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Structural changes in needle epicuticular waxes of Balkan *Abies* species in relation to natural weathering

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ABSTRACT - This study represents the first survey of the structural changes of needle epicuticular waxes of *Abies alba* Mill., *A.* x *borisii-regis* Mattf. and *A. cephalonica* Loudon in relation to natural weathering. In all three studied species, epicuticular wax on the adaxial surface of the needles was in the form of granules, whose amount increased with age of needles, so they were most prominent on the oldest needles. The main wax crystalloids on the abaxial needle surfaces were tubules with a tendency to concentrate within the stomatal complexes and between them in the stomatal rows. As the tubules aged they generally tended to agglomerate and fuse, forming amorphous wax crusts. Although we did not find differences in the micromorphology types of epicuticular wax among the examined species, both the tendency of the amount of granules to increase and the degree of degradation of tubules into amorphous crusts as the result of the natural aging process were well-documented.

Keywords: amorphous crusts, firs, granules, micromorphology, tubules

RESUMO – **Alterações estruturais em ceras epicuticulares de acículas de espécies balcânicas de *Abies* em relação ao intemperismo natural.** Este estudo representa o primeiro levantamento das alterações estruturais das ceras epicuticulares das acículas de *Abies alba* Mill., *A.* x *borisii-regis* Mattf., e *A. cephalonica* Loudon em relação ao intemperismo natural. Nas três espécies estudadas, a cera epicuticular superfície adaxial das acículas estava na forma de grânulos, cuja quantidade aumentava com a idade das acículas, de modo que eram mais proeminentes nas acículas mais velhas. Os principais cristaloides de cera na superfície abaxial da acícula foram túbulos, com tendência a se concentrarem dentro dos complexos estomáticos e entre eles, nas fileiras estomáticas. À medida que os túbulos envelhecem, geralmente tendem a aglomerarem-se e fundirem-se, formando crostas de cera amorfas. Embora não tenhamos encontrado diferenças nos tipos de micromorfologia da cera epicuticular entre as espécies examinadas, a tendência de aumento da quantidade de grânulos e o grau de degradação dos túbulos em crostas amorfas como resultado do processo natural de envelhecimento foram bem documentados.

Palavras-chave: crostas amorfas, abetos, grânulos, micromorfologia, túbulos

INTRODUCTION

Cuticle is a multifunctional interface between the plant and environment, composed of a three-dimensional network of cutin (Kolattukudy 1980, 2001) and integrated and superimposed lipids called "waxes". Plant waxes embedded into the cuticle are called "intracuticular waxes", while waxes superimposed onto the cuticle are called "epicuticular waxes". Numerous studies using SEM (Scanning Electron Microscopy) have shown that most epicuticular waxes form three-dimensional structures, which are characterized by great morphological diversity (e.g., Jeffree 2006; Koch *et al*. 2008; Tomaszewski & Zieliński 2014; Ramos 2015, *etc*.). They are believed to be mainly crystalline in nature, so they are termed crystalloids (Ensikat *et al.* 2006). Crystalloids usually have characteristic shapes (granules, platelets, plates, rodlets, threads, or tubules), sizes (0.5-100 µm), and orientation towards the surface, emerging from the ubiquitous wax film (Koch *et al*. 2008). In general, crystalloids show an irregular distribution, even though they are arranged into characteristic orientation and aggregation patterns in some species (rosettes of platelets, clusters of tubules, clusters of rodlets, longitudinally aggregated rodlets, etc.—as noted in Barthlott *et al*. 1998).

The first classification of epicuticular waxes into different micromorphological types was proposed by Amelunxen *et al*. (1967). Then Barthlott *et al*. (1998) and Jeffree (2006) provided more recent overviews of terminology and micromorphology. The classification by Barthlott *et al*. (1998) included 23 different wax types, taking chemical and morphological features into account, and considered the orientation and pattern of the epicuticular wax structures, while Jeffree (2006) distinguished six main wax types, including a background epicuticular wax film.

Certain wax crystalloids were often positively correlated with the presence of specific components within the wax, leading to several papers dealing with the interrelation between chemical composition and micromorphology of crystalloid types (e.g., Baker 1982; Walton 1990; Bianchi 1995). For example, the secondary alcohol nonacosan-10-ol is the main component of cuticular waxes of species from the Pinaceae family (Riederer 1989, Barthlott *et al*. 1998), and its biological importance is mainly ascribed to the role of shaping the crystalloids in the form of characteristic tubules on the conifer needles (Matas *et al*. 2003; Jeffree 2006; Mitić *et al*. 2017). Tubules are cylindrical, hollow crystalloids with a terminal opening (Barthlott *et al*. 1998). Still, they were often misinterpreted as rodlets in various publications (Hallam 1967, 1970; Johnson & Jeffree 1970; Jeffree *et al*. 1975, 1976), due to the obscured openings or the limited resolution of the SEM. Over time, the tubules on the conifer needles degrade into amorphous wax mass resulting from natural weathering (Wells & Franich 1977; Crossley & Fowler 1986), which may be accelerated by various external factors (Jeffree 2006).

Since the epicuticular wax types and their orientation patterns are characteristic of certain plant taxa and little affected by the environmental conditions, they have been used in plant systematics (e,g., Herbin & Sharma 1969; Hanover & Reicosky 1971; Hennig *et al*. 1994; Barthlott *et al*. 2003). Nevertheless, the micromorphology of wax crystalloids has rarely been analyzed for a group of related conifer taxa. Namely, the influence of different air pollutants on the epicuticular wax structure of conifer needles has attracted more attention from researchers, as these environmental factors significantly accelerate the degradation of wax crystalloids into amorphous crusts (e.g., Cape & Fowler 1981; Huttunen & Laine 1983; Riding & Percy 1985; Crossley & Fowler 1986; Bermadinger *et al*. 1987; Tuomisto 1988; Bačić *et al*. 2005).

Within the Balkan Peninsula, two species of the genus *Abies* Mill. are very important as forest-building species: (i) *A. alba* Mill. (Silver fir), widespread across Central Europe and some parts of Southern and Eastern Europe (Mauri *et al*. 2016); and (ii) *A. cephalonica* Loudon (Greek fir), endemic to the mountains of central and southern Greece (Panetsos 1975). In addition, a third taxon of hybrid origin between *A. alba* and *A. cephalonica* was described by Mattfeld (1926) from the southern Balkans: *A.* x *borisii-regis* Mattf. This natural hybrid shows either intermediate or mosaic morpho-anatomic characteristics of vegetative organs compared to the putative parental species (Chater 1993). Nevertheless, this is a highly polymorphic hybrid whose populations represent a mixture of various morphotypes with different degrees of transition between Silver and Greek fir, posing a significant challenge for botanists determining the boundaries of its natural range (Nikolić *et al*. 2021a; 2021b).

This study represents the first comparative survey of structural differences in epicuticular waxes from adaxial and abaxial needle surfaces of *A. alba*, *A.* x *borisii-regis*, and *A. cephalonica* and concerning natural weathering, including the first results on the micromorphology of epicuticular waxes in Balkan endemic fir species *A.* x *borisii-regis* and *A. cephalonica*. Specifically, our aims in this study were: i) to determine whether there are any differences in the micromorphological structures of needle epicuticular waxes among the studied fir species; ii) to determine structural changes in epicuticular waxes as a result of natural weathering and the degree in which these changes differ among them.

MATERIAL AND METHODS

Plant material

The present study was performed on needles of three age classes (one-, two-, and three-year-old needles) of *A. alba*, *A.* x *borisii-regis*, and *A. cephalonica*, native to the flora of the Balkan Peninsula. Table 1 presents the data on location, habitat description, and voucher information for each selected population. Two needles from the central part of one-, two-, and three-year-old shoot increments were collected from one individual of each species (18 needles in total). One of the two needles of the same age was used for scanning the adaxial surface of the needle and the other one the abaxial surface. Voucher specimens of each species were deposited in the "Herbarium Moesiacum Niš" (HMN) of the Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš (Niš, Serbia). Identification of plant material was done by Dr. Z.S. Mitić and Dr. B.K. Zlatković.

Table 1. Location, habitat description, and vouchers of the studied *Abies* species

Taxon	Locality	Latitude (N)	Longitude (E)	Altitude (m above sea level)	Substratum	Sampling date	Voucher
A. alba	Serbia, Mt. Tara, Predov Krst	40°11'47"	22°10'48"	1096	Limestone	June, 2018	13949 HMN
A. x *borisii-regis*	Greece, Mt. Olympus, above Leptokarya	40° 01'54"	22°30'25"	640	Limestone	October, 2018	13951 HMN
A. cephalonica	Greece, Cephalonia, Mt. Ainos	38°09'37"	20°37'13"	1005	Limestone	July, 2019	13996 HMN

Scanning electron microscopy (SEM)

To examine the micromorphology of epicuticular waxes, the needle surface moisture was allowed to evaporate for the same amount of time, as many researchers have used air-dried samples in order to preserve and dehydrate needles for wax morphology studies. After air-drying, needle samples (six needles per studied species) were mounted sequentially on specimen stubs, sputter-coated with gold, and examined with JEOL JSM 5300 scanning electron microscope. The middle portion of the adaxial and abaxial surfaces of each needle was photographed at magnifications in the range of 50–7.500x.

RESULTS

All three studied *Abies* species were characterized by the absence of adaxial stomata (Figs. 1-3: A-C). Nevertheless, the presence of irregular, often rounded wax structures in the form of granules was observed on the adaxial surface of the needles (Figs. 1-3: D-F). These granules were present along the entire adaxial needle surfaces, but the largest quantities were observed in the central parts (along and near the central mid-vein). An increasing amount of granules with needle aging was a shared characteristic, appearing in all three fir species, so the smallest amount was observed on one-year-old and the greatest on three-year-old needles (Figs. 1-3: A-F).

Stomata arranged in longitudinal rows were observed on the abaxial needle surface, forming two parallel bends (stomatal bands; Figs. 1-3: G-I). The main wax crystalloids on the abaxial needle surfaces were tubules that tend to concentrate within stomatal complexes. Specifically, tubules were quite dense with a reticular aspect inside the stomatal pores but sparsely present on epistomatal rims and surfaces between stomata within the stomatal bands (Figs. 1-3: J-R). Although the tubules are known to be tubular, their tips were permanently occluded (Figs. 1-3: J-R). The surfaces between stomata of the stomatal bands mostly appeared as continuous massive wax coverings with a more or less prominent surface sculpturing, i.e., crusts, but in some places with noticeable granules (Figs. 1-3: J-O).

There were apparent differences in the structure of epicuticular waxes of stomatal areas when one-, two- and three-year-old needles were compared: dense tubules with a reticular aspect within the stomatal pores were usually best-preserved on the one-year-old needles in all three fir species. Additionally, on one-year-old needles of *A. alba* and *A. cephalonica* the epistomatal rims and surfaces between stomata within the stomatal bands were essentially covered in these wax crystalloids (Figs. 1 and 3: M-O). However, in the case of the one-year-old needle of *A.* x *borisii-regis*, tubules were exclusively concentrated within the stomatal pores (Fig. 2: M-O). In contrast, the epistomatal rims and surfaces between stomata of the stomatal bands were already covered by amorphous wax crusts (Fig. 2: M-O). Two-year-old needles were in a more advanced stage of tubule agglomeration, thickening, and fusion compared to one-year-old ones (Figs. 1-3: M-R). Moreover, amorphous crusts may be observed in some places within the stomatal pores of two-year-old needles, which was again most pronounced in *A.* x *borisii-regis*, and less in *A. cephalonica*. On three-year-old needles, the stomata were almost occluded by amorphous crusts, especially in *A.* x *borisii-regis* (Fig. 2: M-O). Therefore, the stomatal pores on the abaxial surface of the fir needles were permanently blocked either by wax tubules or by wax crusts.

Fungal mycelia were detected sporadically on both surfaces of *A. alba* needles of all ages, but most pronounced on three-year-old needles (Fig. 1: J-L). They were not observed on the needles of *A.* x *borisii-regis* and *A. cephalonica*.

DISCUSSION

The leaf surface is primarily covered with a thin continuous layer of amorphous wax, which arises crystalloids of the so-called structural wax. In our study, epicuticular wax structures in the form of granules were observed on the adaxial needle surfaces, which were characterized by the absence of stomata (Figs. 1-3: A-F). Nevertheless, it is debatable whether the granules are crystalloids since contaminants were often misinterpreted as granules and *vice versa* (Barthlott *et al*. 1998). Moreover, for a long time, it was debated whether the characteristic forms of epicuticular waxes and their distribution patterns are controlled by the plant cuticle, by the formation of pores, or by particular areas of increased permeability (Koch & Ensikat 2008).

When examining species of the genus *Pinus* L., Yoshie & Sakai (1985) detected epicuticular wax crystalloids only on the needle surface with stomata. Such a restriction of the crystalloids to the needle surface with stomatal complexes suggested that the structural wax originates associated with the stomatal complex. In accordance, many studies have shown that wax tubules on needles are not distributed evenly in Pinales, considering their tendency to be concentrated near stomata and between them in the stomatal rows (Crossley & Fowler 1986; Kim *et al*. 2011; Ivănescu *et al*. 2008; Mitić *et al*. 2017). Epicuticular wax tubules on *A. alba* needles are deposited only on the abaxial surface where stomatal complexes occur (Bačić & Popović 1998), just as observed in our study. Furthermore, this was a shared characteristic, appearing in Balkan endemic fir species *A.* x *borisii-regis* and *A. cephalonica* as well. Interestingly, the situation could be quite different on *Abies* stems: the tubules are more or less evenly distributed (Tomaszewski & Zieliński 2014).

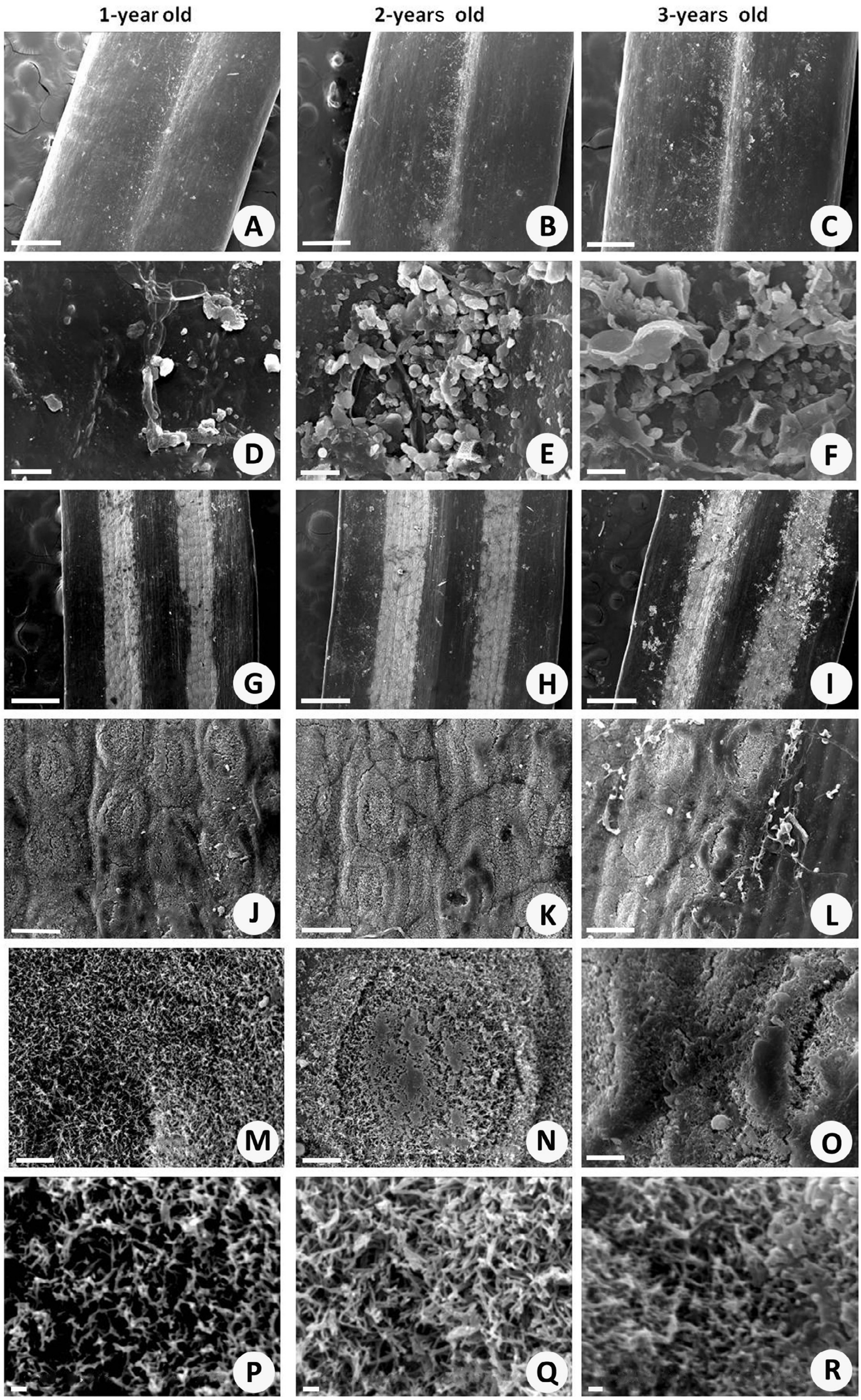

Figure 1. SEM micrographs of *A. alba* epicuticular waxes. **A-F.** Adaxial surface of needles with noticeable granules; **G-I.** Abaxial surface of needles with two stomatal bends; **J-L.** Abaxial stomatal rows; **M-O.** Stomatal pores occluded either by wax tubules or by wax crusts; **P-R**. Agglomeration, thickening and fusion of tubules with needle aging. Bars: **A-I** = 500 µm; **J-L** = 50 µm; **D-O** = 10 µm; **P-R** = 1 µm.

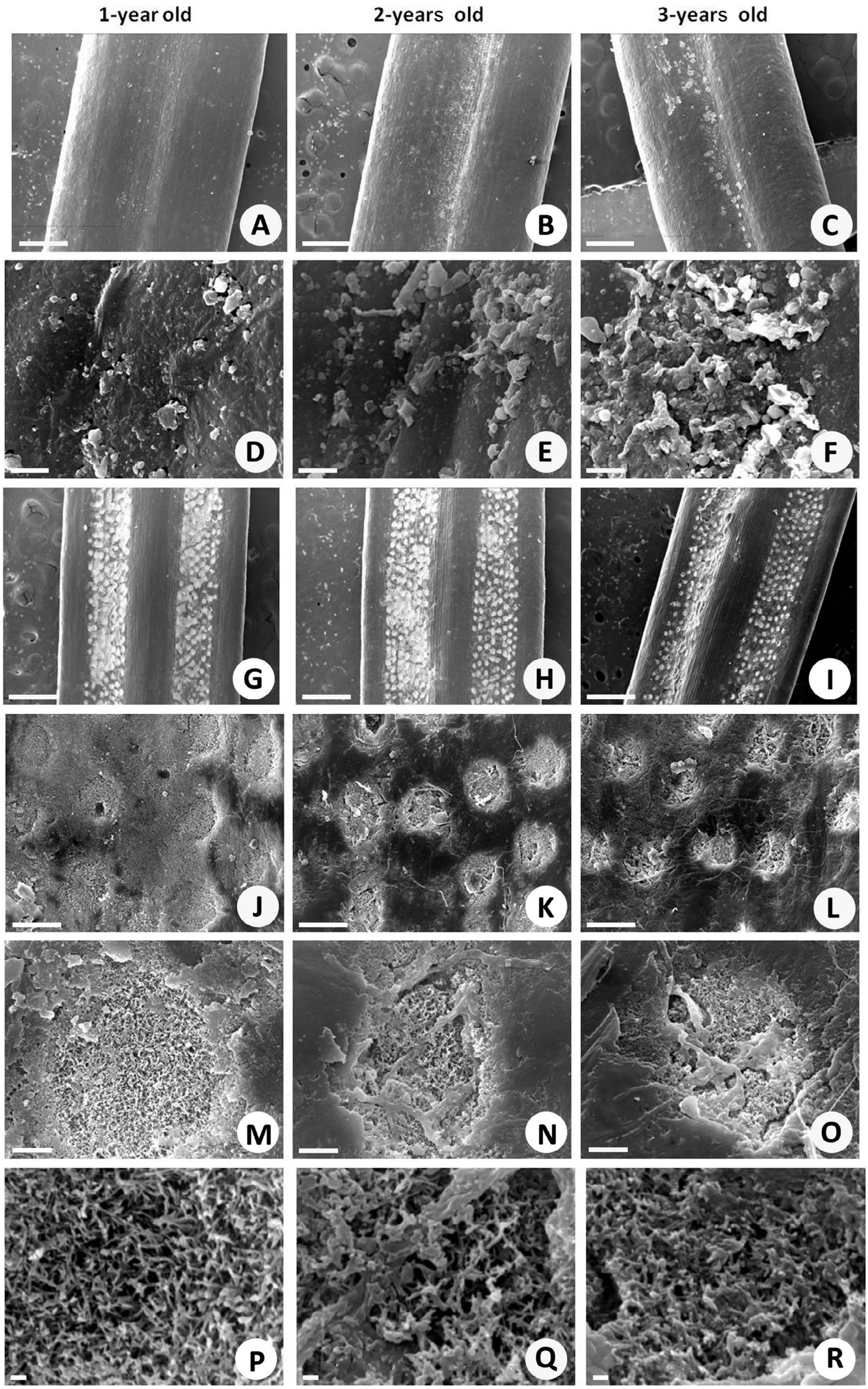

Figure 2. SEM micrographs of *A.* x *borisii-regis* epicuticular waxes. **A-F.** Adaxial surface of needles with noticeable granules; **G-I.** Abaxial surface of needles with two stomatal bends; **J-L.** Abaxial stomatal rows; **M-O.** Stomatal pores occluded either by wax tubules or by wax crusts; **P-R**. Agglomeration, thickening and fusion of tubules with needle aging. Bars: A-I = 500 μm; J-L = 50 μm; D-O = 10 μm; P-R = 1 μm.

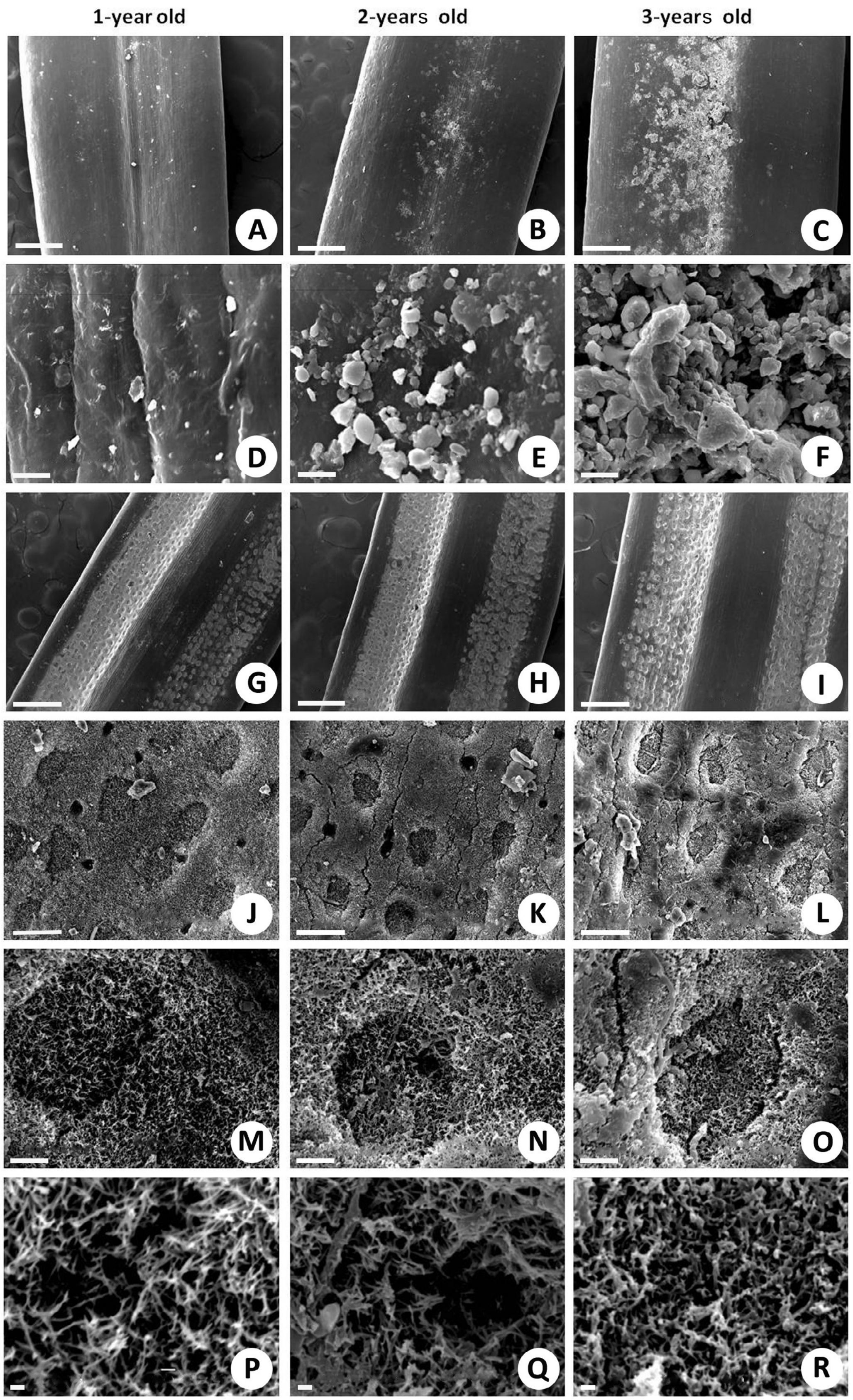

Figure 3. SEM micrographs of *A. cephalonica* epicuticular waxes. **A-F.** Adaxial surface of needles with noticeable granules; **G-I.** Abaxial surface of needles with two stomatal bends; **J-L.** Abaxial stomatal rows; **M-O.** Stomatal pores occluded either by wax tubules or by wax crusts; **P-R.** Agglomeration, thickening and fusion of tubules with needle aging.

Bars: A-I = 500 µm; J-L = 50 µm; D-O = 10 µm; P-R = 1 µm.

Tubules on the abaxial needle surfaces appeared as rodlets in the micrographs presented here (Figs. 1-3: P-R). This was probably an artifact considering that the applied sputter coating technique would probably have obscured the terminal opening of a tubular crystal form (Crossley & Fowler 1986). In conifers, tubules have already been recognized as microstructures of epicuticular wax (Riederer 1989; Hennig *et al*. 1994; Jeffree 2006). Also, crystalloids once described as rodlets in various species are now known to be tubules (Jeffree 2006). In support of this, the secondary alcohol nonacosan-10-ol is the main component of cuticular waxes in many gymnosperms (*Ginkgo*, *Pinus*, *Abies*, *Picea*, *Wollemia*, *Taxus*, etc.; Nikolić *et al*. 2012). Its biological importance is mainly ascribed to the role of the shape of the crystalloids in the form of characteristic tubules on the conifer needles (Matas *et al*. 2003; Jeffree 2006). Currently, there is a joint agreement that the micromorphology of epicuticular waxes depends mostly on their chemical composition (Jeffree *et al*. 1976; Baker 1982; Jeffree 1986; Barthlott *et al*. 1998).

Over time, the tubules were altered to various extent, fused inside the stomatal pores, on epistomal rims and areas between stomata of stomatal bands forming amorphous wax crusts. This leads to forming of a solid wax plug above the pore, entirely or partially occluding the stomata in the older needles. Such changes in the stomata may either limit or obstruct gas exchange and impair protection against water stress. Wells & Franich (1977) reported that the density of tubiform wax tufts on *P. radiata* leaves was greatest near the stomatal pores, where the morphology of the wax changed from tubiform to amorphous along with the maturation of the primary needle. In accordance, our study showed increasing degradation of tubules on *Abies* needles with the needle aging. These changes in the appearance of structural waxes are part of the natural aging process, and gradually all structural wax degrades to granules or amorphous crusts (Mitić *et al*. 2017).

Moreover, the tubules of conifer epicuticular waxes has been observed to be degraded by a different environmental factors, such as mechanical (wind, abrasion), chemical (deposited loads of pollutants), and biotic (the activities of epiphytic microorganisms and insects; Jeffree 2006). Bačić & Popović (1998) have shown the very early beginning of fusion and aggregation of wax tubules on *A. alba* needles and the increasing percentage of amorphous wax stages on very young needles, particularly in the more polluted areas. This indicates of an accelerated wax degradation process, which is not only age-dependent but also influenced and augmented by air pollution coming from industry and road traffic. However, plant material of three *Abies* species in our study was collected from their native populations away from known sources of air pollutants. Thus, the degree of wax degradation apparently had no relation to pollution but varied among species since it was faster and started earlier on *A.* x *borisii-regis* needles than in *A. alba* and *A. cephalonica.* Nevertheless, such accelerated degradation of wax crystalloids in *A.* x *borisii-regis* should be statistically supported in future studies.

During the degradation process there is a gradual increase in humidity, when epicuticular surfaces of conifer needles show signs of weathering and may be colonized by green algae and fungal hyphae. As a consequence, the intensity of photosynthesis decreases, and at the level of the entire tree this reduction may be between 4 and 9% (Peveling *et al*. 1992). In our study, only fungal mycelia were sporadically recorded on *A. alba* needles of all ages, but were most pronounced on three-year-old needles that were characterized by the highest degree of wax degradation (Fig. 1: J-L).

CONCLUSION

The needle surfaces of examined *Abies* species were primarily covered with a thin continuous layer of amorphous wax, on top of which the crystalloids of the so-called structural wax arise. On the adaxial surface of the needles in all three studied fir species, epicuticular wax was in the form of granules, whose amount increased with needle aging, so they were the most present on the oldest (three-year-old) needles. The main wax crystalloids on the abaxial needle surfaces were tubules, concentrating within stomatal complexes and between them in the stomatal rows. Namely, tubules were quite dense with the reticular aspect inside the stomatal pores but sparsely present on the epistomatal rims and surfaces between stomata within the stomatal bands. These are the first results regarding the micromorphology of epicuticular waxes in Balkan endemic fir species *A.* x *borisii-regis* and *A. cephalonica*.

The tubules reached more advanced aggregation, thickening, and fusion stages over time, forming amorphous wax crusts. As a result, the stomata were almost occluded by the wax crusts on three-year-old needles, especially in *A.* x *borisii-regis*. Although we did not find differences in the micromorphology types of epicuticular wax among the examined species, the tendency to increase in the amount of granules and the degree of degradation of structural wax (tubules) into amorphous crusts as the result of the natural aging process was observed. Moreover, these structural changes of epicuticular waxes started earlier and were faster on *A.* x *borisii-regis* needles than in *A. alba* and *A. cephalonica*, which should be statistically supported in future studies.

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