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Differences in the structure of pollen in the ‘Senga Sengana’ and ‘Selva’ cultivars of *Fragaria* × *ananasa*

Różnice w budowie pyłku u *Fragaria* × *ananassa* w odmianach ‘Senga Sengana’ i ‘Selva’

Summary. The strawberry belongs to one of the youngest species of arable crops. The study aimed to analyze pollen's structure in the ‘Senga Sengana’ and ‘Selva’ cultivars of *Fragaria* × *ananassa*. The strawberry is a dicotyledonous plant from the *Rosaceae* family. The article presents research carried out on *Fragaria* × *ananassa* cv. ‘Senga Sengana’ and ‘Selva’ collected during flowering. Pollen morphology was examined using a scanning electron microscope and a traditional optical microscope. The results showed that fresh pollen outnumbered dead pollen, confirming that the ‘Senga Sengana’ cultivar produces larger amounts of fertile pollen. Mature ‘Senga Sengana’ pollen grains are characterized by a specific sculpture of the exine walls. The analysis of *Selva* pollen grains revealed substantial changes in the structure of the sporoderm during its maturation. It becomes thicker and, as the size of the pollen grain increases, the exine layer becomes more elaborate.

Keywords: pollen, strawberries, ‘Senga Sengana’, ‘Selva’

INTRODUCTION

Bees are pollinating insects playing an important role in ecosystems. They contribute to production of fruits by most plants. The share of honey bees in pollination of flowers is about 90%, whereas the rest is attributed to other insects – mainly bumblebees.

The importance of bees in the pollination of fruit trees and shrubs is not only associated with the increase in yields but also an effect on fruit quality. As a result of cross-pollination, better formed fruits with a greater number of seeds, a milder color, and better taste are obtained. Strawberries are one of such fruits [Capri and Marchis 2013, Konieczna and Krupa 2013, Garczyńska and Kostecka 2015]. The strawberry belongs to one of the youngest crop species [Yildiz et al. 2014, Cvetković et al. 2017]. This is a rare example of a fruit plant whose ripe fruit lies on the ground [Tešić et al. 2018]. The strawberry originates from wild strawberries. *Fragaria* × *ananassa* cv. 'Senga Sengana' is a perennial with a spherical, compact habit and robust growth, reaching a size of about 30 cm and producing quite numerous runners. The number of runners produced depends on soil conditions [Jung et. al. 2013]. The leaves are medium to large in size, with dark green leaf blades, which have smooth and shiny edges with broadly double serrations [Sargent et al. 2006]. A particularly important feature is the long and stiff petiole and the oval shape of the middle leaf. The flowers are numerous on highly branched loose inflorescences [Sargent et al. 2007]. The fertility of the cultivar is very high, making it one of the most prolific strawberry cultivars [Sargent et al. 2008]. The ripening period is quite late, which prevents frost damage. The cultivar also has a fairly long flowering period (about 25 days) [Mishra et al. 2015]. Chang et al. [2000] demonstrated that all fruits produced by flowers pollinated by *Apis mellifera* were well-formed, whereas those pollinated by *Apis cerana* showed a proportion of deformed fruits [Roselino et al. 2009].

Developmental processes such as changes in the pigmentation, senescence and abscission of flower organs, and growth and development of ovaries are controlled by pollination, promoting the reproductive success of plants [Wang and Lin 2000, Iqbal et al. 2017]. Completely fertilized flowers generate well-formed fruits, which mature within a short time. Strawberry plants have hermaphrodite flowers [Sargent et al. 2009]. However, the female reproductive organ becomes receptive before the male element, i.e. pollen, in the same flower is available [Zorrilla-Fontanesi et al. 2011]. This feature of strawberry flowers favors cross-pollination. Bees facilitate cross-pollination and are necessary for the development of strawberry fruits [Seeram 2008, Roselino et al. 2009].

Microsporogenesis is a reducing process that uses meiotic division to form microspores in the initial phase, which then develop into pollen grains, i.e. male gametophytes [Lu et al. 2014]. This process takes place in anthers of sexually reproducing plants [Seong et al. 2019]. Microsporogenesis involves cells of the sporogenic tissue called microsporocytes or microspore stem cells filling the microsporangium [Aharoni et al. 2004, Sargent et al. 2009]. These cells are formed through numerous mitotic divisions of sporogenous tissue cells. The duration of the meiosis process is varied and specific the species in which it occurs [Sargent et al. 2012]. In angiosperms, pollen is the male gametophyte. It is a strongly reduced organism surrounded by a wall with a unique structure [Hafidh et al. 2016]. The pollen grain wall, called sporoderm, is one of the most complex and the most durable cell walls [Peterson et al. 2010]. It contains various patterns of sculpture that differ between species [Jordan 2006]. In angiosperms, the sporoderm consists of two layers: external exine and internal intine [Vitten 2008]. Complex phenomena involving the microsporogenesis process produce fertile pollen grains [Roselino et al. 2009]. Mature and properly developing pollen grains are able to germinate into the pollen tube on the pistil stigma [Kilarski 2012]. Plant fertility is determined mainly by genetic mechanisms but also remains under the control of

external environmental factors such as bees, which increase pollination and improve fruit quality [Delaplane et al. 2013].

Pollen grains arising from microspores (male spores) of seed plants contain a heavily reduced male gametophyte. Pollen grains are usually spherical or ellipsoidal in shape, and their size varies greatly [Sujeet et al. 2017]. The wall of pollen grains has two layers. The inner layer is called the intine. It consists mainly of hemicellulose, cellulose, and pectins. Protein elements are also incorporated into the interior, but they are activated only after pollination [Skupień and Oszmiański 2004]. The outer layer, called the exine, is saturated with sporopollenin, i.e. a lipid substance, which gives the wall hardness and durability, including resistance to rot. It contains small amounts of lignin, proteins, and polysaccharides. It is resistant to mechanical and chemical factors. In the interior of pollen, two cells occupy most of the space: a large vegetative cell, also known as a tubular cell, and a smaller generative cell [Stevanović et al. 2019].

Currently, there are no research publications presenting strawberry pollen grains; therefore, the present study was focused on the structure of pollen grains, which may be helpful in palynological assessment of honeys with strawberry pollen grains.

The aim of the study was to trace the microsporogenesis and development of pollen grains in *Fragaria* × *ananassa* cv. ‘Senga Sengana’ and ‘Selva’ as well as the morphological features and structure of pollen grains.

MATERIALS AND METHOD

For embryological studies, anthers from *Fragaria* × *ananassa* cv. ‘Senga Sengana’ and ‘Selva’ were sampled at various stages of development. This material was collected from plants growing in the breeding collection of the Botanical Garden of Maria Curie-Skłodowska University in Lublin. The anthers were taken randomly from one hundred representatives of the ‘Senga Sengana’ cultivar on two dates. The first collection took place in the first decade of May, while the second collection took place in the fourth decade of May. The two harvest dates were intended to provide more material for better repeatability. The anthers of *Fragaria* × *ananassa* cv. ‘Senga Sengana’ and ‘Selva’ were isolated from flower buds and fixed with the paraffin method.

The anthers *Fragaria* × *ananassa* cv. ‘Senga Sengana’ and ‘Selva’ were placed in two fixers:

1) Navashin fixer (CrAF) – combination of formalin, acetic acid, and chromic acid,

2) Carnoy’s fixer – combination of ethyl alcohol and acetic acid.

Then, the fixed material was deaerated in a vacuum pump. The dyed material was rinsed with distilled water and then dehydrated in increasing concentrations of ethyl alcohol. The dehydrated sections were supersaturated with paraffin, using increasing concentrations of paraffin in acetone until pure paraffin was obtained. The liquid paraffin with the plant material was poured into aluminum molds and allowed to solidify. Blocks with the material were cut on a rotary microtome. The sections prepared in this way were glued to slides using Haupt adhesive and ethyl alcohol. Before using the correct dye, the paraffin was removed from the sections by washing the slides with the sections in a series of xylene and ethyl alcohol solutions. The preparation crushing method was used to stain the remaining preparations. Flower anthers stored in Carnoy’s

fixative were placed on a slide. Then, a few drops of the coloring reagent were added. The material in the dye drop was gently warmed over the burner flame. The preparations prepared in this way were closed with a slide, crushed, and viewed under a light microscope. The following dyes were used in the research: bright green, safranin, acetocarmine, aniline blue, DAPI, and Alexander's reagent.

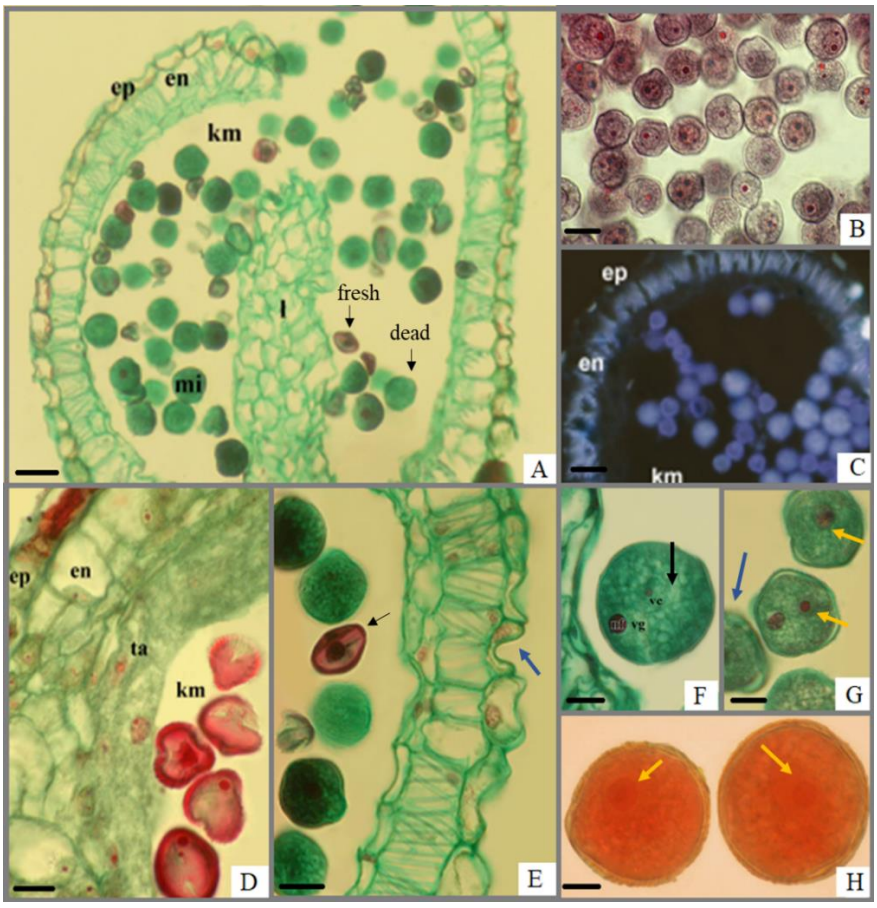
Strawberry pollen viability was checked by staining according to the Alexander method. This method makes it possible to distinguish fresh pollen from dead pollen. Flower bud anthers stored in Carnoy's fixative were placed on a glass slide. Then a few drops of the coloring reagent, malachite green, acid fuchsin, or orange G were added (Alexander's reagent forms a given dye in combination with 95% ethyl alcohol, acetic acid, and glycerol). The material in the dye drop was gently heated over the flame of the burner. Such preparations were closed with a glass slide, crushed, and examined under a light microscope. Malachite green cannot penetrate the cell wall. It stains cell walls and lipid envelopes green. Fuchsin, on the other hand, can penetrate biological membranes and stains the cell protoplast purple. Orange G is used to contrast the cytoplasm. In the staining image, fresh pollen grains exhibit green-stained cell walls and purple-stained protoplasts. Dead pollen grains are completely green. Fresh and dead pollen grains were counted in each field of view, giving a total number of not less than 300 pollen grains. The percentage of staining was determined by dividing the number of stained pollen grains by the total number of pollen grains in the field of view and expressed as a percentage. Data were analyzed using statistical software Sigma Stat. For statistical analysis of data we used one way ANOVA. Fresh and dead pollen within each species were compared using the t test ($P < 0.05$).

The study of the structure and development of *Fragaria* × *ananassa* cv. 'Senga Sengana' and 'Selva' was carried out using light field light microscopy (Nikon OPTIPHOT-2), fluorescence microscopy, and scanning and transmission electron microscopy.

In order to study the anthers under the electron microscope, the procedure of fixing and embedding the anthers in the LR-WHITE polymer was used. The dissected anthers of *Fragaria* × *ananassa* 'Selva' were fixed in a mixture of 3.5% glutaraldehyde and 3.5% paraformaldehyde in 0.1 M phosphate buffer (PBS) at pH = 7.2 at room temperature for 10 h. The fixed material was washed three times for 15 min with 0.1 M phosphate buffer and placed in a 4% aqueous solution of osmium tetroxide for 24 h at room temperature. After rinsing with distilled water, the material was dehydrated in a series of increasing concentrations of alcohol and acetone. The anthers were saturated in LR-White artificial resin and acetone mixtures. The material saturated with pure resin was sealed in gelatin capsules filled with the LR-White polymer and placed in an incubator for 24 h reaching the temperature of 55°C. The material embedded in the LR-White polymer was cut into semi-thin sections with a thickness of 1.5–2 μm with glass knives using a Reichert Ultracut S ultramicrotome. The anthers embedded in the LR-White polymer were cut into 65–70 nm thick ultrasounds using glass knives and then a diamond knife. The sections were placed on copper grids covered with a mold and dried in a Polon laminar chamber. Ultrathin sections were contrasted with uranyl acetate and lead citrate (Reynolds reagent). The preparations were observed and imaged using the transmission electron microscope (Zeiss Leo 912 AB).

RESULTS

Photograph A (Fig. 1-A) shows a longitudinal section through the anther of *Fragaria* × *ananassa* cv. ‘Selva’ strawberries. The layers of the anther walls, i.e. the epidermis and endothecium, and pollen chambers with microspores are visible. In photograph B (Fig. 1-B), there is a cross section through the anther. There are also microspores with clearly visible nucleoli. The anther wall is made of several layers. The most common are four layers.



c – cytoplasm, ep – epidermis, endothecium, tapetum, km – pollen chamber, nfg – generative cell nucleus, nfc – vegetative cell nucleus, sp – sporoderm, v – vacuole, vg – generative cell, zp – pollen grain

Fig. 1. Anther of *Fragaria* × *ananassa* ‘Selva’. A – longitudinal section stained with bright green, scale, 350×; B – cross section stained with safranin, scale 750×; C – stained with DAPI, scale 250×, D, E – stained with bright green, scale 750×; F – stained with bright green, scale 1500×, G – stained with bright green, scale 1100×; H – structure of two microspores stained with aceto-carmine, scale 1500×.

Photograph C (Fig. 1-C) shows a microsporangium with visible thickening of endothecium cells. Weak and slightly differentiated fluorescence after the use of DAPI is visible in some pollen grains filling the loculus and epidermal cell walls. Very strongly fluorescent leaf-like lumps are visible in the endothecium cells. In the anther of the strawberry shown in photograph D (Fig. 1-D), there are four or five layers building the wall of the anther. The outermost layer, i.e. the epidermis, is composed of large cells. These cells are elongated and flattened. They form a layer covering the rod. Photograph E (Fig. 1-E) shows the structure of the anther wall (marked with a blue arrow) surrounding ripe pollen grains (marked with a black arrow). Strip-shaped lumps are visible in the walls of the endothecium cells. The exact structure of the microspores is presented in photographs F, G, and H (Fig. 1-F,G,H). There are numerous microspores in a single pollen chamber. Individual microspores contain visible nucleoli. The cytoplasm (marked with a black arrow) of these cells is dense and stained gray-red. Some of the microspores have irregular shapes (marked with a blue arrow). The nuclei inside the microspore nuclei are stained red (marked with orange arrows).

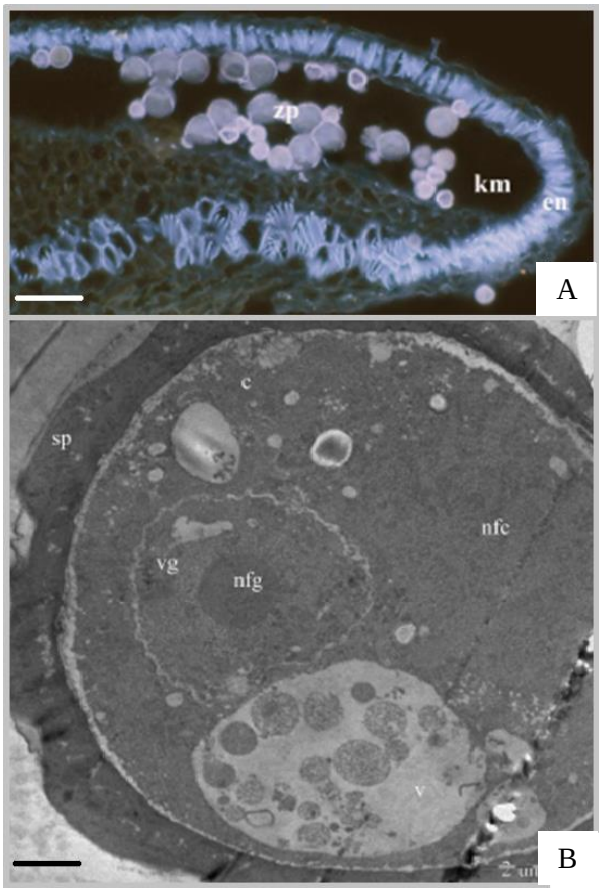
Photograph A (Fig. 2-A) shows a visible microsporangium with different sized pollen grains. Strong fluorescence of the wall beads is visible in the endothecium cells. Photograph B (Fig. 2-B) is a cross-section through the 'Selva' pollen grain at the two-cell stage. The pollen grain is surrounded by a thick sporoderm. The cytoplasm inside the pollen grain contains a few vacuoles and hardly visible organelles. A generative cell is visible inside the cytoplasm. The generative cell is surrounded by a callose wall. Inside, a nucleus with a large nucleolus is visible. On the right, the nucleus of the vegetative cell is barely visible.

Photographs A and B (Fig. 3-A, 3-B) show two pollen grains with a characteristic core of exine. The outer layer of the sporoderm is made of lamellar and band-like polysaccharide elements with the addition of fats or proteins. Cracks and gaps in the structure are often observed in this layer; therefore, the lack of color reaction in the place of porus is related to the discontinuous layer of the exine and the completely reduced intine.

Photographs C and D (Fig. 3-C, 3-D) show normally developed spherical pollen grains with a developed sporoderm, the nucleus of the vegetative cell, and the nucleus of the generative cell. The whole content of the viable pollen grain of *Fragaria ananassa* cv. 'Senga Sengana' is stained red with acetocarmine, while pollen that shows irregularities in development and structure is yellow. Such degenerated pollen grains are often smaller and heavily shrunk, with substantial changes in the structure of the sporoderm. A dead pollen grain without the cytoplasm is shown in the upper right corner. Mature pollen grains of *Fragaria* × *ananassa* cv. 'Senga Sengana' are shown in photographs E and F (Fig. 3-E, 3-F) (images from the scanning microscope).

The results obtained show that the anthers of the strawberry cultivars studied in this work have a similar structure to those found in other angiosperm species. In pineapple strawberries, the structure of the anther wall depends on its development. In older developmental stages, the anther wall layers have a more complex structure and are more diverse. In summary, the pollen of the 'Senga Sengana' cultivar was mainly two-celled, but single-celled pollen with a normally formed sporodermal wall was observed as well.

Typical for dicotyledonous plants, the internal tapetum represented the secretory and microsporogenesis type (male meiosis), with simultaneous cytokinesis observed in angiosperms. The results showed that fresh pollen outnumbered the dead pollen, confirming that the ‘Senga Sengana’ cultivar produces larger amounts of fertile pollen (Tab. 1). In the anther loculus, there are approx. 300–350 pollen grains. In the viability test, fresh pollen grains are turn stained purple and, have a regular spherical, regular shape with three movements pori and larger sizes than dead grains. Non-viable grains are characterized by much smaller sizes; they turn green as a whole and their shapes are clearly flattened (Fig. 1-A, 3-D).



c – cytoplasm, nfc – vegetative cell nucleus, nfg – generative cell nucleus, sp – sporoderm, v – vacuole, vg – generative cell

Fig. 2. A – cross-section through the anther of *Fragaria* × *ananassa* ‘Selva’ stained with DAPI, scale 450×, B – cross-section through the pollen grain of *Fragaria* × *ananassa* ‘Selva’ in the two-cell stage stained with uranyl acetate and lead citrate. Transmission electron microscope (TEM)

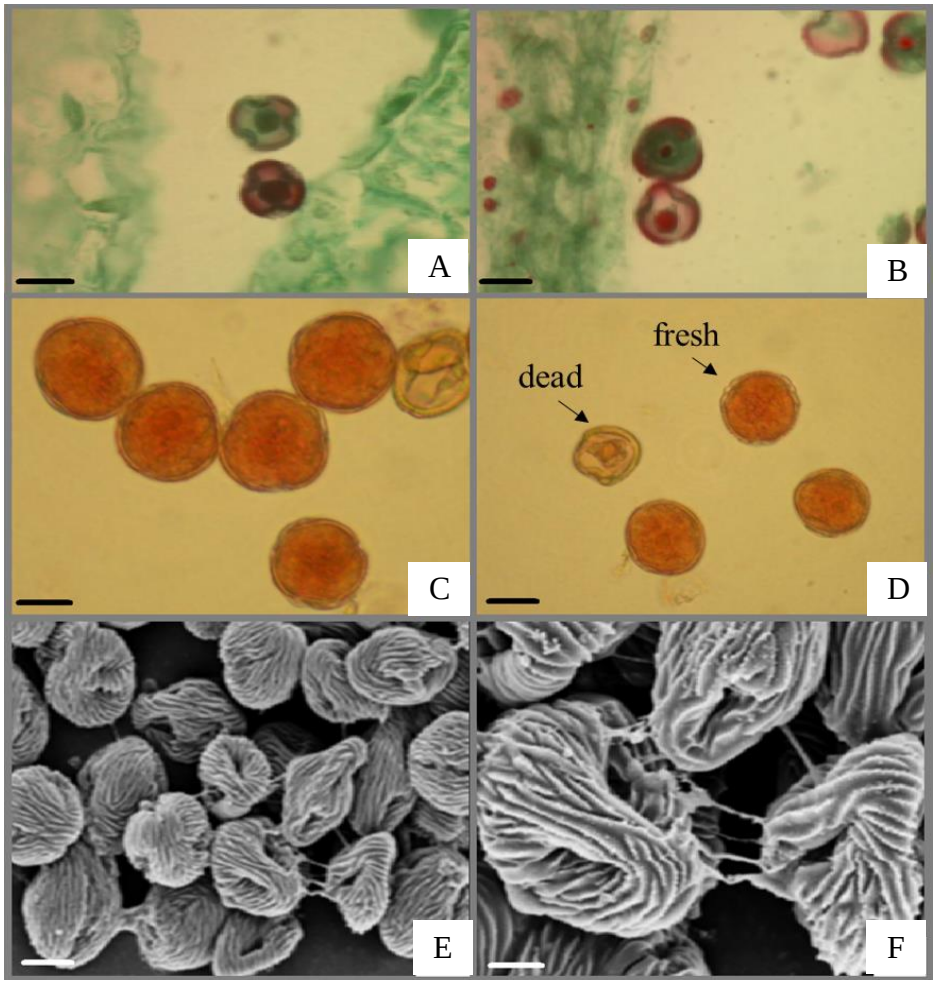


Fig. 3. Anther of *Fragaria* × *ananassa* cv. 'Senga Sengana'. A, B – mature pollen grain with a red-colored central nucleus of the vegetative cell, longitudinal section, stained with bright green, scale 750×; C, D – stained with acetocarmine, scale 750×; E, F – the outer part of the exogenous wall structure of mature heavily dehydrated pollen grains, scanning microscope (SEM); E (scale 4 000×), F (scale 10 000×)

Tab. 1. Percentage of stained fresh and dead pollen in both cultivars. Data are means (±S.E.) for five slides with two fields of view each (N = 10)

Cultivar of strawberry	Fresh pollen (%)	Dead pollen (%)	*t*	*P*
'Selva'	56 ±1.1	44 ±1.4	0.43	0.783
'Senga Sengana'	73 ±3.1	27 ±1.6	0.91	0.001

The mean percentages of stained fresh and dead pollen grains within each species were compared using t tests

Mature pollen grains of ‘Senga Sengana’ are characterized by specific wall sculpture. The analysis of the ‘Selva’ pollen grains demonstrated substantial changes in the structure of the sporoderm during its maturation. It becomes thicker and, as the size of the pollen grain increases, the exine layer becomes more elaborate and creates specific sculpture. The resulting two-cell pollen grains can take different shapes from oval, elongated, or triangular to round. The latter are characteristic of the pineapple strawberry cultivar ‘Selva’. In addition to the particular sculpture of the exine surface of ‘Senga Sengana’ pollen grains, there are also characteristic wall cavities visible as the collapsing external structure of the walls, which is most likely caused by the gradual dehydration of pollen grains in the mature stage.

DISCUSSION

As reported by Dybova-Jachowicz and Sadowska [2003], the exine of pollen grains has many diagnostic features, and the surface sculpture is one of the most important indicators. The pollen grain of the strawberry cultivar ‘Senga Sengana’ has protruding elements of sculpture, with which is protruding elements, as the so-called “positive” sculpture. The exine part of the sporoderm in this strawberry cultivar can be described as streaky, lamellar, and grooved. Such surface sculpture allows pollen grains to attach easily to the stigma [Jasnowska et al. 2008].

Strawberries are pollinated not only by the *Apis mellifera* species but also by other bees, i.e. *Trigona recursa*, *Paratrigona lineata*, and *Nannotrigona testaceicornis*. The presence of *N. testaceicornis* on flowers indicates that this bee species is attracted to strawberry flowers. Maeta et al. [1992] showed that flowers should only be visited four times by individuals of this species to develop well-formed fruits. Many authors [Roselino et al. 2009] have studied the pollination success of two stingless bee species. The differences in their results were associated with the mode of accumulation of accumulated pollen and nectar by bees. *Nannotrigona testaceicornis* is a small-sized bee species [Roselino et al. 2009]. Moreover, the influence of bees on the shape and sculpture of pollen grains should be investigated in the future. Bumblebees and honey bees are important pollinators, but bumblebees are more efficient than *A. mellifera* in pollinating fruit and vegetables in greenhouses [Li et al. 2006]. The introduction of *A. mellifera* hives during the flowering period significantly improved the setting of many fruit species [Li et al. 2006]. Bee pollination not only improves seed setting and the quality of crops such as fruits, vegetables, and forage crops, but also greatly facilitates the yield of fruits and oilseeds, including beans, sesame, sunflower, and rape. The effectiveness of bee pollination is related to their foraging behavior and the length of visits to pollinated plants, the number of visits, and the amount of pollen carried by the bees [He et al. 2019].

CONCLUSIONS

The innermost layer present in the pollen chamber is the lining layer otherwise called tapetum. It is one of the most important layers in the microsporangium. The proper functioning of the cells of this layer has a positive effect on the development of pollen

grains. The viability of pollen is a systematic feature and falls within range from several hours to several days. The amount of fresh and dead pollen can depend on the plant species. Pollen viability and pollen transfer efficiency in part determine the reproductive success of a species. It is unclear exactly which factors determine pollen viability in the tested strawberry species. The exact structure of strawberry pollen grains presented in the paper may facilitate palynological analyses of honeys and pollen deposition.

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Streszczenie. Truskawka należy do jednych z najmłodszych gatunków roślin uprawnych. Celem badań była analiza budowy pyłku u *Fragaria* × *ananasa* odmian ‘Senga Sengana’ oraz ‘Selva’. Truskawka należy do roślin dwuliściennych, do rodziny różowatych. W artykule przedstawiono badania przeprowadzone na pylnikach *Fragaria* × *ananassa* odmian ‘Senga Sengana’ oraz ‘Selva’ zebranych w okresie kwitnienia. Morfologię pyłków badano za pomocą mikroskopu elektronowego i mikroskopu optycznego. Pyłek odmiany ‘Senga Sengana’ był głównie dwukomórkowy, ale zaobserwowano także pyłek jednokomórkowy. Typowy dla roślin dwuliściennych wewnętrzny tapetum był typu wydzielniczego i mikrosporogenezy. Wyniki wykazały, że żywy pyłek przeważa nad martwym, co potwierdza, że odmiana ‘Senga Sengana’ produkuje większe ilości płodnego pyłku. Dojrzałe ziarno pyłku ‘Senga Sengana’ cechuje się specyficznym rzeźbieniem ściany. Po przeanalizowaniu budowy ziarna pyłku odmiany ‘Selva’ stwierdzono, że w miarę jego dojrzewania sporoderma wykazuje znaczne zmiany w budowie.

Słowa kluczowe: pyłek, truskawka, ‘Senga Sengana’, ‘Selva’

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