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Investigation on the Relationship between Morphological and Anatomical Characteristic of Savoy Cabbage and Kale Leaves and Infestation by Cabbage Whitefly (*Aleyrodes proletella* L.)

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Abstract: The cabbage whitefly (CW), *Aleyrodes proletella* (L.) (Hemiptera: Aleyrodidae), is an important pest in *Brassica oleracea* L. crops. Little is known about the mechanisms of resistance to CW of savoy cabbage and kale cultivars. Light microscopy (LM) and scanning electron microscopy (SEM) analysis were used to determine the relationship between the morphological and anatomical features of savoy cabbage (*Brassica oleracea* L. convar. *capitata* (L.) Alef. var. *sabauda* L.) and kale (*Brassica oleracea* L. convar. *acephala* (DC.) Alef. var. *sabellica* L.) leaves and host suitability to colonization by CW. Two kale cultivars, "Redbor" and "Starbor", and two savoy cabbage cultivars, "Gloriosa" and "Alcosa", that differed in the degree of infestation by *A. proletella* were taken for histological analysis. The lowest infestation by all forms of *A. proletella* was observed on savoy cabbage cultivar "Alcosa" and kale cultivar "Starbor". The reduced colonization by cabbage whitefly may be related to the structure of the epidermis and the anatomical features of the leaf. The leaves of "Starbor" and "Alcosa" had more folds in the epidermis, less numerous but larger stomata, and a more compact mesophyll structure compared to "Redbor" and "Gloriosa". In both analysed species, there was no clear relationship between the thickness of the abaxial epidermal layer, thickness of the lamina, number of vascular bundles, and degree of infestation by the cabbage whitefly. This study identified promising sources of resistance to *A. proletella* among cultivars of savoy cabbage and kale. Varying infestation by CW was associated with morphological and anatomical characteristics of leaves. Further study is needed to confirm the relationship between insect resistance and leaf surface and morphological characteristics of leaves in a broader range of *Brassica* spp.

Keywords: cabbage whitefly; *Brassica oleracea* L. var. *sabauda*; *B. oleracea* L. var. *sabellica*; histology; leaf surface ultrastructure; morphological trait

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1. Introduction

The cabbage whitefly (*Aleyrodes proletella*) (CW) is an important agricultural pest species that has been a common problem for many commercial crops over the last decade [1,2]. The main host plants are *Brassica*, such as Italian cabbage, Brussels cabbage, cauliflower, broccoli, and kale [3]. Larvae and adults of the cabbage whitefly feed on the underside of the leaves. In addition to reducing the size and quality of the crop, the plants can be infested with eggs and larvae, and a large amount of honey dew is excreted during feeding by adults and larvae [4].

CW control is based mainly on spraying with insecticides. One of the main strategies for sustainable agriculture is the implementation of resistant or more tolerant cultivars that require less chemical protection [5]. The damage level caused by CW to plants of *Brassica* varies with the cultivar [2], indicating a different level of resistance to this pest. The identification of resistant varieties and characterization of the mechanisms underlying this resistance enables breeding of insect-resistant cultivars. Plant pest resistance is a complex process that includes induced and constitutive resistance on the one hand and direct and indirect resistance on the other. The basis of plant resistance to pests may be the production of secondary metabolites, such as terpenoids, alkaloids, anthocyanins, phenols, quinones, and glucosinolates that may either kill or retard the development of herbivores [6–11]. There is also evidence that plant defense against herbivores is associated with leaf surface and leaf morphological characteristics, such as the type and size of trichomes, trichome density, epidermal thickness, cell wall thickness, color of leaves [9,10,12,13], crystals [14], waxes, and toughened cuticles [15,16]. Structural defenses include also changes in cell wall thickness as a result of lignification and suberization [17]. An essential factor in anti-herbivore defense is also structural cohesion of mesophyll [13,18]. For some herbivorous insects, the selection of host may be affected by both chemical and morphological defenses. For example, the resistance of dwarf-casher plants (*Anacardium occidentale* L.) to whitefly (*Aleurdicus cocois*) is associated with an increased number of trichomes and accumulation of high levels of phenolics in leaves [9]. Shibuya et al. [19] and Rustamani et al. [20] reported that a high number of trichomes on the leaf surface may serve as a physical barrier to sucking insect pests, limiting the mobility of nymphs. Pubescence may affect oviposition by preventing the eggs attachment to leaves [17] and the movement behavior of the mites [21]. Secondary metabolites released from glandular trichomes can function as toxins and deterrents [22]. According to Broekgaarden et al. [23], resistance in *B. oleracea* is highly dependent on plant age and compounds present in the phloem.

In the presented study, we determined the (i) micromorphology of the leaf epidermis, (ii) internal leaf structure, and (iii) possible relationship between morphological and anatomical features of savoy cabbage and kale leaves and their level of infestation with cabbage whitefly.

2. Materials and Methods

2.1. Plant Materials

Research on the susceptibility of plants to CW colonization was conducted on two varieties of savoy cabbage, "Alcosa" and "Gloriosa", and two varieties of kale, "Redbor" and "Strabor". These cultivars showed differences, within the species, in the degree of infestation by CW in a previous field studies conducted in 2017. The selected cultivars differed also in the degree of leaf corrugation and their color (Figure S1). Plants were planted in the experimental field of the National Institute of Horticultural Research in Skierniewice, Poland (GPS: 51°57′50.6″ N 20°10′15.2″ E) on 24 April 2019. Experimental plots with 12 plants of each variety were replicated 5 times (at least 5 m^2 per plot) according to a randomized block design. The assessment of the number of adults, whitefly larvae, and eggs were made on 10 plants from each plot of the replication on the day of harvest, 8 August 2019. The counting of adult whiteflies was conducted early in the day when temperatures were lower, and the adults were less active. The infestation level of the pest was expressed as the mean number of adults, larvae, and eggs per plant.

2.2. Stomata Count

Samples of the abaxial epidermis (for each genotype), isolated from the third leaf, at a 10-cm distance from their tips, were mounted on slides for microscopic observations and stained with toluidine blue according to the procedure of Dyki and Habdas [24]. For each genotype, the number of stomata per 1 mm^2 (n = 5 replication/genotype) and length of stomata (n = 5 replication × 100 stomata/genotype) were determined. The observations were performed using a Nikon Eclipse 80i microscope (Eclipse 80i, Nikon, Tokyo, Japan) with the program NIS-Elements BR ver. 2.30 (Nikon Instruments Inc., Tokyo, Japan), at 100 and 400 times magnification.

2.3. Scanning Electron Microscopy (SEM)

For studies with SEM, the fragments of third leaf (10 × 5 mm) were fixed with a CrAF mixture/fixative (chromic acid, acetic acid, and formalin), then dehydrated in an alcohol series (70, 80, 90, and 100%), desiccated with critical point drying CO_2, and sputter-coated with gold [25]. For each genotype, 3 replications were made. The micromorphology of the leaf surface was analyzed using the scanning electron microscope JEOL JSM 6390LV, in Mossakowski Medical Research Centre, Polish Academy of Sciences in Warsaw.

2.4. Histological Observations

For histological observation, 10 × 5 mm pieces of the third leaves were cut. Five samples were collected for each genotype. The material was fixed in CrAF solution for 48 h at room temperature, dehydrated through an increasing alcohol series (70, 80, 90, and 100%), and embedded in paraffin according to Ruzin [26]. Cross sections (10 µm thick) were cut with a rotary microtome (Leica, Wetzlar, Germany) and stained with safranin (1% prepared in ultrapure water) followed by fast green (1% prepared in 95% ethanol). The sections were mounted in Canada balsam and analysed using a light microscope (LM) (Eclipse 80i, Nikon, Tokyo, Japan) with imaging software NIS-Elements Br ver. 2.30 (Nikon Instruments Inc., Tokyo, Japan) for photo documentation and measurements. For each sample, the thickness of the lamina, spongy and palisade mesophyll, and abaxial epidermal layer were determined. For statistical analysis, three replicates were used for each genotype, and each replicate consisted of 20 measurements.

2.5. Statistical Analyses

The results of the experiments were subjected to a one way analysis of variance (one-way ANOVA). For the stabilization of variance, logarithmic transformation was used when necessary. The means were separated using a Newman-Keuls test at $\alpha = 0.05$. Statistical analyses were performed with the STATISTICA v.13 program (StatSoft, Tulsa, OK, USA).

3. Results

3.1. Host Preference of *A. proletella* on Cultivars of Savoy Cabbage and Kale

Among the analyzed varieties of savoy cabbage, "Gloriosa" was more infested by CW than "Alcosa". About 70 larvae, 300 eggs, and 300 adults per plant were found on "Gloriosa", while only single adults, eggs, and larvae were recorded on "Alcosa". Significant differences were also confirmed statistically in the colonization of kale by CW. The number of larvae, eggs, and adults on "Redbor" was twice that of "Starbor" (Figure 1).

3.2. Morphology of Abaxial Leaf Surface Using LM and SEM

Adult forms feed and lay eggs, which hatch later into crawling nymphs on the underside of the leaf (Figure 2), therefore, our research focused on the study of the abaxial epidermis.

Scanning electron micrographs revealed that the abaxial surfaces of kale and savoy cabbage leaves are smooth and lack trichomes, however, their ultrastructure and degree of arching in stomata guard cells differed between the cultivars of the two species (Figure 3a–h). The epidermis was less folded in "Redbor" (Figure 3a,b) and "Gloriosa" (Figure 3e,f) as compared to "Starbor" (Figure 3c,d) and "Alcosa" (Figure 3g,h). The thick cuticle covered stomata in "Redbor" and "Alcosa", and it was more distinct than in the other two cultivars. Moreover, in "Redbor", the cuticle was wrinkled and formed striations.

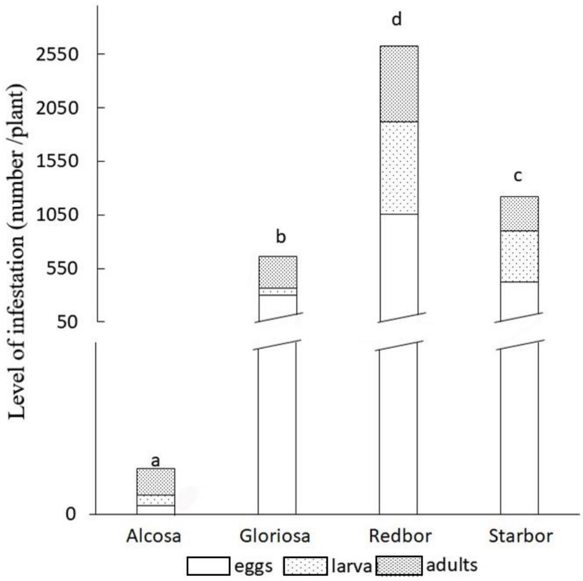

Figure 1. Infestation of kale and savoy cabbage cultivars with cabbage whitefly analyzed by one way analysis of variance (one-way ANOVA followed by Newman-Keuls test: $p < 0.05$). Means (all developmental stages together) with the same letter are not significantly different.

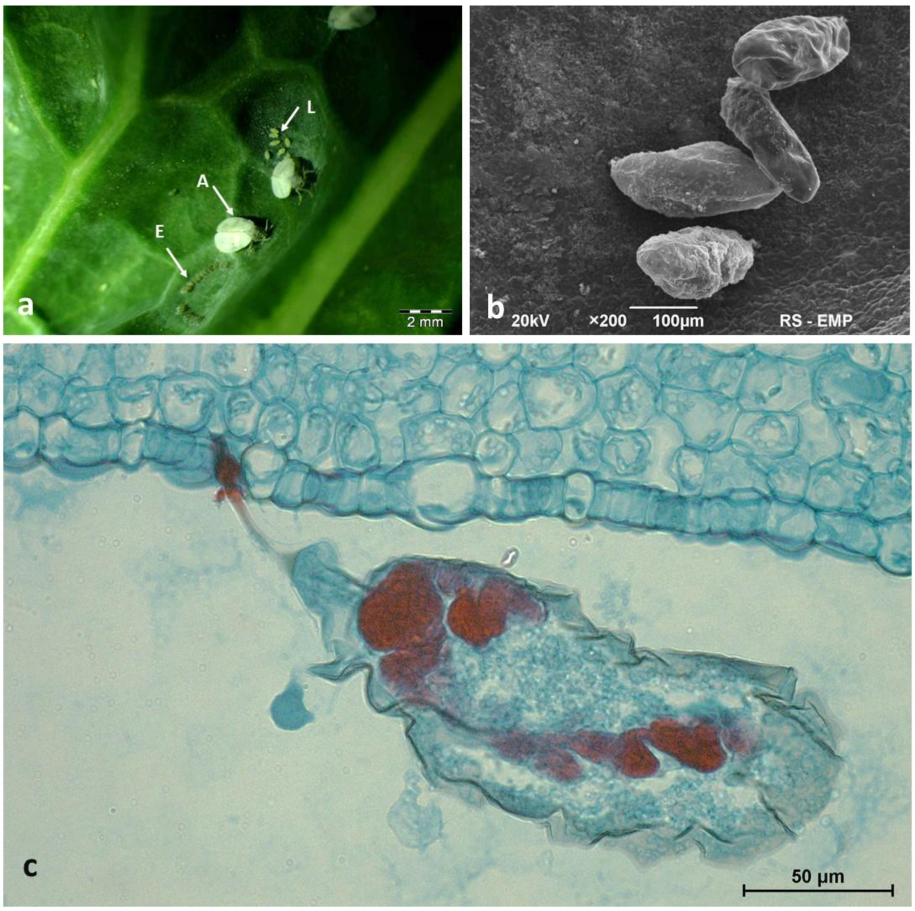

Figure 2. Cabbage whitefly (*Aleyrodes proletella*) on the underside of the leaves of savoy cabbage. (**a**) Adult forms (A), eggs (E), and larvae (L) in the stereoscope microscope, (**b**) Larvae in SEM, (**c**) Adult form of *A. proletella* inserting its mandibular stylet in the leaf epidermis. Cross-section of leaf stained with safranin-fast green and imaged with Light microscopy (LM).

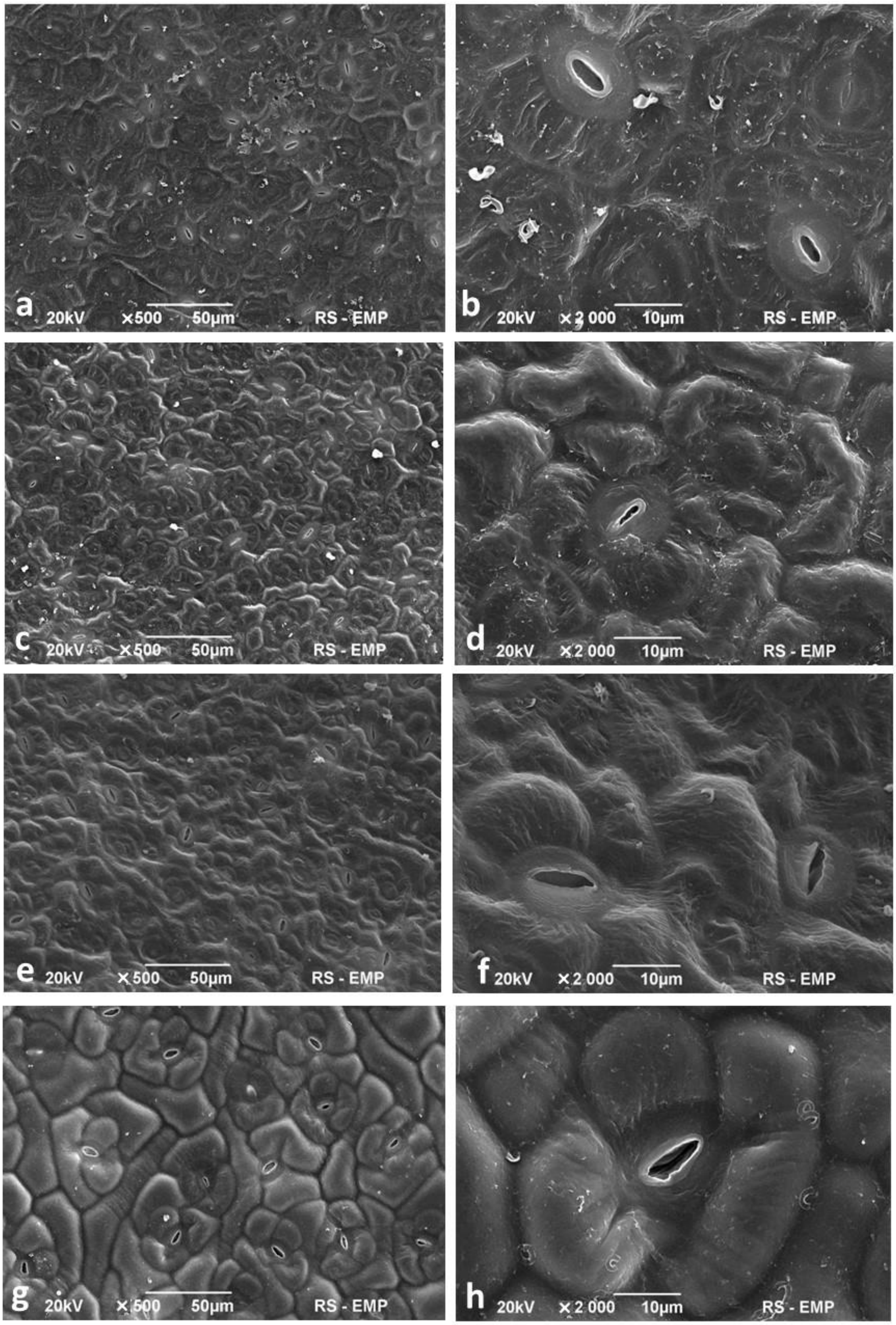

Figure 3. Scanning electron micrograph of the abaxial epidermis. (**a**,**b**) kale cultivar "Redbor", (**c**,**d**) kale cultivar "Starbor", (**e**,**f**) savoy cabbage "Gloriosa", (**g**,**h**) savoy cabbage "Alcosa".

Stomata cells in *Brassica* are anisocytic, meaning stomata are surrounded by three cells, one of which is usually smaller than the other two (Figure 4a–d). In both species, the stomata were present in the adaxial and abaxial epidermis (Figures 5 and 6). Stomata of the lower epidermis varied significantly in size ($F_{3, 8}$ = 17.205, p = 0.00075) and number per 1 mm^2 ($F_{3, 16}$ = 15.770, p = 0.00005) (Figure 4e,f). The density of stomata was higher in "Redbor" (413.4 per mm^2) and "Gloriosa" (356.6 per mm^2) than "Starbor" (309.6 per mm^2) and "Alcosa" (233.8 per mm^2) (Figure 4e). However, the former cultivars had significantly shorter stomata (16.1 µm in "Redbor" and 18.2 µm in "Gloriosa") than "Starbor" (20.56 µm) and "Alcosa" (22.7 µm), respectively (Figure 4f).

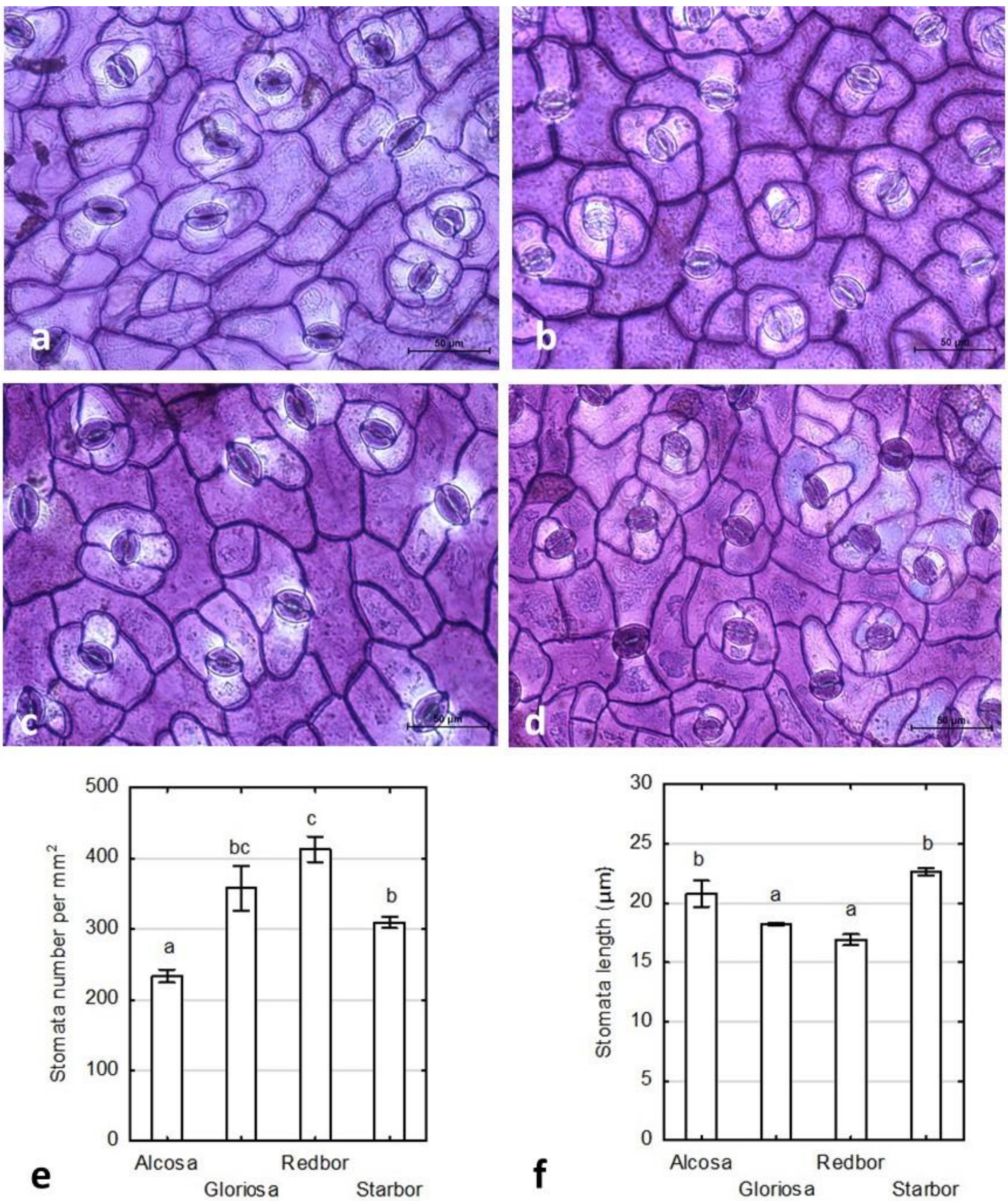

Figure 4. Abaxial epidermis of savoy cabbage and kale cultivars. (**a**–**d**) Optical micrographs stained with toluidine blue (LM). (**a**) "Alcosa", (**b**) "Gloriosa", (**c**) "Redbor", (**d**) "Starbor", (**e**,**f**) Morphological characteristics (mean ± 1SE) (one-way ANOVA followed by Newman-Keuls test: $p < 0.05$). Means with the same letter are not significantly different. (**e**) Number of stomata per 1 mm^2, (**f**) Length of stomata in µm.

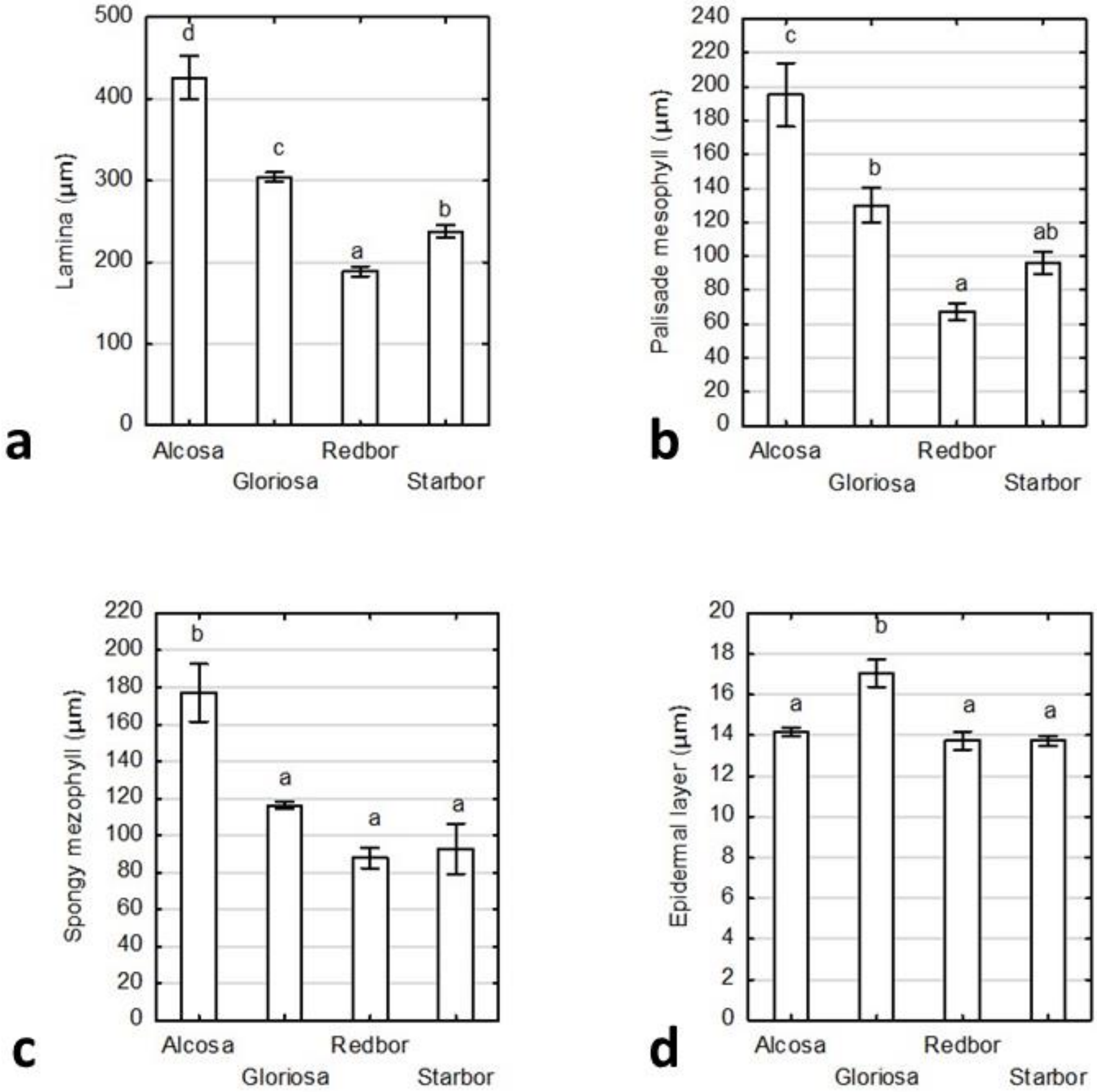

Figure 5. Anatomical characteristics (mean ± 1SE) of leaves of savoy cabbage and kale cultivars (one-way ANOVA followed by Newman-Keuls test: $p < 0.05$). Means with the same letter are not significantly different. (**a**) Thickness of lamina, (**b**) palisade mesophyll, (**c**) spongy mesophyll, and (**d**) abaxial epidermal layer.

3.3. Internal Leaf Structure Using LM

LM examination of cross sections of leaf fragments revealed significant differences among the analyzed cultivars with respect to the thickness of the lamina ($F_{3,8} = 50.538$, $p = 0.00002$), palisade mesophyll ($F_{3,8} = 22.952$, $p = 0.00028$), and spongy mesophyll tissues ($F_{3,8} = 14.538$, $p = 0.00133$) (Figure 5a–c). Savoy cabbage "Alcosa" and kale "Redbor" had the thickest and thinnest lamina, respectively, of the tested cultivars. The examined cultivars differed in the thickness of particular cell layers in leaves that was related with the thickness of the lamina (Figure 5a–c). Both the palisade and spongy mesophyll were significantly thicker in "Alcosa" leaves. We found varietal differences in the thickness of the abaxial epidermal layer ($F_{3,8} = 13.546$, $p = 0.00168$), however, there were no significant differences between either of the cultivars of kale or savoy cabbage "Alcosa". The epidermis in 'Gloriosa' was slightly thicker compared to other cultivars (Figure 5d). In both species, the cells of the lower epidermis differed in shape and size, which were tightly arranged into a single layer. The characteristic feature of both species was the presence of gigantic, strongly vacuolated cells among smaller ones in the epidermal layer (Figures 6 and 7).

We also found significant differences among tested cultivars in cell arrangement both in the palisade and spongy mesophyll and cell compactness, as well as the number of vascular bundles (Figures 6 and 7). The palisade mesophyll consisted of a varying number of cell layers that ranged from 3–4 in "Redbor" to 5–6 in "Alcosa". Cells of the palisade mesophyll differed in length and width. In "Alcosa" and "Starbor", the palisade mesophyll cells had distinctly larger dimensions and were rounder, whereas in the leaves of "Gloriosa" and "Redbor", the cells were longer, having typical features of the palisade mesophyll. In leaves of "Starbor" and "Alcosa", the spongy mesophyll was compact with cells tightly arranged and small intercellular spaces localized mostly above the stomata, whereas in "Redbor" and "Gloriosa", the cells of this tissue were loose with spacious intercellular spaces (Figures 6 and 7). In the cross-sections of the leaves of savoy cabbage observed at the same magnification, larger and more numerous vascular bundles were recorded in "Gloriosa", whereas no differences in respect to the size and number of vascular bundles was observed in the kale cultivars.

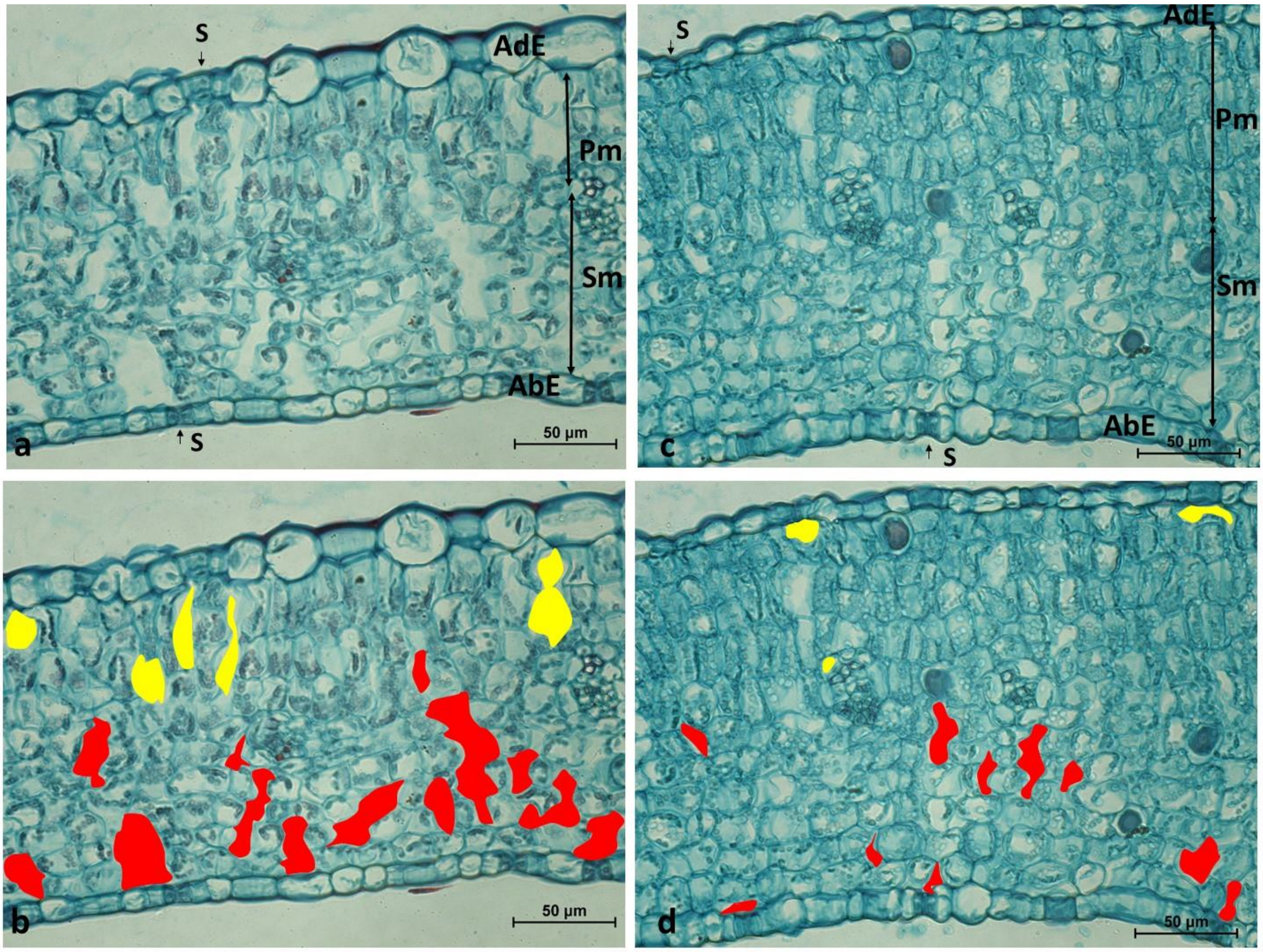

Figure 6. Cross-section of kale leaves stained with safranin-fast green and imaged with light microscopy. (**a**,**b**) cultivar "Redbor"—susceptible to the cabbage whitefly, (**c**,**d**) cultivar "Starbor"—resistant to the cabbage whitefly. (**b**,**d**) visualization of intercellular spaces in palisade mesophyll (yellow) and spongy mesophyll (red). Cultivars Abbreviations: AdE—adaxial epidermis, AbE—abaxial epidermis, Pm—palisade mesophyll, Sm—spongy mesophyll, S—stomata.

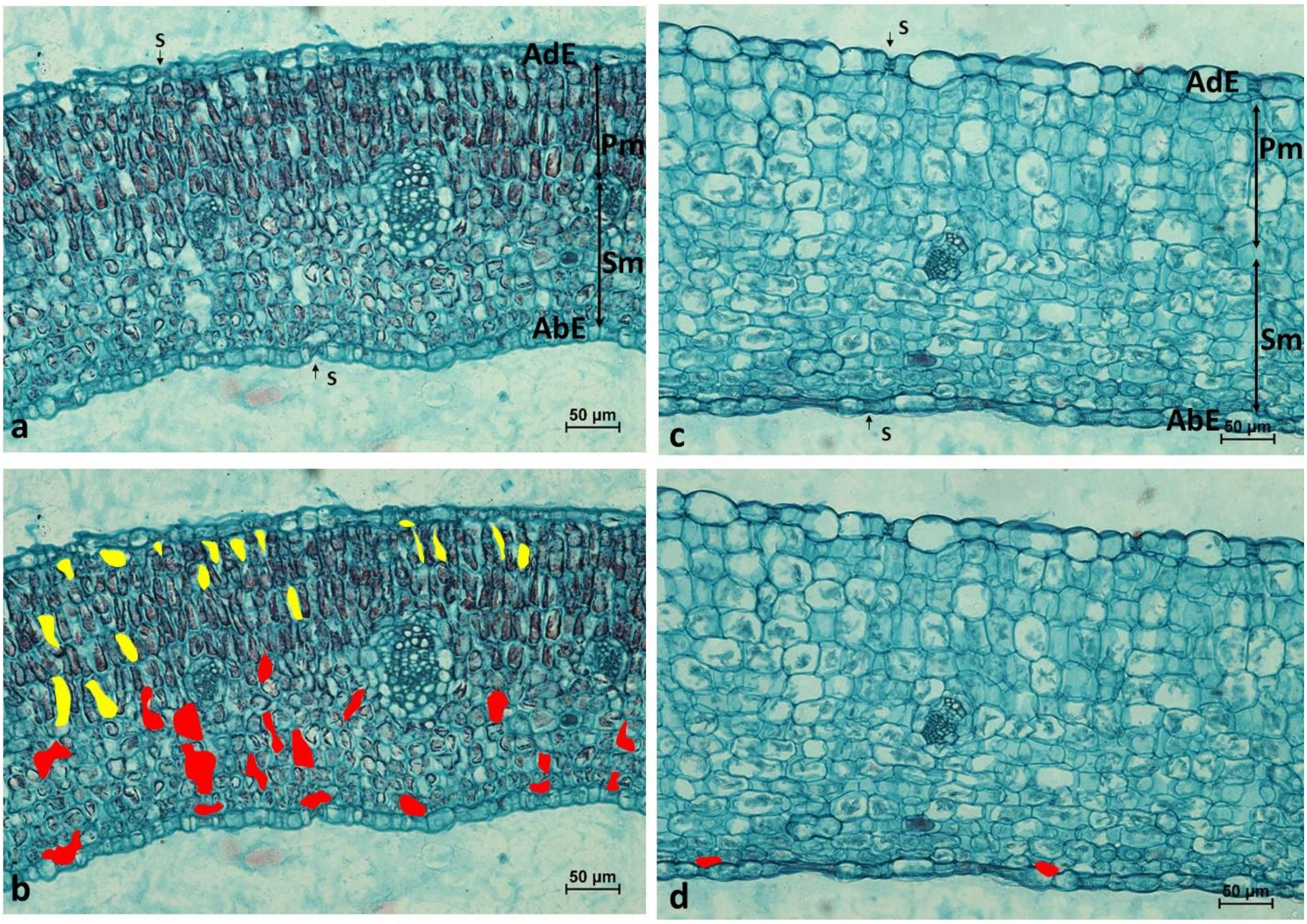

Figure 7. Cross-section of savoy cabbage leaves stained with safranin-fast green and imaged with light microscopy. (**a**,**b**) cultivar "Gloriosa"— susceptible to the cabbage whitefly, (**c**,**d**) cultivar "Alcosa"— resistant to the cabbage whitefly, (**b**,**d**) visualization of intercellular spaces in palisade mesophyll (yellow) and spongy mesophyll (red). Abbreviations: AdE—adaxial epidermis, AbE—abaxial epidermis, Pm—palisade mesophyll, Sm—spongy mesophyll, S—stomata.

4. Discussion

All tested cultivars of *B. oleracea* L. are a host of *A. proletella*, however, they clearly differed in infestation with adults, eggs, and larvae of CW. We showed that, in field conditions, savoy cabbage is more effective in maintaining low numbers of *A. proletella* than kale. Among analyzed cultivars, Savoy cabbage cv. "Alcosa" had the lowest number of eggs, larvae and adults whereas kale "Starbor" was the most heavily infested. These results confirmed the data obtained earlier in relation to other plants of the Brassicaceae family. Łabanowski [4] observed significantly higher numbers of the vegetable whitefly on broccoli "Parthenon", than on the "Monaco" and "Monflor" varieties. Similarly, whitefly cabbage colonized the "Bering", "Monaco" and "Raft" varieties of kale to a large extent, while the "Gohan" and "Graffiti" varieties were inhabited to a lesser extent. The differences in susceptibility of 7 cauliflower varieties to the whitefly were demonstrated by Muñiz and Nebreda [27]. Hondelmann et al. [11] found the differences in CW infestation of 16 Brussels sprout cultivars. Host plant resistance is a desirable and environmentally friendly alternative to control pests compared to using insecticides [19]. The present study focused on the role of physical barriers and leaf anatomy on the differences in plant infestation by CW in *Brassica*. Plant defence against insect herbivores can be associated with a number of factors, including production of secondary metabolites and morphological structures [7,24]. Currently, there is very little information in the literature about the type of secondary compounds in *Brassica* leaves that could modulate host selection of CW. The leaf surface undoubtedly plays a role in the preference of plant cultivars by herbivores. Mechanical barriers to feeding are formed on the leave surface by structural traits, such as waxy cuticle, trichomes, thorns, spines, and cell wall thickness and lignification, and they have been reported to defend the plants against many insects [28–30]. Stoner [15] and Chamarthi et al. [30] reported that there is a relationship between insect resistance and the glossiness of the leaf surface caused by reduced quantity or/and different chemical composition of the wax. The surface of *Brassica* leaves analyzed in our study were smooth and lacked trichomes, however, the analyzed varieties differed in the epidermis arrangement. Cultivars with low infestation by *A. proletella* had more epidermis folds. "Alcosa" (the less populated cultivar) was characterized by the lowest abaxial stomatal density, and the stomata cells were covered with a thick layer of cuticle.

Direct defenses may also be mediated by morphological and anatomical traits that affect the herbivore's biology, e.g., thicker or hardened leaves (sclerophylly), incorporation of minerals in plant tissues, and compactness of tissues [17,18,30]. In our study the thickness of the epidermal layer seems to be insignificant in the defense against CW. All cultivars tested showed similar thickness of the abaxial epidermal layers, despite displaying different levels of infestation by CW. According to Warabieda et al. [18], the resistance of apple cultivars to *Tetranychus urticae* is linked to the thickness of the cuticle rather than epidermal thickness. Reduced infestation is related to the thickness of the lamina and compactness of the mesophyll, among others. Smaller intercellular spaces and a tight cell arrangement of both palisade and spongy mesophyll were observed in savoy cabbage cv. "Alcosa" and kale cv. "Starbor", on which, within each species, lower numbers of all forms of CW were found. Similarly, the presence of small intercellular spaces within the spongy mesophyll of apple cultivars was related to low infestation with spider mites [18]. We did not confirm this relationship between lamina thickness and the degree of infestation by CW in analyzed cultivars, whereas populations of the whitefly *Bemisia tabaci* were positively correlated with the thickness of leaf lamina in cotton [31] and eggplant [32]. In carnation, the selection of cultivars resistant to two-spotted spider mite (*Tetranychus urticae* Koch) was based on the internal structure of the leaf, where the resistant plants had thicker abaxial palisade tissue ($\geq$120 µm) as compared to the susceptible plants [13]. As a sap-sucking insect from the phloem sieve elements, the cabbage whitefly needs to insert its mandibular stylet through the epidermal layer, deep in plant tissues of the leaves [33]. It seems that the compact structure of the mesophyll, may hinder penetration and makes it difficult to reach the vascular bundle. Conversely, Broekgaarden et al. [23] suggested that resistance to the whitefly in *Brassica oleracea* is not related to morphological traits but rather the compounds present in the phloem that interfere with sap ingestion.

The present paper is a prelude to forthcoming research on the biochemical basis of resistance in *Brassica* to *A. proletella*, especially the accumulation of phenolics in the leaves. We also plan to confirm the presence of the relationship between CW resistance and leaf surface and morphological characteristics in a broader range of *Brassica* species and cultivars.

5. Conclusions

Due to the constant tendency in agriculture to limit the use of plant protection products, the use of non-chemical methods of preventing the development of abundant pest populations becomes a necessity. The selection of plant varieties with specific anatomical and morphological features may be one of the elements enabling the cultivation of brassica plants in integrated and ecological system of producing these vegetables.

The present study focused on the role of physical barriers in resistance of savoy cabbage (*B. oleracea* L. var. *sabauda*) and kale (*B. oleracea* L. var. *sabellica*) to *A. proletella.* From this study, we concluded that savoy cabbage cultivar "Alcosa" and kale cultivar "Starbor" were most effective in maintaining a low number of *A. proletella*. A comparative histological analysis of cultivars of different susceptibility to CW showed the link to morphological and anatomical characteristics of the leaves. The increased resistance of savoy cabbage and kale plants to the cabbage whitefly may be associated with the greater density of mesophyll cell. The layout of the cuticle, folding of epidermal cells, and stomata size and density may be another factor determining susceptibility to CW.

Supplementary Materials: The following are available online at https://www.mdpi.com/2073-4395/11/2/275/s1, Figure S1: Morphological characteristics of leaves. (a) "Alcosa"—an early cultivar of savoy cabbage which produces compact green head, leaves are strongly crinkled, (b) "Gloriosa"—a medium/late cultivar of savoy cabbage with dark-green, corrugated leaves, (c) "Redbor"—cultivar of kale characterized by a purple-greenish color and frilly, curled leaves. (d) "Starbor"—cultivar of kale with finely curled, dark blue-green leaves with long petioles. Bars represent 2 mm.

Author Contributions: G.S. and A.M.-C. conceived and designed the experiments. A.M.-C., G.S., W.W., U.K. and D.R. performed the experiments. W.W. performed statistical analysis. A.M.-C. and G.S. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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