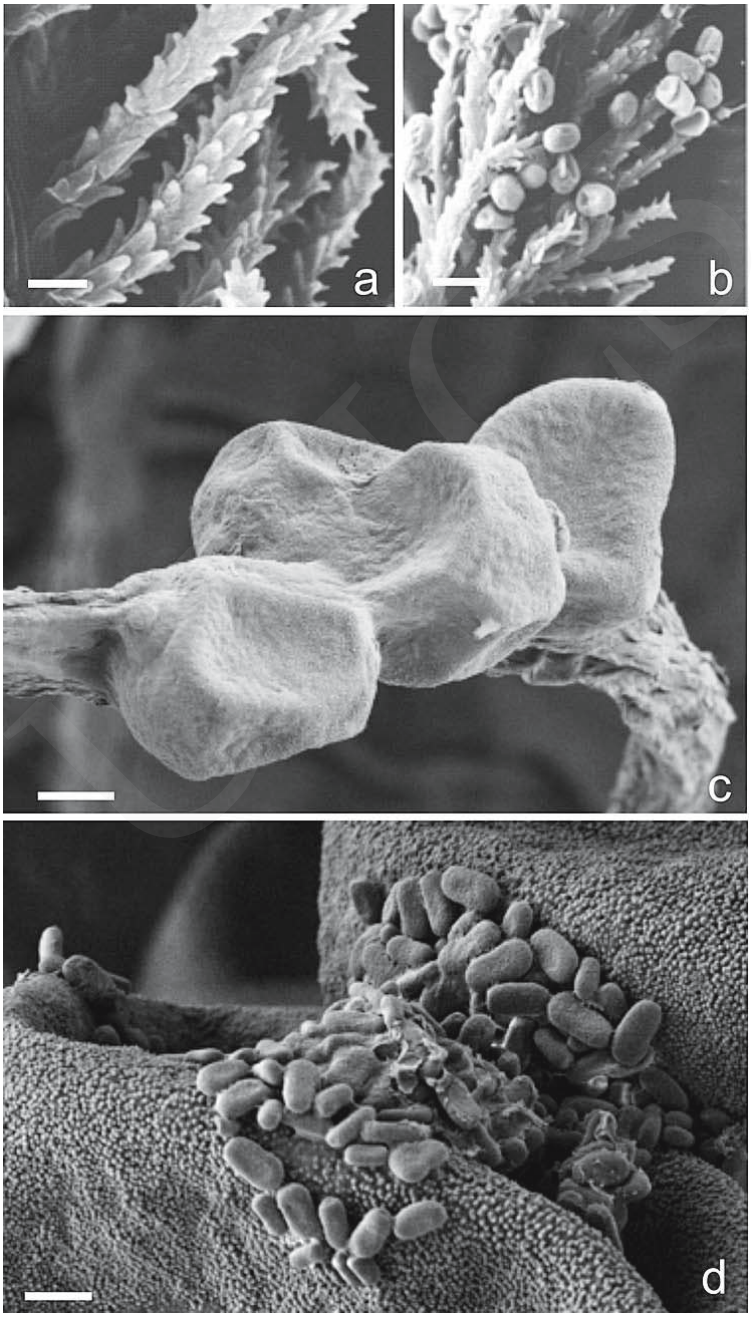

Fig. 1. a – rye pistil taken in the period of anthesis phase Ls 250. SEM; b – abundant pollination of the whole surface of the stigma of the self-incompatibility Amilo cultivar. SEM; c, d – pollen grains deposited on the tops of the stigma of the self-incompatibility Ls193 line. SEM. Scale bars = 1 μm

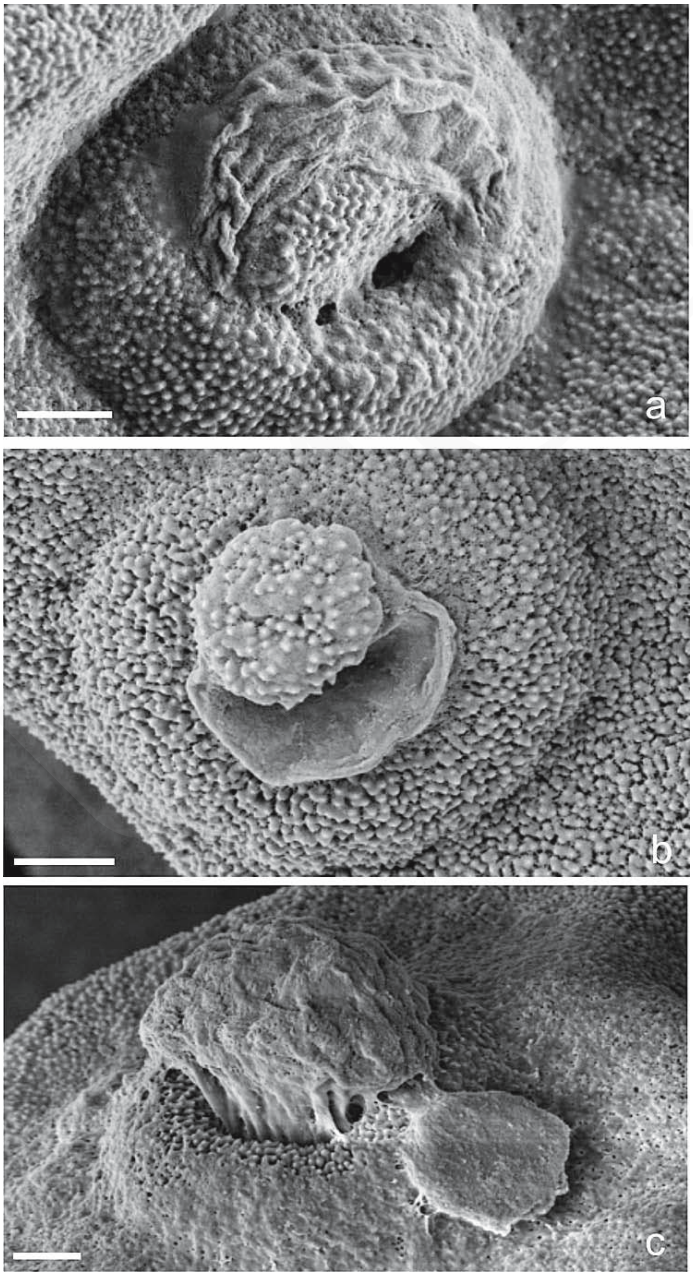

Fig. 2. a – pollen deposited on the stigma of the self-incompatibility Kier cultivar. SEM; b – the stigma of self-incompatible plant of Amilo cultivar with germinating pollen after staining with aniline blue. Fluorescent microscopy; c – visible anther's wall with pollen grains turned with the pori towards tapetum. Amilo cultivar. SEM; d – pollen grains with the tapetum leftovers within anthers of the self-compatible Ls225 line. SEM. Scale bars = 1 μm

and cultivars, except the Ls193 and Ls225 lines, the pollen was evenly distributed on the stigmata. In the Ls193 line, most of the pollen was placed on the upper part of the stigmata, and little amountwas in the central part. Whereas, in the Ls225 line, the largest accumulation of pollen in the central part of the stigmata's lobes was observed.

Next, the germination of pollen grains *in vivo* into pollen tubes on the surface of stigmata was examined. In self-incompatible Amilo and Kier cultivars, in spite of large and even pollination, only a few pollen grains germinated into pollen tubes (Fig. 1c). In the self-compatible line, on the surface of stigma, pollen tubes germinating inside the pistil's tissues appeared. The largest number of germinating pollen grains was observed in the Ls193 and Ls225 lines.

At the anthesis phase, in all the tested rye lines and cultivars, in each pollen sac within anther a peripheral arrangement of pollen was observed, i.e. pollen grains created a regular cylinder adjoined to the anther's wall, whereas the centre of the pollen sac loculus was devoid of pollen. Interestingly, within pollen sacs between particular pollen grains a thin layer of degraded tapetum (Fig. 2d), only (especially) in self-compatible Ls193 line was observed. These unresorbed tapetum was located near the radial wall and created a gluing layer. Such a substance was observed neither in the anthers of self-compatible Ls190, Ls250 lines, nor in the case of self-incompatible Amilo and Kier cultivars. In mature pollen sacs, free pollen grains were located, and tapetum was fully resorbed in mentioned above lines and cultivars.

The observation on pollen germination showed that on the stigmata of self-incompatible lines, only few of the placed grains germinated into pollen tubes; for the rest of it, callose plugs appeared both in the short growing pollen tubes and in the porus of the grains adjoined to the stigma cells but not germinating. Some of the pollen tubes were left on the stigma surface with large amounts of callose plugs. Whereas, in self-compatible lines, pollen tubes grew under stigma and headed towards the ovule in ovary.

The study on the ability of mature pollen grains to germinate into pollen tube in *in vitro* conditions showed that pollen grains started to germinate already after 30 min. The percentage of germinating pollen grains was high, about 80%. Mostly small, poorly formed grains did not germinate. Successive steps of germination pollen grain into pollen tube are shown on Figure 2 a-c.

DISCUSSION

The process of pollination, that is deposition of pollen on the stigma, is a very important stage of generative reproduction of flowering plants. The family Poaceae includes typical anemophilous species. Anemophily evolutionary was the earliest mechanism of moving of pollen, but it is often a secondary adaptation to

environmental conditions, e.g. in a region of a shortage of pollinating insects, plants may evolve from entomophilous into anemophilous. The pollen of an anemophilous plant is carried by wind in different directions, and does not always land on a proper stigma. To increase the chances, plants have created many adaptations enabling effective pollination. These include: huge amount of produced pollen for one ovule and extended surface of stigmata. In the examined both self-compatible and self-incompatible lines of *Secale cereale*, the dusting of stigma was abundant (12, 31). The structure of stigma was conductive, with high segmentation and numerous multicellular papillae, creating a large prehensile surface. Hundreds of pollen grains were placed on the stigmata, and they remained there even for up to 2 weeks. Among tested in this study self-compatible lines, the strongest pollination was observed in Ls193, Ls225 and Ls250 lines. Among analyzed four lines and two cultivars, different degrees of pollination were observed. This diversity was not connected with the feature of auto- or allogamy. However, the placement of pollen on the receptive surface was diverse.

In Ls193 line, abundant pollination appeared only on the tops of stigmata, the central and lower part was devoid of pollen, and in Ls225 line most of pollen was placed in the central part of the stigma. In four examined self-compatible lines the large number of pollen germinated in pollen tubes on stigma surfaces. In self-incompatible Amilo and Kier cultivars, stigma pollination appeared mean, but only few pollen grains created pollen tubes. At the anthesis phase, within mature anthers of self-incompatible Amilo cv., free pollen grains were observed, whereas tapetum was fully resorbed. In the case of two out of four examined self-compatible lines, unused tapetum laid in pollen sacs and between pollen grains. However, the presence of the leftovers of tapetum did not hinder spilling the pollen out of the pollen sacs, while in every inbred line a high pollination of stigmata was observed.

The placing of a pollen on a stigma is the first stage of recognition between male gametophyte (pollen grain) and sporophytous stigma. In case of a stigma dry in character, so-called pollen coat plays a crucial role in this process. Research carried out on *Arabidopsis*, proved that adhesion of pollen to the stigma's cells was possible thanks to lipophilic molecules present in the exine, and not in the pollen coat (33). Lipids played a crucial role also in the initial stage of the growth of pollen tube on the stigma. This fact has been proven by research conducted on mutants with defective lipid canals, the grains of which were unable to germinate. When triacylglycerols were added, hydration of pollen grains on the stigma appeared, and germination of such pollen into pollen tube was possible (22).

Literature data show that when pollen grains had been placed on stigma, their hydration occurred. This process may be facilitated by water canals in the plasmatic membranes of stigma's cells. The theses of Ikeda (16) prove that the reaction of

the rejection of self-incompatible pollen consists of the lack of possibilities for hydration of a pollen falling on a dry stigma in plants from Crucifereae family.

In reply to the recognition of pollen as incompatible, the inhibition of the growth of pollen tube appears (14**)**. The inhibition of the growth of pollen tube may occur:

– on the stigma, e.g. *Secale cereale, Papaver rhoeas*, *Brassica chinensis*,
– in the style, e.g. *Petunia violacea*, *Abutilon hybridum*,
– in the ovule, e.g. *Gasteria verrucosa*, *Reseda odorata*.

The recognition and rejection of the incompatible pollen grain in rye takes place on the surface of stigma. Incompatible pollen grain usually do not germinate at all, or short pollen tubes do not grow into the tissues under the stigma. So-called callose response is a cytological picture of the inhibition of incompatible pollen grains (23). In *Sinapis alba* in the aperture of pollen or on the surface of germinating pollen tube, and in the wall of papillae, to which pollen grain is adjoined, callose is synthesized (29).

Earlier embryological publications show that the germination of pollen depends on the pollen type – three-celled pollen grains germinate faster than the two-celled ones (4). In three-celled pollen, needed reserves of RNA and proteins are accumulated, necessary to pollen grain germination and the initial growth of pollen tube. Whereas, there are no such reserves in two-celled pollen, thus they have to be synthesized during pollen tube germination. The ability of pollen to germinate depends also on the blooming time and the position of the flower from which comes the pollen. In plants such as *Zea mays* or *Oryza sativa* most of the vital pollen develops in the florets which are opened the earliest, whereas, the next flowers from the same inflorescence produce less and less of the vital pollen. Usually, mature pollen only keeps its ability to germinate for a short period of time. Depending on the species, the pollen losses its vitality from a few hours to a few days e.g. *Zea mays*, *Triticum* spp., *Secale cereale* pollen keeps its ability to germinate for 5–7 days (24).

After the pollination on the receptive part of stigma, different substances are released from pollen intine, e.g. proteins of gametophytic origin, which react with the proteins from the receptive regions of the stigma. The research on *Brassica oleracea* conducted via transmission electron microscope by Vithanage and Knox (30) shows, that pollen proteins do not filter into papillae cells. This crucial moment for the expression of the reaction of compatibility or rejection occurs on the surface of stigma within the first few minutes. The penetration of stigma cells takes place only after the adjunction of pollen with the stigma wall as an effect of compatible pollination. Pollen grain is “taken” mostly in the region where its hydration performed by wall takes place, in this region cuticule is discontinuous. In the experiment carried out by the authors, it was proved that the shift of labelled

proteins to the region of the tops of papillae occurs, while cuticule is not an effective barrier. This fact suggests that after the initial hydration of a pollen and the release of proteins from the wall, they can go back to the stigma's cells and locate in plasmolema (7).

The character of pollen coat and the way it is formed on the pollen grain surface are a very important taxonomic feature. Regardless of the fact that it is so-called tryphine or pollenkitt, its function is very important during the period when it is adjoined to the stigma and during the first reactions of recognition. The use of isolated exine coating in *in vitro* experiences proves that in many species of plants this layer is responsible for the stigma's surface activation and adhesive features (28, 6).

Many authors point out the characteristic placing of the pollen in the anther of grass and all of them agree that in *S. cereale* all pori of pollen grains are turned towards the tapetum. In the later thesis from (20) Kirpes and co-authors slightly modify their former opinions, and claim that some of the pollen grains (small part of them) are placed precisely with the porus towards tapetum (3). These descriptions agree with our microscope observations of *S. cereale*. The location of pollen in all the examined pollen lines was "peripheral", but not all the pollen had porus adjoined to tapetum. This is one of the features characteristic of all the species belonging to Poaceae family. Only two species: *Anomochloa marantidea* and *Pharus lappulaceus* constitute an exception. Summing up the research concerning pollination both in self-compatible and self-incompatible lines of *S. cereale*, one may state that:

– the plants of both forms produce large amounts of vital pollen,

– the pollination of stigmata is abundant but it differs in the placing of pollen grains on the stigmata,

– regardless of significantly stronger pollination of the stigmata of self-incompatible plants, only few grains create pollen tubes,

– it is interesting that in some of the self-compatible plants, leftovers of not used tapetum are present; however, it does not make releasing of pollen from the anthers difficult.

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