From Dimness to Glossiness—Characteristics of the Spring Rapeseed Mutant Form without Glaucous Bloom (*Brassica napus* L.)

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Received: 1 September 2020; Accepted: 5 October 2020; Published: 14 October 2020

**Abstract:** As a result of the treatment of “Vikros” spring canola with the chemical mutagen ethyl methanesulfonate (EMS), a high-protein mutant form without glaucous bloom (wax bloom) on leaves, shoots, and siliques was isolated. Segregation into glossy and glaucous forms was always observed in the progeny of glossy plants from self-pollination, and the proportion of glaucous plants could reach up to 25%. The progeny of glaucous plants were homogeneous and did not segregate. If during the period of seed germination and seedling development the soil did not dry out and remained moist, and the average daily temperature did not exceed 16 °C, then the amount of glossy plants could reach 99%. Glossy plants possessed qualities valuable for breeding forage varieties, such as the increased content of protein in seeds (more than 30%), and change phenol metabolism, что приводит к уменьшению содержания липина и синапина в сравнении с оригинальным сортом. In addition, plants without wax coating showed weakened shoot growth, decreased pollen fertility and seed production, and reduced lignin content in the shoots. Glossy mutants are of interest for the obtaining of fodder low-sinapine and low-lignin varieties of spring rapeseed.

**Keywords:** chemical mutagenesis; *Brassica napus* L.; wax bloom; morphological features; lignin; sinapine; seed coat
1. Introduction

The plant cell walls perform two main functions ensuring its integrity—maintaining mechanical hardness and protecting against damage by biotic and abiotic factors. Cuticle wax and lignin serve to these goals. Wax protects the plant from superfluous transpiration, UV irradiation as well as provides a barrier against pathogens and insects [1]. Disruptions in the formation of the wax layer can undermine survival, impair growth, delay development, and lead to a reduction of yield [2]. In annual crops sensitive to lack of moisture, the flowering period is shortened, and stem and seed production decreases [3]. Wax accumulates on the surface of plant aerial organs in the form of crystalloids or films [4]. The crystalloids are responsible for a glaucous dim bloom on the shoots and leaves of Brassica napus varieties and its relative species: Arabidopsis thaliana and Brassica oleracea L. [5]. Wax chemical composition may vary depending on the organ, stage of development, physiological state of the plant, and environmental conditions [4,6–8]. The basis of cuticular wax is constituted by fatty acids consisting of very long carbon chains (VLCFA) C_{20–34} and various compounds including primary and secondary alcohols, esters, alkanes, aldehydes, and ketones. [9]. Palmitic (C_{16:0}) and stearic (C_{18:0}) fatty acids are the precursors for VLCFAs [10,11]. It has been shown that the main role in maintaining the impermeability of the cuticle and response to water insufficiency play VLC alkanes of plant wax. Hormone as abscisic acid can be modulated wax biosynthesis. [12].

Cutin has similar biochemical features to lignin, one of the widespread compounds in second cell wall vascular plants. These compounds determine protection against pathogens, microorganisms as well as the formation of vascular systems in plants. Thus, the biosynthetic pathway leading up to the formation of suberin involves the fatty acid and phenylpropanoid pathways and is intimately associated with the biosynthesis of cutin and lignin. [6,13].

Lignin, which is associated with lignification, is another important component of plant cell walls, where the cellulose and hemicellulose are the basis of the cell wall [14]. It is a natural phenolic polymer with a high molecular weight, complicated structure, and composition. Lignin fills the pores between polysaccharides and accounts for approximately 20% of the total mass of the secondary cell wall [15]. Lignin begins to be deposited after cell differentiation is completed under the secondary thickening of the walls [16,17]. In dicotyledonous plants, a series of enzymatic reactions lead to the formation of monomethylated guaiacol (G) units from coniferyl alcohols, and dimethylated syringyl (S) units from synapyl alcohol [18]. Lignin biosynthesis genes are expressed mainly in vascular tissues in various stages of development, as well as in the apical shoot meristem (SAM), epidermal cells, and flower organs [15]. Most of the mutations in the lignin biosynthesis genes are responsible for the deformation of vascular system elements that lead to a hindered supply of nutrients to plant organs and, as a result, to retardation and deterioration of their growth up to dwarfism [19–21]. The effect of a reduction in lignin synthesis on growth and development has been described for different plant species: tobacco (Nicotiana tabacum), alfalfa (Medicago sativa), and others [19]. On the other hand, too much lignin in seeds reduces the nutritional value of the feed because it makes it difficult to digest. In addition to lignin, sinapine, an ester of choline synapate, has a negative effect on feed quality [22]. Lignin and sinapine are formed in different branches of the same phenylpropanoid synthetic pathway. Sinapine predominates in the seeds of the Brassicaceae family oilseed crops, including B. napus [23]. Sinapine is known to deteriorate the quality of rapeseed protein in forage [24].

Rapeseed (Brassica napus subsp. oleifera (Moench) DC) is the second most important oilseed crop in the world. For Russia, it is of particular interest, since it can be cultivated in a temperate climate with a low sum of effective temperatures. The seeds of modern varieties of spring rapeseed contain 24–27% crude protein that makes it also one of the main forage crops. Rapeseed is a natural allopolyploid (2n = 38), whose characteristics are determined by the genomes of two related species: Brassica rapa subsp. oleifera (DC.) Metzg.–genome A and Brassica oleracea subsp. capitata (L.) Metzg.–C genome [25]. These two Brassica genomes originated from an ancient ancestor related to Arabidopsis thaliana [26,27]. The use of chemical mutagens to induce variability in allopolyploid species, including rapeseed, is especially effective, since they do not cause mass death of the material [28], and the self-compatibility characteristic
of many polyploids allows the resulting recessive changes to be preserved. This method, developed by I.A. Rapoport, became widespread in the world [29–31]. To improve important technological traits of rapeseed, which does not show significant genetic diversity, chemical mutagenesis has become a common method [32–36].

As a result of seed treatment rapeseed cv. Vikros have identified forms with various changes in morphological characters (42 types of changes in total). Based on these forms, plant lines were created that manifested such traits as shatterproof resistance, shooting lodge resistance (lodging), with wide or very long siliques, variegate, and different color seeds (yellow, orange, red, purple), and others. In one of the forms, with low oil content and high protein in seeds obtained in the variant with EMS treatment at a concentration of 0.2%, the leaves, shoots, and pods have a yellowish-green color atypical for rapeseed and without glaucous bloom. The plants were also stunted and early maturing, but seed productivity was reduced.

This study aimed to examine the morphological and biochemical characteristics of the mutant form of spring rapeseed without glaucous bloom and to reveal the possible connection of this trait with impaired growth and reduced content of lignin and sinapine in seeds. It was important to determine whether the waxless on vegetative organs and siliques are associated with changes in phenolic metabolism and whether the absence of glaucous bloom can serve as a morphological marker for identifying breeding lines with a reduced concentration antinutrient compounds such as lignin and sinapin.

2. Materials and Methods

2.1. Plant Material

The work was carried out in the Kropotovo Biological Station (Moscow, Russia) and the Federal Williams Research Center of Forage Production and Agroecology (Moscow, Russia). The mutant forms and lines obtained by A.V. Shirokova at the Kropotovo Biological Station in 2010–2015 were used in this research.

2.2. Characteristics of Wild-Type Plants

The without erucic acid, slightly dehiscing and productive (2.5–3.0 t·ha⁻¹) cultivar of spring canola, “00”, B. napus L. “Vikros” (3480, Russian Federation), bred at the Federal Williams Research Center of Forage Production and Agroecology, was selected. Plants are 110–115 cm high and 35–40 cm in diameter; the number of I order shoots is about 3–5 pcs, and the II order ones up to 10 pcs. The shoots are strong; the leaves are bluish-green with a glaucous waxy bloom. The flowers are bright yellow, up to 1.7 cm in diameter. The siliques are glaucous-green, on average 7.5 cm long, 0.5 cm wide. The mass of 1000 seeds is 3.2–3.5 g. Seeds are dark gray, almost black, 1.6–1.8 mm in diameter, 22–26 seeds per pod on average. The average content of crude protein in seeds is 22–26%, oil—43–45%, fiber—7.5–9%, glucosinolates—13–15 µmol·g⁻¹ of seeds.

2.3. Seed Treatment with Chemical Mutagens and Obtaining of Mutant Lines

The seeds of “Vikros” was treated with chemical mutagens water solution with concentration (%): ethyl methansulfonate (EMS)-0.3; 0.2; 0.04; 0.03; 0.02; dimethyl sulfate (DMS)-0.08; 0.06; 0.02 and diethyl sulfate (DES)-0.06; 0.05 for 16 h. The control variant seeds were soaked in water. In each variant, 120 seeds were treated in two replicates. Young seedlings M₁ from treated seeds were grown in the greenhouse and were planted outdoors. To get seeds and next progeny plants, flower buds were isolated for self-pollinated. The seeds were collected from each plant separately, mutant lines M₂–M₅ were grown in the greenhouse and were planted in experimental plots.

Subsequent M₂–M₅ generations were also obtained by sowing seeds in a green-house, young plants at the age of 25–30 days were planted in the experimental field. Inflorescences were isolated with lutrasil bags at the beginning of flowering. Seeds were collected separately from each plant. To assess
the possible effect of inbreeding on plant variability, along with plants of the “Vikros” (wild type–WT) cultivar, I₁–I₄ plants from self-pollination of WT plants were grown as a second control for four years. The breeding lines of “glossy” plants used in the experiment differed among themselves in seed productivity, drought tolerance, and the degree of manifestation of growth violations under high moisture conditions. The glaucous plants “accompanying” glossy forms in the progeny of “glossy” plants are phenotypically similar to WT plants but differ by the branching degree and yield. The differences between the glaucous and glossy descendants of the same line mainly concern the habitus and the content of protein, oil, and fiber in the seeds. The biometric data (plants high and width, number of different order shoots, number of seeds in siliques, seed production) were obtained from 10 plants.

In this work, plants of M₆ generation of each of 8 lines (lines 1159, 1163, 1165, 1168, 1169, 1171, 1172, 1175) were planted in 40–45 pieces in outdoor. 30 plants of lines 1171 and 1169 were planted on a plot with regular watering, and 12 plants from lines 1168, 1169, 1171 were planted in a ground greenhouse. M₅ plants from all 8 lines were grown by direct sowing outdoor. The ratio of “glossy: glaucous” plants was calculated before planting. The plants were covered with 40 g m⁻² spandbond (LUTRASIL17, Weinheim Germany).

2.4. Biochemical Analysis

To determine the content of oil, protein, and fiber, a 2 g seed sample was taken from 10 plants of each line. The oil content in seeds was determined by the oil-free residue method [37]. The oil was extracted from the crushed seeds with 2 mL of hexane for 3 h. Then the filtered oil was methylated with sodium methoxide by a standard protocol used to obtain fatty acid methyl esters. The fatty acid composition was determined with a chromatograph Crystal 2000 M using a capillary column (ZB FFAP, 25 mm length, Phenomenex, Torrance, CA, USA) by standard technique (National State Standard R 51483-99 (ISO 5508-90). The fatty acids identification was performed according to the standard protocol.

The total nitrogen content was determined photometrically with a subsequent recount to protein using a coefficient of 6.25 [37]. The crude fiber content was determined by the Weende method.

The content of lignin, cellulose, and hemicellulose in shoots and seeds was determined according to the standard method [37]. To determine the ratio of cellulose, hemicellulose, and lignin in shoots, we used samples from plants of four lines (lines 1168, 1169, 1171, 1175). Young lateral shoots 12–15 cm long, formed in the leaf axils on the main stem at the end of the growing season, were cut from 3–5 plants of each line and combined into one sample. In the control variant, young shoots of the II order from WT plants were used. To determine the ratio of lignin, cellulose, and hemicellulose in seeds, 0.5 g of seeds from six glossy and four glaucous plants obtained from each of the lines No. 1168, 1169, and 1171, subjected to self-pollination, were taken separately. The seeds of WT plants (cultivar “Vikros”) were used as a control. The oil-free material of seeds and shoots was dried, ground, and a sample of 0.5 g was used for analysis according to the standard procedure [37].

Phenylpropanoids were extracted from 0.5 g crushed reddish-brown seeds with 96% C₂H₅OH acidified HCl (97:3). Samples (2 µL) were analyzed on an Agilent Technologies chromatograph (model 1100); chromatographic column 2.1 × 150 mm, with octadecyl silyl sorbent, grain size 3.5 µm, “ZORBAX-SB C-18”. Detection parameters: wavelength 330 nm; scanning time 0.5 sec; standard sinapic acid (Sigma-Aldrich D7927, Missouri, USA). We used three biological and three analytical replicates for all analyses.

Chromatographic conditions: feed rate of the mobile phase 0.25 mL·min⁻¹; column thermostat temperature 35 °C (Table 1).
Table 1. Gradient regime of chromatography.

| Time, min | A% H$_2$O (0.1% H$_3$PO$_4$) | B% MeOH |
|-----------|-------------------------------|----------|
| 0.0       | 90                            | 10       |
| 8.0       | 70                            | 30       |
| 25.0      | 20                            | 80       |
| 26.0      | 0                             | 100      |
| 30.0      | 0                             | 100      |
| 30.1      | 90                            | 10       |
| 35.0      | 90                            | 10       |

2.5. Scanning Electron Microscopy

Pollen grains and seed coats were studied by scanning electron microscope JEOL, JSM-6380LA, accelerating voltage 20 kV; IB-3 Ion Coater (EIKO, Shawnee, KS, USA), the thickness of Au layer was 20 nm.

To obtain seed coat surface and slice photos, coats sections of seeds from six plants was placed onto an SEM stub, which is covered by carbon adhesive tape (Double-Sided Carbon Tape, 8 × 20 mm, EMS Cat#77817-08-AL, Shanghai, China). The coats no less than 30 seeds were examined from the self-pollination of each of the six plants belonging to the M$_6$ generation of each line (lines 1168, 1169, 1171, 1175). Magnifications ×100–×2000 were used. The coat thickness was determined using the SEM Control User Interface Version 7.11 Copyright 2004 JEOL Technics LTD. Pollen from two upper anthers from three young flowers from I order inflorescences was taken in four wild-type plants and mutant lines 1171 and 1169. This pollen was placed on a stub onto disks from SPI Supplies Division of Structure Probe, Inc., West Chester, PA, USA.

Color images of seeds were obtained using an SMZ 1500 stereomicroscope (Nikon Instruments, Inc., New York, NY, USA), HR Plan Apo 0.5× Nikon objective, with a Nikon digital camera with DS-Fi2.

The data were statistically processed with the programs Microsoft Excel 2007 and SigmaPlot 12.2. The means and their SEs are reported in tables. The significance of differences was calculated with Student’s t-test. The means significantly different at $p \leq 0.05$ are indicated with different letters.

3. Results

We obtained the mutant glossy forms, which were distinguished by the absence of a glaucous bloom on the leaves, shoots, and siliques. Herewith, plants differ sharply from the wild type in the color of vegetative organs and siliques. In addition, the mutant forms have a yellowish shade in the color of shoots, leaves, and siliques (Figure 1).

3.1. Obtaining of Mutant Glossy Plants

Mutant plants were identified after 0.2% EMS treatment in the M$_2$ progeny of one plant. In the flowering phase 30 such plants out of 36 (83%) were observed, but when the siliques ripen, 12 plants had a light bloom appeared on the leaves. Few seeds have been formed in isolated inflorescences of the remaining 18 glossy plants, some of them germinated in siliques. When sowing in the spring, single seedlings were only obtained from the seeds of these plants.

Eleven plants of the M$_2$ generation produced viable seeds and plants, but only one of them generated the glossy forms in the M$_3$ generation (Figures 1 and 2). Segregation “glossy”:“glaucous” corresponded to the Mendelian ratio 3:1 (39:12).

“Glossy” forms were absent in the glaucous plants in the M$_4$-M$_5$ generations, while segregation was observed in the glossy plants (Figure 2). Moreover, the amount of glossy plants varied in the M$_4$ generation from 75% to 85%, while their number was higher and amounted to more than 98% in two lines of the M$_5$ generation (lines 1168 and 1169). Their number varied from 74% to 87% in the plants of the remaining six lines. The tendency persisted in M$_6$ progeny. So, the glossy lines produced from 3% to 10% of glaucous plants in their progeny, while the glaucous lines generated a homogeneous progeny with a glaucous bloom.
Figure 1. Morphological features of the leaf surface, fragments of the abaxial and adaxial leaf surfaces, the surface of stems and siliques of mutant forms (M₆) of spring rapeseed differing in the presence/absence of bloom. Gloss and the absence of a bloom on the surface of leaves, shoots, and siliques in glossy plants are clearly visible.

Figure 2. The pattern of the segregation of mutant lines of spring rapeseed (M₃–M₅), differing in the presence/absence of glaucous bloom. Mutant lines are designated as “glossy”. Segregation was absent in the progeny of glaucous plants.
3.2. Influence of Abiotic Factors (Humidity, lighting, Heat) on the Manifestation of the “Glossy” Trait

Observations have shown that the number of glossy plants depends on the conditions of seedling development during the period from seed germination to the development of the first four true leaves. Glossy seedlings are attacked by pathogens and die to a greater extent than do glaucous plants during temperature and moisture changes. To maintain soil moisture and protect it from drying out, the boxes with seedlings that had been prick out in the two cotyledons phase were covered with spunbond for 10 days. As a result, the amount of glossy plants of the M5 generation was 92–99% or more, while in the M4 generation their number did not exceed 87%. A similar ratio of about 86:14% (glossy:glaucous plants) was observed among seedlings that were pricked out seven days later and escaped from covering.

In field conditions, with regular watering, even at a daytime air temperature of more than 27 °C, no wax coating was formed on the leaves of flowering plants. On the contrary, with prolonged drought during the fruiting period, a weak grayish bloom appeared along large veins on adult leaves in 4–15% of plants of line 1175. However, both the shoots and the yellowish-green pods remained “glossy” in color. With a decrease in night temperatures to 8–10 °C in 8–12% of glossy plants grown by direct sowing in open ground (lines 1165, 1171), the appearance of weak glaucous bloom was noted on adult rosette leaves along the main veins. Plants did not form bloom in the greenhouse.

3.3. Morphological Features of Glossy Mutant Plants

Differences between glossy and glaucous plants are already observed at the germination stage. When wild-type seeds were germinated, the root appeared on the third day, and the cotyledon leaves were unfolded after 10–12 h. Seeds of “glossy” plants were swelled faster than those of glaucous mutants and wild-type plants, but germination was prolonged. The radicle could appear earlier than in wild-type seedlings, already on the second day, but the cotyledonous leaves were unfolded 24–36 h after the root appears. Strong elongation and curvature were characteristic of the hypocotyl of the “glossy” seedlings. Rare bristly pubescence was noticeable on the petioles and abaxial side of the first four true leaves of seedlings. The bristles were located not only on the main vein but also on the surface of the leaves.

Differences in quantitative traits were noted between wild-type plants and mutant lines (Table 2), associated with the peculiarities of their growth and development. In wild-type plants, the main stem is erect, productive shoots of I order are formed first in axils of the leaves closest to the main inflorescence, and then in axils of the third or fourth lower leaves. On the contrary, in mutant plants, the bulk of I order shoots grows first from the axils of the cotyledons and the lower leaves of the main stem. The main stem of glossy plants is shortened. In conditions of sufficient moisture, it is erect and the plants are narrow. In drought, the main stem of the glossy plants is curved, “lays down”. In such plants, shoots of III order develop, but siliques are not formed on them or are deformed with underdeveloped seeds. The total number of shoots in glossy plants varies greatly and can exceed 40; they are formed during the entire growing season. Glaucous plants have fewer shoots than glossy plants, but full siliques are formed on all shoots, and seed productivity is several times higher (Table 2).

Wild-type plants, usually, dry up and die off after the siliques ripen; their shoots are strongly lignified by this time. Young shoots can grow on first-order shoots in single plants during prolonged rains and low air temperatures. On the base of the main stem from axils of the lower leaves of glossy plants, young shoots grow even after the siliques are ripe.

Glossy plants develop faster, bloom about five to seven days earlier than glaucous plants, both mutant and wild type. This is because the rosette of leaves takes longer to form in glaucous plants. Inflorescences of glossy plants contained up to 35–45 flowers, while the wild type has an average of 70 flowers (Figure 3). Spontaneous self-pollination was found to be disruptive since the filaments of the four long stamens are often shorter than the style; the anthers are located below the stigma and dehisce weaker than in the wild type plants. Glossy plants produce about 3–4 times less pollen grains than wild-type plants, and fertile pollen is only 20–30%. The sterile grains were deformed.
Table 2. Quantitative traits of wild type and mutant glossy (Gs) and glaucous (Glc) plants from M₅ mutant lines.

| Line Number | Plant Height, cm | Plant Width, cm | Number of Shoots, pcs. | Branching Order, Silique/Spout Length, cm | Number of Seeds in Silique, pcs. | Seed Production, g Plant |
|-------------|------------------|-----------------|------------------------|-------------------------------------------|----------------------------------|--------------------------|
| Wild type   | 93.9 ± 4.3 a     | 28.3 ± 5.1 a    | 4.5 ± 0.8 a            | 6.3 ± 0.5 a                               | 7.3 ± 0.4 b/0.9 ± 0.2 a         | 25 ± 3.8 a               | 20.9 ± 1.7 a             |
| 1159 (Gs)   | 75.4 ± 8.4 b     | 31.7 ± 10.7 a   | 5.6 ± 1.4 b            | 18.7 ± 3.2 b, *10.1 ± 0.6                 | 5.4 ± 0.5 b/0.8 ± 0.2 a         | 13 ± 2.1 b               | 3.6 ± 1.0 b              |
| 1169 (Gs)   | 80.1 ± 6.6 c     | 19.3 ± 5.6 b    | 8.8 ± 1.9 b            | 15.2 ± 2.2 b, *13.5 ± 2.0                 | 6.2 ± 0.5 a/0.8 ± 0.1           | 21.8 ± 3.3 a             | 3.9 ± 1.0 b              |
| 1171 (Gs)   | 85.7 ± 9.1 a     | 37.9 ± 9.1 c    | 6.8 ± 1.7 b            | 16.3 ± 2.5 b, 12.4 ± 3.5                  | 6.0 ± 0.9 b/0.9 ± 0.3           | 20.0 ± 5.0 c             | 6.7 ± 1.9 b              |
| 1171 (Glc)  | 88.3 ± 5.7 a     | 28.8 ± 7.3 a    | 6.4 ± 1.4 b            | 12.2 ± 1.1 c, *2.6 ± 0.7                  | 7.4 ± 0.5 a/1.1 ± 0.4           | 25.2 ± 3.1 a             | *11.6 ± 3.5 c            |
| 1175 (Glc)  | 91.2 ± 6.3 a     | 27.8 ± 3.3 a    | 5.2 ± 0.9 b            | 10.3 ± 1.5 c, *6.2 ± 1.4                  | 7.5 ± 0.5 a/1.0 ± 0.3           | 24.6 ± 2.2 a             | 14.6 ± 2.4 c             |

The data were represented as mean values ± SE (n = 10), p ≤ 0.05 Means and their SEs are reported. Different letters (a, b, c) indicate significant differences between the means at p ≤ 0.05. *indicated values for branches of the III order.
Under long-drawn drought conditions, fewer siliques are formed on the plant. They are bending and curved on the upper shoots, shorter than in wild-type plants, and contain up to 22 seeds, a third of which are underdeveloped. Not only the siliques and seeds are deformed, but also the placentae themselves. They can be thin, filamentous, and twisted, or thickened in the part that connects to the septum (median plate). The siliques of both glossy and glaucous mutant plants are resistant to dehiscence. Manual harvesting requires effort to unhusk the seeds out of them. The silique valves are thinner in glossy plants, and, on the contrary, thicker and denser in glaucous plants than in WT plants.

Viable seeds are almost black, with brown siliques, round, flat, small, and medium in diameter, from 1.3 to 1.7 mm. Up to 10–40% of seeds with defects are formed. They are small in diameter: 1.0–1.2 mm, with a thin reddish coat, often angular. There were no external signs that would make it possible to distinguish and separate the glaucous and glossy forms by seeds. When rainy cool weather sets in, seeds germinate directly in the silique (up to 60%) of glossy plants. Seed productivity was higher in plants with a wax bloom (Table 2).
Differences in the color of the mutant seeds surface region were associated with the thickness of their envelope. The seed coats are deformed in the reddish areas, and the coat is thinner than in the dark areas (Figure 4). The walls of isodiametric cells form a foveate surface with raised borders and are arranged in rows on the surface of wild-type seeds. Various types of structural disturbances were observed on the seed coat surface of glaucous and glossy plants. They were most significant in the hilum region on the seeds of glossy plants (Figure 4, lines 1168, 1171).

**Figure 4.** The surface and thickness of the seed coat of wild-type plants, glaucous (1171a), and glossy plants (1171b, 1168). Bar 50 µm; line 1168 chip 100 µm.

In addition, differences in the thickness of individual layers of the seed coat were observed in mutant and wild-type plants. In wild-type plants, the outer part of the seed coat is 2–3 times thicker and formed by large cells, while in mutant seeds, the thickness of the inner and outer layers is almost the same. The thickness of various parts of the seed coat varies from 25 to 75 µm in mutant plants without a glaucous bloom, while it is more uniform and ranges from 35 to 50 µm in wild-type plants.
3.4. Comparative Study of Lignin, Cellulose and Hemicellulose Content in Shoots and Seeds of Wild Type and Mutant Plants

The amount of cellulose in the shoots is the most stable and differs little in mutant and wild-type plants (Table 3). At the same time, the amount of hemicellulose in the shoots of glossy plants is noticeably higher—more than 1/3 and more variable. On the other hand, the lignin content in the shoots of glossy plants is about 1/3 lower than that of wild-type plants. Herewith, the differences between plants with a glaucous bloom of both wild type and mutant forms are insignificant.

Table 3. Content of lignin, cellulose, and hemicellulose in shoots and seeds of WT and mutant plants.

| Genotype  | Lignin, % (± SE) | Cellulose, % (± SE) | Hemicellulose, % (± SE) |
|-----------|------------------|--------------------|------------------------|
| in shoots |                  |                    |                        |
| Wild type | 5.92 ± 0.38 a    | 24.2 ± 1.22 a      | 6.3 ± 0.52 a           |
| Glaucous  | 6.2 ± 0.59 a     | 25.7 ± 1.14 a      | 5.6 ± 0.62 a           |
| Glossy    | 4.02 ± 0.32 b    | 27.2 ± 0.89 a      | 10.2 ± 1.18 b          |
| in seeds  |                  |                    |                        |
| Wild type | 6.27 ± 0.31 a    | 10.32 ± 0.63 a     | 8.02 ± 1.08 a          |
| Glaucous  | 5.68 ± 0.39 a    | 8.22 ± 0.68 b      | 10.82 ± 1.20 b         |
| Glossy    | 3.42 ± 0.38 b    | 8.52 ± 0.8 b       | 14.4 ± 2.18 c          |

Lignin content is estimated as the percentage of acid detergent fiber in terms of dry matter. Data were represented as mean values ± SE, p ≤ 0.05. Different letters (a, b, c) indicate significant differences between the means at p ≤ 0.05.

In general, the ratio between lignin and polysaccharide content is 1/5 in wild-type plants, while this ratio is 1/9 in glossy plants. The differences in the content of lignin and hemicellulose in the seeds of mutant and WT plants were even more pronounced. The lignin content was 1/3 higher in seeds with a dark coat (WT), and hemicellulose content was almost half that in seeds of glossy plants. On the whole, the ratio of lignin and polysaccharides was 1:2 in seeds of wild-type plants, and it was 1:4 in seeds of mutant plants without wax coating.

3.5. Comparative Analysis of the Content of Sinapine and Sinapic Acid in the Seeds of Wild-Type Plants and Mutant Plants without Wax Bloom

Twenty-five phenylpropanoid compounds were found in seed extracts of wild-type plants, and twenty-four were found in brownish-red seeds of plants without glaucous bloom (Figure 5). The total amount of these compounds in the seeds of wild-type plants was 911 mg 100 g⁻¹ of seeds, and 204 mg 100 g⁻¹ ones were detected in mutant plants, that is, 4.5 times less. As the content of sinapine and sinapic acid in seeds is of practical importance for rapeseed breeding, data on these compounds are presented here.

Sinapine predominates in the seeds of plants, both glossy and glaucous, but it is three times less in mutant seeds (Table 4).

Table 4. Content of sinapine and sinapic acid (mg 100 g⁻¹) in seeds of WT and glossy plants.

| Substance  | Retention Time, t min⁻¹ | Wild Type    | Line 1169   |
|------------|-------------------------|--------------|-------------|
| Sinapine (5)| 12.58                   | 313.2 ± 7.5  | 94.2 ± 2.3  |
| Sinapic acid (15) | 18.17 | 42.3 ± 1.0 | 13.38 ± 0.3 |

(5) and (15)—numbers of compounds on the chromatogram in Figure 5.
At the same time, the progeny of plants with constant bloom did not segregate into glaucous and glossy plants and changes in the ratio between glaucous and glossy caused by increased moisture and shading were insignificant. The content of C16:0 increased by 6% in comparison with the wild type was found in the M4 seeds, but by the M6 generation, it decreased. The seeds of mutant plants without bloom, line 1163 almost twice as compared to the wild type.

### 3.6. Biochemical Characteristics of Seeds of Wild-Type Plants and Mutant M4 Plants

Comparison of the content of main storage compounds showed that the seeds of mutant plants, both with and without bloom, accumulate, in contrast to the wild type, much more protein, and the amount of oil decreases by 5–18% (Table 5). In addition, the content of crude cellulose increases significantly, in line 1163 almost twice as compared to the wild type.

#### Table 5. The content of fiber, protein, oil, and essential fatty acids in the seeds of WT plants and M5 mutant lines.

| Line   | Content, % | Fatty Acids |
|--------|------------|-------------|
|        | Crude Fiber | Oil | Protein | C16:0 | C18:0 | C18:1 | C18:2 | C18:3 |
| Wild type | 8.9 ± 0.9 | 45.3 ± 1.2 | 25.8 ± 1.1 | 3.7 ± 0.2 | 0.6 ± 0.2 | 64.5 ± 0.8 | 19.7 ± 0.6 | 9.6 ± 0.3 |
| 1159 | 8.6 ± 1.8 | 36.7 ± 1.2 | 31.8 ± 1.9 | 4.2 ± 0.6 | 1.4 ± 0.4 | 58.9 ± 1.3 | 23.6 ± 1.0 | 9.0 ± 0.5 |
| 1169 | 8.2 ± 1.2 | 35.6 ± 2.8 | 29.9 ± 3.0 | 4.6 ± 0.3 | 1.5 ± 0.3 | 61.7 ± 2.2 | 21.7 ± 1.5 | 6.9 ± 0.3 |
| 1171 | 9.8 ± 1.1 | 28.7 ± 1.3 | 31.3 ± 2.0 | 5.2 ± 0.5 | 2.1 ± 0.2 | 59.4 ± 2.4 | 23.4 ± 1.6 | 7.1 ± 0.2 |
| glossy |           |     |         |     |   |   |   |   |
| 1171 | 10.7 ± 1.8 | 33.7 ± 3.1 | 32.8 ± 2.3 | 4.01 ± 0.4 | 1.2 ± 0.03 | 59.0 ± 1.1 | 21.6 ± 0.8 | 10.5 ± 0.3 |
| 1175 | 12.0 ± 2.8 | 39.7 ± 1.4 | 32.3 ± 2.1 | 3.9 ± 0.2 | 1.2 ± 0.1 | 62.9 ± 0.7 | 21.3 ± 0.7 | 8.4 ± 0.6 |
| glaucous |         |     |         |     |   |   |   |   |

Differences in the content of essential unsaturated fatty acids in seeds of wild-type and mutant plants were insignificant. The content of C16:0 increased by 6% in comparison with the wild type was found in the M4 seeds, but by the M6 generation, it decreased. The seeds of mutant plants without glaucous bloom contain C18:0, while in the seeds of wild-type plants it is usually absent or insignificant in the seeds of wild-type plants.

### 4. Discussion

Forms without bloom prevailed in the progeny from self-pollination of “glossy” M3–M5 plants, but their number varied from 75% to 98% that indicates the dominant nature of this trait inheritance. At the same time, the progeny of plants with constant bloom did not segregate into glaucous and glossy, which indicates a recessive character of this trait (Figure 1). Variations in the number of glossy plants and changes in the ratio between glaucous and glossy caused by increased moisture and shading.

![Figure 5. HPLC-chromatograms of alcohol extracts of wild-type seeds (a) and red seeds of plants without wax bloom, line 1169 (b). Wavelength 330 nm; peaks: 5-sinapine, 15-sinapic acid.](image-url)
indicate that bloom formation is dependent on these external conditions. Moreover, the influence of conditions is noted at the initial stages of ontogenesis of mutant forms, at the stages of germination and development of the first to fourth leaves. With high soil moisture and the absence of bright sunlight, the number of glossy plants is higher than when the soil dries out in bright sunlight. It may also be due to the reduced viability of glossy seedlings in unfavorable conditions. On the other hand, a drought can contribute to the formation of a weak bloom on old leaves along the main vein at a later stage of plant development, during the ripening of siliques, displacing the ratio of glossy and glaucous forms.

The influence of external conditions, primarily drought and light, on the change in the thickness and wax composition has been revealed in many plant species, including various *Brassicas* [38]. Wax of brussels sprouts (*B. oleracea var. gemmifera*) contained more fatty acids at high temperatures. In barley, the deposition rate and density of the wax increased in bright light, in maize leaves are more abundant accumulated long-chain wax components [39,40]. The wax deposition had been increased in older leaves of *B. oleracea var. capitata*, which is one of the parental species of rapeseed [41]. At the same time, the formation of wax at high moisture and low light intensity is one of the problems when obtaining plants by tissue culture. Thus, wax deposition on cabbage (*B. oleracea*) and nasturtium (*Tropaeolum majus*) plants increased by reducing the relative moisture from 100% to 35% and from 98% to 30%, respectively [39].

Usually, the trait “no wax bloom” is controlled by recessive genes; the information on dominant mutations is rare [42]. Most mutations do not cause the accumulation of intermediate compounds due to the blocking of certain specific metabolic stages. Two recessive genes *lw1* and *lw2* block the formation of wax in wheat. The effect of the *lw1* locus does not depend on the genetic background or tissue and is associated with a slight increase in n-alkanes and primary alcohol content [43]. All 22 wax-deficient *cer* mutants of *A. thaliana (eceriferum)* studied to date have been found to be recessive. Moreover, the differences in the degree of glossiness (*cer1-cer10* are very glossy, while in *cer11-cer22* the gloss is muted) are associated with the types of wax crystals, their number, and morphological changes. Both recessive and dominant waxless “glossy green” mutants are known for *Brassica oleracea var. capitata*. In *Brassica oleracea var. capitata*, both recessive and dominant waxless “glossy green” mutants are known. Phenotypically, these forms are similar, but the chemical differences can be very significant. Thus, in recessive mutants, the amount of primary alcohols is reduced, while in dominant mutants several classes of different compounds are not detected at once [44,45]. A waxless glossy trait in *Brassica rapa* L. ssp. *pekinensis* is under the control of a single recessive genetic locus. [43]. In the *GLOSSY* dominant mutant of rapeseed for the *BnaA.GL* gene, not only shoots and siliques, as in *eceriferum* mutants of *A. thaliana*, are devoid of bloom but also leaves [42].

The wax deficiency mutation is known to be pleiotropic. For example, in *eceriferum* mutants of *A. thaliana* the wax deficiency is accompanied by a number of other disorders, such as growth inhibition, wilting, postgenital fusions of organs (sepals, pedicels, siliques, leaves), pollen sterility, self-incompatibility [46–48], weakening of pubescence [49]. At the same time, the manifestation of such morphological disturbances decreases with increasing moisture. In our glossy plants, the absence of a glaucous bloom is also accompanied by a violation of apical dominance and a weakening of the growth of the main stem, which leads to its curvature and shortening. Stamen filaments and anthers are shortened in flowers. Their dehiscence is difficult, pollen grains are deformed that lead to their sterility and reduces seed productivity. Resistance to silique dehiscence, “elasticity”, a decrease in the thickness of valves and seed coat, and deformation of placenta and seeds were noted. Under normal humidity (rainy weather, watering), mature plants exhibit increased branching and repeated flowering, and lack of seed dormancy. In general, low content of triacyl glycerides in seeds was also noted, but the content of stearic, and, especially, palmitic fatty acids the precursors of wax VLCFAs was significantly higher than that of the wild type (Table 5). This may indicate changes in the synthesis of fatty acids in glossy plants.

The changes caused by the decrease in the lignin content in glossy plants probably contributed to the disturbances in the development and growth of shoots, stamens, siliques, seeds, low pollen fertility,
and impaired seed germination. Thus, the low content of lignin in the seed coat significantly reduces the rate of their germination [50]. The cell walls of the peduncle vessels in the ref8 mutant A. thaliana was found to form poorly, and the vascular lumens were blocked in many places that caused the growth inhibition and sterility [20]. Inhibition of lignin accumulation was also revealed to cause impairment of apical dominance and male sterility in C4H mutant A. thaliana [51]. A decrease in cell wall thickness compared to the wild type, which also caused dwarfism, habitus changes, reduced flower size, and low seed productivity in another ccr1g mutant have been observed [21,52]. Perhaps, the mechanical solidity of the main shoot in our glossy rapeseed plants is reduced due to a lack of lignin, which caused growth arrest and lodging. It was also possible that the vessels conductivity was disturbed, and the siliques were not formed (deformed) due to a lack of nutrients. Similar violations are known in many species [19]. Herewith, a decrease in the amount of lignin in shoots was accompanied by an increase in hemicellulose content (Table 3). Similar changes have been described in irx4 and ccr1g mutant A. thaliana plants with a lignin deficiency [21]. In seeds of glossy plants, the changes in the content of lignin/hemicellulose were more visible. Therefore, we assume that it is the decrease in the lignin content in glossy mutants led to disturbances in the structure of the seed coat (Figures 2 and 4). The increase in the amount of hemicellulose serves as a “compensation” for maintaining the solidity of mechanical tissues.

In addition to the reduced content of lignin in seeds, a significant, threefold, compared with the wild type, a decrease in the total content of phenylpropanoids, including sinapine, was also found (Table 4). The seeds of the “glossy” mutants were found to contain sinapine significantly less than in the wild-type plants. Previously, it was shown that the number of minor components, sinapate conjugates, may slightly decrease in the seeds of A. thaliana mutants, but a decrease in the content of sinapine is not observed [23,53,54]. This imparts a noticeable practical value to our glossy mutants. For breeding purposes, glossy mutants may be useful to produce lines with reduced content of the anti-nutritious sinapine and lignin in seeds. In addition, a yellowish shade in the color of leaves, shoots and siliques may also be associated with an increased content of flavonoids in these organs that, in general, indicates significant changes in the biosynthesis of this class of compounds in glossy mutants.

The absence of a dormant period and the germination in seed siliques in our rapeseed mutants may be also associated with disorders in the accumulation of abscisic acid (ABA), as described in Arabidopsis thaliana [55]. A. thaliana eceriferum plants, which are deficient in wax, especially the ser10 mutant with increased gloss, showed similar wilting symptoms to those of mutants deficient in ABA [46]. ABA has been shown to influence cuticle formation and waxy composition on tomato leaves and fruits [56]. ABA-induced the expression of BnKCS1-1, BnKCS1-2 genes, involved in wax biosynthesis in canola plants [12].

We also observed another agronomically beneficial feature of the waxless mutant plants. Excessive moisture, with heavy rains of a plant, causing damage to the roots from hypoxia for our conditions is often more critical than the drought. Glossy mutants tolerate increased soil moisture better and thrive faster under these conditions.

It is possible that in our glossy mutants the germination of seeds with a thin coat in siliques is caused by a low ABA content, and not only by a wax deficiency.

5. Conclusions

We obtained mutant “glossy” plants that generally had a reduced content of various compounds: wax, fatty acids, lignin, and sinapine. Further study of these “glossy” mutants will reveal the nature of the observed changes. In addition, the absence of a waxy bloom on vegetative organs and siliques, combined with a habitus disruption, can serve as a marker of low content of lignin and sinapine in seeds. The use of mutant glossy lines with high protein content in breeding programs will possibly develop the valuable low-sinapine forage varieties.

Author Contributions: Conceptualization, A.V.S.; data curation A.V.S., L.M.K., O.N.K., T.N.N. and E.N.B.; formal analysis, O.B.S., and A.A.A.; investigation, A.V.S., V.T.V., N.V.Z., G.P.Z., H.K.K., L.M.K., O.N.K., T.N.N. and E.N.B.; project administration A.V.S.; resources, A.V.S. and V.T.V.; methodology, O.N.K. and N.V.Z.; visualization E.N.B.;
supervision, A.V.S.; writing—original draft, A.V.S.; writing—review & editing E.N.B. All authors have read and agreed to the published version of the manuscript.

**Funding:** The reported study was supported by a grant, from the Russian Foundation for Basic Research 17-29-08034, and within the framework of the scientific assignments: 0108-2018-0001 (AVS, OBS, IBR RAS); 0082-2014-0015, 17-117032750201-9 (ONK, ICP RAS); AAAA-A19-119041890054-8 (NVZ, TNN; IPP RAS), 0574-2019-002 (ENB; ARRIAB RAS) and 18-11802149011-5 (ENB, MBG RAS) of the Ministry of Science and Higher Education of the Russian Federation.

**Acknowledgments:** To the staff of the all-faculty electron microscopy laboratory, Biology Faculty of Moscow State University (Center for collective use “electron microscopy in life sciences”), and A.G. Bogdanov personally for valuable advice and assistance in working with the SM. To senior researcher of the botanical garden of Moscow State University T.A. Ostroumova for valuable advice.

**Conflicts of Interest:** The authors declare no conflict of interest.

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