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Tsveta Ganeva*, Miroslava Stefanova, Dimitrina Koleva, Segundo Ríos Ruiz

Isolation and recrystallization of epicuticular waxes from *Sorbus* and *Cotoneaster* leaves

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Abstract: Wax morphology and chemical composition are widely accepted to be important for the protective properties of the leaf's surface and also valuable characteristics in plant systematics. The leaves of *Sorbus domestica* L. and *Cotoneaster granatensis* Boiss., species of two large genera with intricate taxonomy referred to subtribe Pyrinae, Rosaceae (formerly subfamily Maloideae), were studied by scanning electron microscope (SEM) and performing different methods of wax isolation. The aim of the study was to acquire a suitable, cost and time effective method for wax removal. Chloroform and methanol extractions and freeze-embedding method for direct isolation of the wax crystals were applied. Immersing the leaves for 3 minutes in chloroform was sufficient to extract the waxes whereas the efficiency of the methanol solvent was lower. Wax layers with well-preserved structures of the crystals from both upper and lower epidermis were successfully transferred to artificial surfaces. The recrystallization experiment demonstrated that waxes from chloroform extracts could recrystallize in *in vitro* conditions on artificial surfaces. The crystals showed same micromorphology as on the intact leaves. Results of this study could be applied in further analytical researches of the waxes of *S. domestica* and *C. granatensis* or other species of the subtribe Pyrinae.

Keywords: Rosaceae, epidermis, wax extraction, freeze-embedding, recrystallization

1 Introduction

The cuticle, covering the aerial surface of plants, is a protective layer against water and polar molecular loss. The plant waxes that are integrated in the cuticle (intracuticular) and superimposed on it (epicuticular) increase plant resistance to various pathogens and insects and have strong influence on the wettability and self-cleaning properties of the leaves [1-3]. The structural diversity of the epicuticular waxes has been extensively investigated by scanning electron microscopy (SEM) [1,4,5]. According to the classification of Barthlott et al. [5], 23 wax types with fairly constant shape, size and orientation are recognized. Many successful recrystallization experiments demonstrated the crystal habit of the waxes therefore termed wax crystals [2,6-14]. Now it is widely accepted that the wax micromorphology depends on the chemical composition rather than environmental factors [4,5], and for that reason they can be used as a valuable character in plant systematics [5,11,12].

In the present study leaves of *Sorbus domestica* L. and *Cotoneaster granatensis* Boiss., two species of different genera of the subtribe Pyrinae, Spiraeoideae, Rosaceae (formerly subfamily Maloideae) [15,16], were studied by SEM. The epicuticular waxes observed on both surfaces were subjected to different methods of isolation. The aim of the study was to acquire a suitable, cost and time effective method for wax removal that could be applied in further analytical researches of the waxes.

2 Methods

2.1 Plant material

Cuticular waxes were isolated from mature leaves of *Sorbus domestica* L. and *Cotoneaster granatensis* Boiss. plants, cultivated at Botanical Garden Torretes, situated in Ibi, Spain.

***Corresponding author Tsveta Ganeva,** Department of Botany, Faculty of Biology, Sofia University "St. Kliment Ohridski", Sofia 1164, Bulgaria, E-mail: tsveta_ganeva@yahoo.com
Miroslava Stefanova, Dimitrina Koleva, Department of Botany, Faculty of Biology, Sofia University "St. Kliment Ohridski", Sofia 1164, Bulgaria
Segundo Ríos Ruiz, Instituto de Biodiversidad CIBIO, Universidad de Alicante, Alicante, Spain

2.2 Isolation of waxes

The extraction of waxes was carried out by immersing the whole leaves for 3 minutes at room temperature (approx. 22-24˚C) in organic solvents - chloroform (99.0% Pancreac) and methanol (99.8% Pancreac).

The epicuticular waxes were mechanically isolated from the upper and lower surfaces of the leaves following the freeze-embedding method of Ensikat et al. [13]. Glycerol was used as soluble embedding liquid. After thawing, the waxes were transferred on artificial surfaces - plastic plates.

2.3 Recrystallization procedure

For each species the waxes were extracted from 20 mature leaves through immersing for 3 minutes in 40 ml chloroform at room temperature in still air. The solutions were filtered and divided into four 25-ml glass vials with placed artificial (inorganic) surfaces – plastic plates. As the chloroform was allowed to evaporate in still air at 20-23˚C, the plates were analyzed by SEM.

2.4 Scanning electron microscope analysis

All samples (leaves without treatment, treated leaves and the plastic plates) were air dried, attached to aluminum specimen stubs by double-sided carbon tape. After being sputter-coated with gold (BLAZER SCD 004 sputter coater), the specimens were examined by scanning electron microscope (SEM) JEOL JSM-840.

3 Results

3.1 Micromorphology of waxes

In SEM observation of *S. domestica* the upper and lower epidermis of the leaf had prominent cuticular ridges (Fig. 1A, 1B). Both surfaces had thick wax coverings of tubular epicuticular waxes (Fig. 1C, 1D). The tubules varied in between 0.5 – 1 μm lengths on the upper surface and exceeded 2 μm on the lower one. In cross section the tubules were circular with approximately 0.2 μm outer diameter.

In *C. granatensis* both leaf surfaces were fully covered by tubular epicuticular waxes (Fig. 2). The upper surface

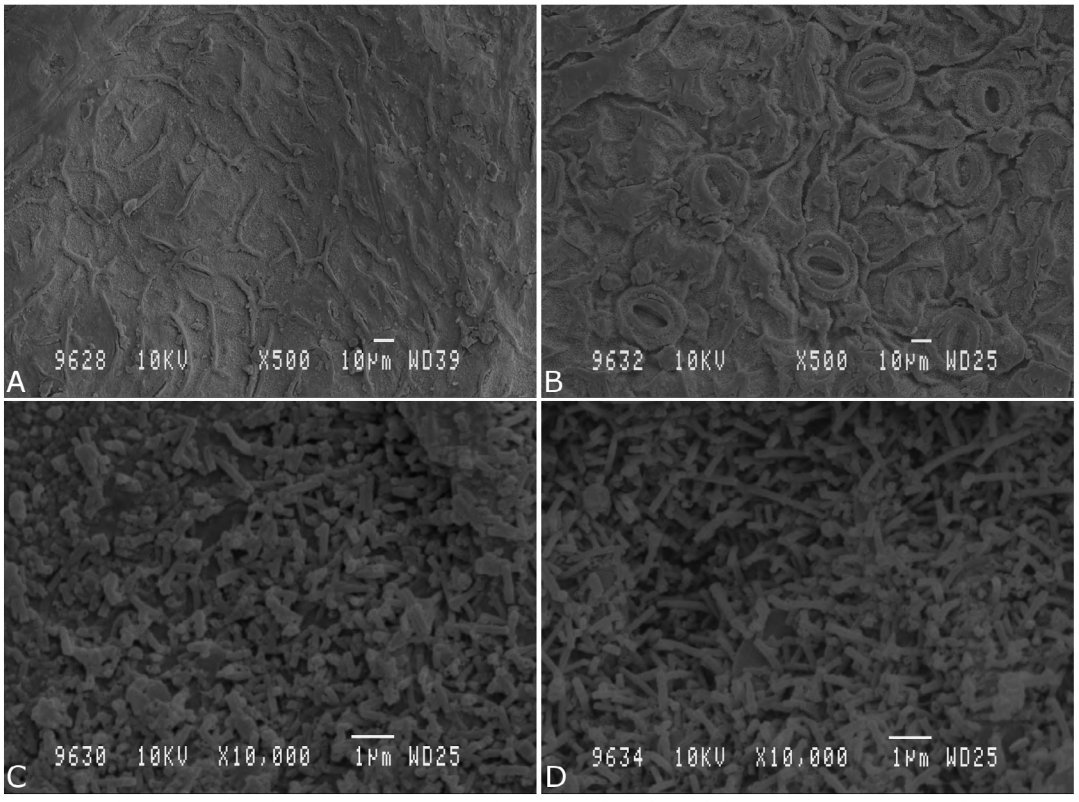

Figure 1 SEM of *S. domestica* leaf surface. Micromorphological characteristics of upper epidermis (A), lower epidermis (B), upper epidermis tubular waxes (C), and lower epidermis tubular waxes (D).

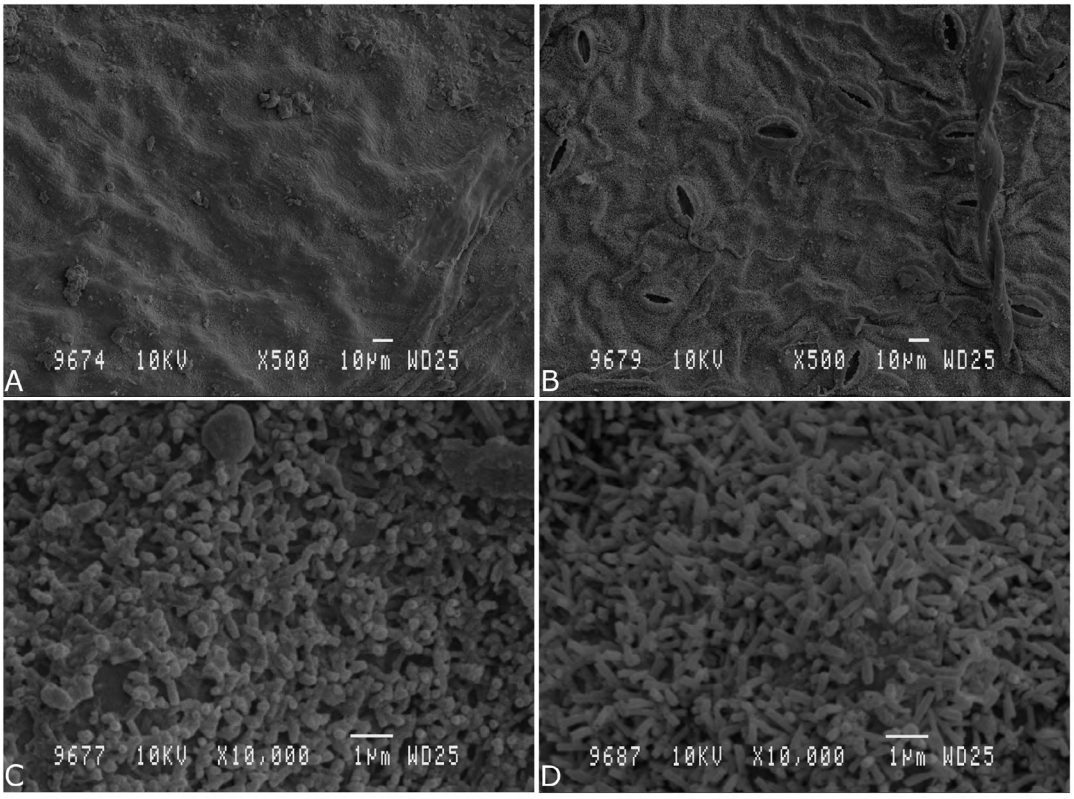

Figure 2 SEM of *C. granatensis* leaf surface. Micromorphological characteristics of upper epidermis (A), lower epidermis (B), upper epidermis tubular waxes (C), and lower epidermis tubular waxes (D).

was smooth (Fig. 2A) whereas the lower had papillae (Fig. 2B). The tubules were with 0.5 µm length on the upper surface and between 0.5 – 1 µm lengths on the lower one. The tubules were circular in cross section with 0.2 µm outer diameter (Fig. 2C, 2D).

3.2 Isolation of waxes

The leaves of *S. domestica* and *C. granatensis* were subjected to organic solvent treatments with chloroform and methanol. The waxes were fully extracted from both leaves surfaces of the species after 3-minutes dipping in chloroform (Fig. 3). After 3-minutes of methanol treatment of *S. domestica* leaves, there were no noticeable changes of the wax covering on the upper and lower epidermis and a significant amount of wax crystals remained (Fig. 4A, 4B). Whereas the crystalline tubules were extracted from *C. granatensis* leaves under the same treatment and only the underlying wax film covered the both surfaces (Fig. 4C, 4D).

The application of direct isolation of the epicuticular waxes without using solvents, but after freezing and transferring on artificial carrier material, was efficient for *S. domestica* and C. *granatensis*. Almost entire wax layers – underlying film with crystalline waxes – were obtained from the upper and the lower surfaces (Fig. 5). The crystals were with well preserved shape and orientation. The wax layers had some holes, wrinkles and crevices because of the ridges and papillae of the epidermis. The epicuticular waxes were almost fully removed from the upper epidermis of *C. granatensis*, whereas large amounts of wax remained around the stomata and other areas on the lower epidermis.

3.3 Recrystallization of wax tubules

The epicuticular waxes of *S. domestica* and *C. granatensis* after isolation with chloroform recrystallized from the solution on an artificial surface in *in vitro* conditions. The waxes consisted of a continuous underlying layer and tubular projections (Fig. 6). The obtained tubules showed same shape, similar size and orientation as the corresponding *in vivo* crystals on the leaf surface. The waxes extracted from *S. domestica* recrystallized inhomogeneous on the plastic plates (Fig. 6A). The most similar to those found on the plant surfaces *in vitro*

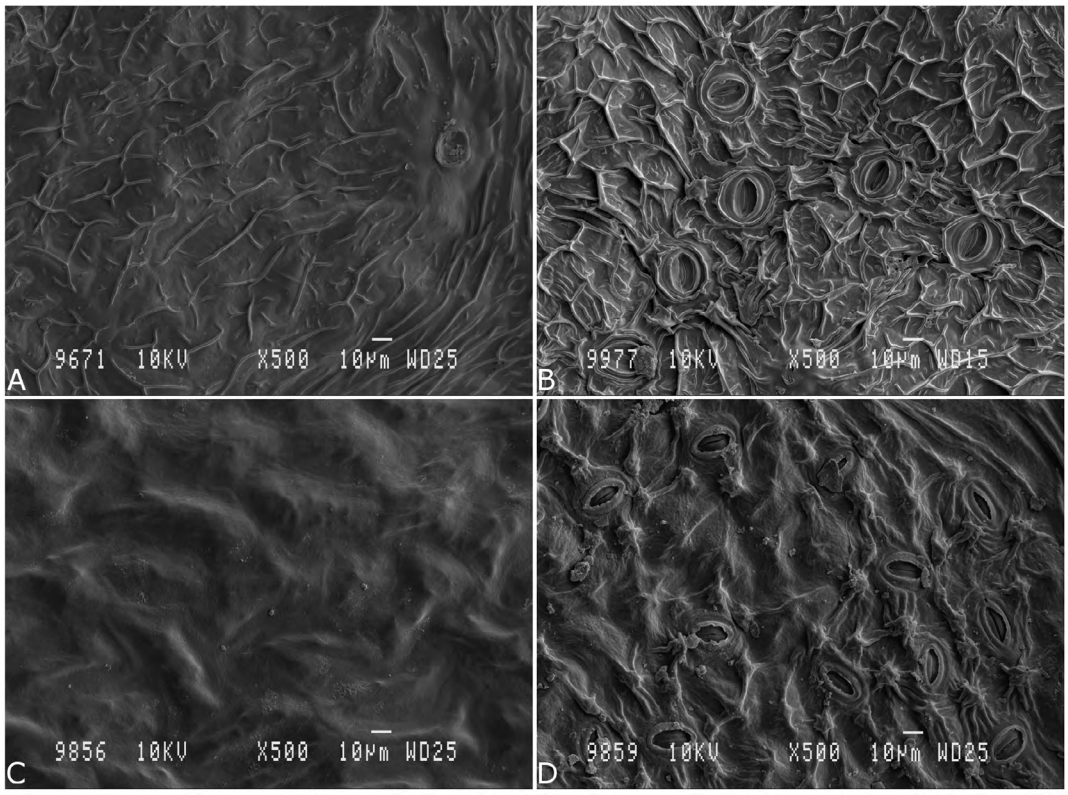

Figure 3 SEM of leaf surface after 3-minutes of being treated with chloroform. Micromorphological characteristics of *S. domestica* upper (A) and lower (B) epidermis, and *C. granatensis* upper (C) and lower (D) epidermis.

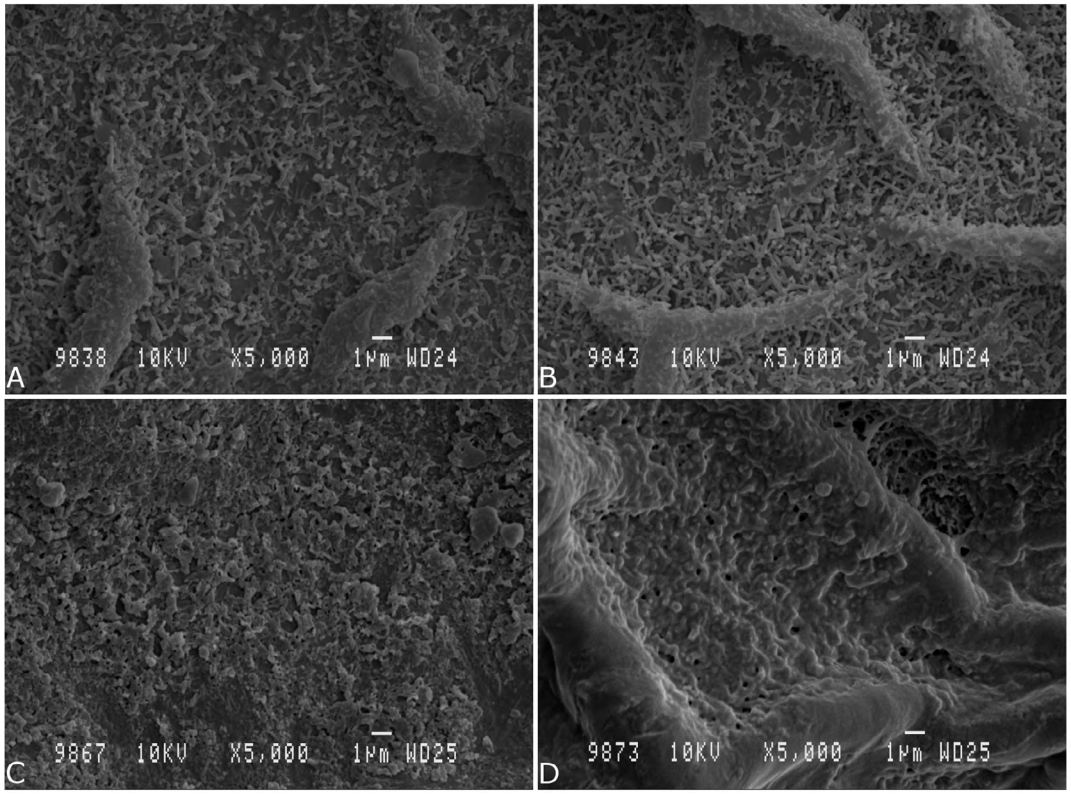

Figure 4 SEM of leaf surface after 3-minutes of being treated with methanol. Micromorphological characteristics of *S. domestica* upper (A) and lower (B) epidermis, and *C. granatensis* upper (C) and lower (D) epidermis.

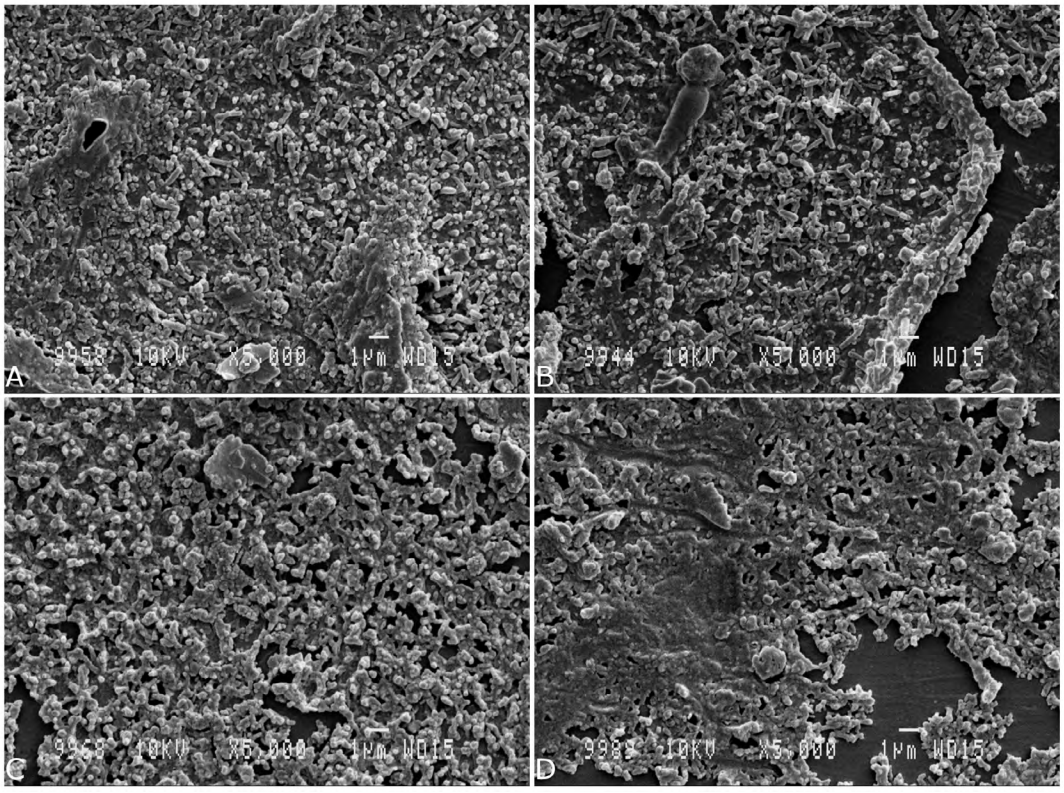

Figure 5 SEM of epicuticular wax layer isolated by freeze-embedding method and transferred on artificial surface (plastic plate). Micromorphological characteristics of wax layers from *S. domestica* upper (A) and lower (B) epidermis, and *C. granatensis* upper (C) and lower (D) epidermis.

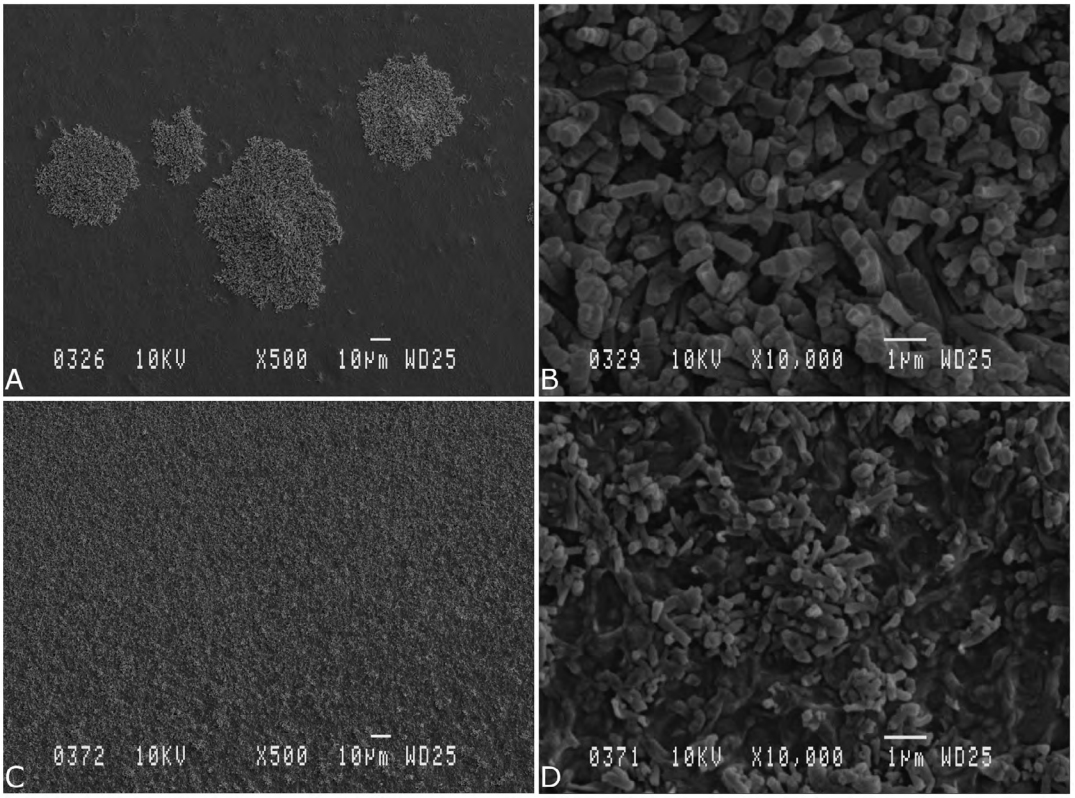

Figure 6 SEM of recrystallized on artificial surface (plastic plate) epicuticular wax tubules from *S. domestica* (A, B) and *C. granatensis* (C, D) leaves.

tubules were observed in the homogeneous areas of this pattern (Fig. 6B). Some of the tubule crystals were with irregular ridged outer surfaces or fused longitudinally and with unclear terminal openings.

The recrystallization of the waxes from *C. granatensis* revealed better results in forming homogeneous layer that coated the plastic plate (Fig. 6C). The observed *in vitro* tubules showed densitities, shapes, sizes and orientations identical to the micromorphology of the crystals found on the intact leaf surface (Fig. 6D).

4 Discussion

The observed tubular waxes on *S. domestica* and *C. granatensis* leaves were unbranched and without specific orientations. Except for their length there were no significant differences in the micromorphology of the waxes from the upper and lower surfaces and between the two species. In the classification of Barthlott et al. [5], tubules were described as cylindrical hollow crystals with a terminal opening and uniform dimensions. The authors [5] divided 2 types of tubules due to their chemical composition, but as they pointed out both types were very similar in appearance and hardly distinguishable in SEM. Barthlott et al. [5], Meusel et al. [12] and other authors occasionally found transitional forms between tubules and coiled rodlets or platelets in their surveys of the epicutucular wax morphology, also branching and cluster formation. Any of these peculiarities could not be reported in the present study. Also no mixtures with other crystalloid types of waxes – plates, platelets or rodlets on the surfaces of *S. domestica* and *C. granatensis* leaves were observed.

The wax types and their chemical compositions can be useful characters in plant systematics [5,11,12]. In a leaf epidermis study of *Cotoneaster* species, Uzunova and Mladenova [17] observed fine granular wax on the upper epidermis and rods of wax on the lower epidermis of *Cotoneaster nebrodensis* Koch., *C. integerrimus* Med. and *C. niger* Fries. However, later investigation of the same species showed tubular waxes on both surfaces [18]. Different types of epicuticular waxes were established on the leaf surface of *Amelanchier* Medik. [19], *Malus* Mill. [20], *Pyrus* L. [18], *Sorbus* L. [18], *Cydonia* Mill. [21], *Crataegus* L. [22], *Mespilus* L. [17] and *Pyracantha* Roem. [17] species. Further analyses of the waxes could contribute to clarifying the relationships among the genera in subtribe Pyrinae.

Although various organic solvents – chloroform, hexane, benzene, methanol, ethanol, acetone etc. and their mixtures were used for wax extraction, the chloroform solvent was most generally used throughout numerous studies [2,6,8,9,11,12,23,24]. According to McWhorter et al. [8], 30-seconds washing in chloroform removed essentially all the wax from the leaf surface. However, in our study a 30-second extraction in chloroform was insufficient. We assume that the diversity in the relief of the studied surface and the indumentum could impede the wax extraction and increase the time necessary for treatment. In present study methanol appeared to be an inappropriate solvent for the waxes as it was found out for *Brassica* [6]. The disadvantage in using organic solvents for isolation of epicuticular waxes is that the resultant extract also includes the intracuticular waxes [25]. However, this method is commonly used to study the properties of the cuticle in studies of the chemical composition and mechanisms of crystallization of the waxes [2,6,9,11,12]. In this study, the 3-minute chloroform treatment of the leaves was sufficient to extract the waxes of *S. domestica* and *C. granatensis*.

The applied freeze-embedding method allowed removal of the waxes of the upper epidermis separately from the lower epidermis of *S. domestica* and *C. granatensis* and isolation of only pure epicuticular waxes but not intracuticular ones. Better results were achieved with the smooth surface than surfaces with ridges and papillae. According to Ensikat et al. [13], who first introduced this method for direct isolation, it could be applied to fruits, stems or larger leaves on a large number of plant species and for investigations of single crystals. Consequently, our results could be useful for further studies of the waxes' chemical composition, physical properties, and resistance to various environmental influences.

Tubular waxes with the same micromorphology as the crystals on the leaf surface of *S. domestica* and *C. granatensis* were observed after extraction with chloroform and recrystallization on artificial substrate. The waxes from *C. granatensis* formed homogeneous layers on the plastic plates while the waxes extracted from *S. domestica* recrystallized inhomogeneously. Koch et al. [2] investigating the growth process of nonacosan tubules also observed inhomogeneous circular growth pattern and named it "coffee-drop effect". The authors [2] suggested this was due to the evaporation of the solution and the most homogeneous part was the one which dries last. According to Jetter and Rieder [9], the conditions of crystallization could modify the habit or arrangement of the resulting crystals. Bergmadinger-Stabentheiner [26] observed differences in tube dimensions of recrystallized waxes on spruce needles due to different recrystallization conditions. According to Meusel et

al. [12], the recrystallized tubules are often larger than the *in situ* ones, especially in diameter. The artificial surface upon which tubules recrystallized seemed to have no influence on the process of wax deposition as we observed identical crystals on glass supports (data not shown). The same results were reported by Jeffree et al. [6], Jetter and Rieder [9], Koch et al. [2] and others. Our study, together with previously reported recrystallisations of tubules in different plant species [6,9,12,24], once again supports the evidence of a close relationship between wax micromorphology and chemical composition [4,6,11,26]. However, many questions remain about the influence of the chemical compounds and recrystallization conditions (rate of evaporation, temperature, wax concentration or surface nature) on wax microstructure and formation.

5 Conclusions

The study results show that 3-minutes chloroform treatment fully removes the epicuticular wax tubules from *S. domestica* and *C. granatensis* leaves. The freeze-embedding method is efficient for separate isolation of the epicuticular waxes from upper and lower epidermis of the leaves. The recrystallization experiment demonstrated that waxes from chloroform extracts could recrystallize in *in vitro* conditions on plastic plates. The obtained results could be applied in further analytical researches of the waxes of *S. domestica* and *C. granatensis* or other species of subtribe Pyrinae.

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Conflict of interest: Authors declare nothing to disclose.

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