Feddes Repertorium **119** (2008) 7–8, 644–654 DOI: 10.1002/fedr.200811188 Weinheim, Dezember 2008

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Discrimination of *Pinus eldarica* MEDW. and its two new species by epicuticular wax, lignin content, electrophoretic isozyme and activity of peroxidase

With 7 Figures

Summary

Mondell pine (*Pinus eldarica* MEDW.) is a rare and drought resistant pine, found naturally only in semi desert environment southeast of Tbilisi, Georgia, and has been probably introduced to Iran more than 800 years ago. This species has gradually altered both in shape and growth rate in Nashtifan-Khaf, and generated two species nova which so called as ball shaped and conical, according to characteristics of their shape and smaller stature. This study was conducted to discriminate these species nova using epicuticular wax features and certain biochemical characteristics i.e., peroxidase electrophoretic pattern (PAGE), the activity of soluble (SPO), ionically (IPO) and covalently (CPO) wall-bound fractions of peroxidase, lignin and total protein content. Observation via scanning electron microspopy showed differences that in adaxial and abaxial surface of needles, and also seed surface, in ball shaped type with the others. But none of the biochemical characteristics had significant difference in these three types. The electrophoretic analysis showed that the peroxidase isozyme is not suitable marker for discriminating the species nova from wild type, in spite of obvious micro- and macromorphological differences. Results of current study, propose the use of molecular markers and cytogenetic studies in order to specify cause of species nova occurrence in the study area.

Zusammenfassung

Die Unterscheidung von *Pinus eldrica* MEDW. und ihrer beiden neuen Arten durch epikutikulares Wachs, Ligningehalt, elektrophoretische Isozyme und Peroxidaseaktivität

Die Mondell-Kiefer (*Pinus eldarica* MEDW.) eine seltene und trockenresistente Kiefer, findet sich am natürlichen Standort nur in einer Halbwüsten-Umgebung südöstlich Tbilissi (Georgien) und ist möglicherweise vor mehr als 800 Jahren in den Iran eingewandert. Diese Art hat sich teilweise sowohl in ihrer Form als auch in ihrer Größe in Nahstifan-Khaf verändert. So entstanden zwei neue Arten: eine so genannte Ball- und eine konische Form, so werden ihre Form und ihre kleinere Statur charakterisiert. In dieser Studie wird versucht, diese neuen Arten zu trennen mittels der Eigenschaften von Epikutikularwachs und einigen biochemischen Merkmalen, als da sind: peroxidase electrophoretic pattern (PAGE), die Lösungsaktivität (SPO), ionikonische (IPO) und kovalenten (CPO) Grenzfraktionen der Peroxidase, den Lignin- und Proteingehalt. Beobachtungen mittels Elektronenmikroskopie zeigten Differenzen in der adaxialen und abaxialen Oberfläche der Nadeln, ebenso der Oberfläche der Samen und der Ballformen untereinander. Aber kein biochemisches Merkmal erbrachte signifikante Unterschiede bei diesen Typen. Die elektrophoretische Analyse bewies, dass die Peroxidase und Isozyme keine brauchbaren Kennzeichen für die Unterscheidung der neuen Arten von den Wildformen liefern trotz eindeutiger mikro- und makromorphologischer Unterschiede. Aus vorliegender Studie resultiert die Möglichkeit, dass die molekularen Kennzeichen und zytogenetischen Untersuchungen geeignet sind, neue Arten im Untersuchungsgebiet zu bestimmen.

 0014-8962/08/7-812-0644

Introduction

Mondell Pine (*Pinus eldarica* MEDW.) is a drought tolerant pine with narrow range of distribution confined to a semi desert environment southeast of Tbilisi, Georgia, Transcaucasia. This pine grows on the eastern extremity of Choban-Dagh Range, along the south side of the Iori River. This pine is considered to be an Oligocene relict that previously, had occupied a larger area (MIROV 1967). Systematic position of *P. eldarica* has not yet been univocally made clear; in fact in GAUSSEN (1960), NAHAL (1962), DEBAZAC & TOMASSONE (1965) opinion it is a geographic variety of *P.* brutia TEN., while according to KOLESNIKOV (1963) it is a separate species (CALAMASSI et al. 1988). However, about more than 800 years ago, this species have been introduced to Iran and cultivated in northeast, east and center of the country. Crown shape, growth rate and most of morphological features of some individuals of Mondell pine, have been altered during times and two distinct forms have been generated from it besides its original form. One, is smaller and have a lower growth rate and is called ball shaped type, corresponding to its shape, and the other one bears middle stature and is known as conical type, referring to its conical crown shape, In the present paper, they are referred as elder, ball shaped and conical.

Epicuticular waxes cover the external side of the leaf epidermis of all higher plants. They are essential structural elements of the surface which have fundamental functional and ecological importance for the interaction between plants and their environment (BARTHLOTT et al. 1998; ENSIKAT et al. 2006). Epicuticular waxes exhibit great micromorphological diversity therefore they have systematic and taxonomic importance (BARTHLOTT et al. 1998) and also in some wax types, a correlation exists between the chemical composition and the morphology of the organ (ENSIKAT et al. 2006).

The characterization of plant germplasm has traditionally been used for the study of morphological traits. However, recently the use of molecular techniques have become widespread in the study of biochemical characters. These techniques provide a better estimation of the genetic diversity and structure of populations (WEISING et al. 1995; ANDRÉS et al. 1999).

Isozymes are closely related to gene products and their electrophoretic mobility, resulting from differences in size and shape of enzyme molecules, are good indicators for genetic diversity (RAHMAN et al. 2000). Many researches have used electrophoresis of isoenzymes as an efficient tool for classification and discrimination between species of higher plants (ELLSTARND & LEE 1987; APAVATJRUT et al. 1999). Studies on isoenzymes have been accomplished on a great number of pine species from North America and also some Mediterranean pines, *Pinus sylvestris* populations and *P. halepensis-brutia* complex in Europe (SCHILLER et al. 1986; SCALTSOYIANNES et al. 1994; ANDRÉS et al. 1999; RAHMAN et al. 2000).

The present study was conducted to compare and discriminate wild type with conical and ball shaped types, using the epicuticular wax layer and its micromorphological variability on stem, needles and seeds, peroxidase activity and variation of its isozymes as well as lignin and protein content.

Material and methods

Seedling samples of the same age of Mondell pine with its conical and ball shaped types (Fig. 1) were collected from Nashtifan, east of Iran in March of 2006. This area is located in 34°25′50″ N and 60°9′58″ E at 865 m a.s.l. and for the first time the great morphological changes in Mondell pine has been observed there. These species nova are able to be increased by sexual reproduction, so villagers grow them for sell. The plants were transferred to greenhouse and kept there under controlled conditions until the time of sampling in the next spring (May). SEM analysis:

Great care was taken to pick the samples from similar locations on seedlings to have samples with identical age, physiological situations, etc. After air drying and mounting on aluminium stubs, 0.5 cm in diameter pieces of needle and stems were coated with gold film in a sputter coater (SCD 005, BAL-TEC Corporation, Switzerland), and were observed by a scanning electron microscope (SEM) (XL30, Philips, Netherlands) operated at 25 kV (BARTHLOTT et al. 1998; TOMASZEWSKI 2004). Following BARTHLOTT et al. (1998), an attempt was also conducted in order to do classification between studied pines by comparing and assigning the observed characteristics of wax.

Polyacrylamide gel electrophoresis of peroxidase

Frozen needle samples (which collected in spring and summer seasons) were homogenized in 5 ml of cold extraction buffer containing 0.1 *M* Tris-HCl (pH = 8), 0.5 *M* NaCl, 5 m*M* 1.4-Dithio-Dl-thretitol (DTT) and 5 m*M* EDTA. Samples were then centrifuged at 12,000 × *g* for 20 min at 4 °C. Equal volumes of the samples (30 µg) were loaded on a native discontinuous polyacrylamide gel (5% stacking, 12.5 separating gel; pH = 6.8) followed by 20 mA for 4 h in Tris-Glycine buffer (pH = 8.3). Gel was stained for peroxidase using the method GRAHAM et al. (1964) (TANKSLEY & ORTON 1986).

Peroxidase activity

Peroxidase (PO) was extracted and determined in three fractions; the soluble (SPO), ironically- (IPO) and covalently- (CPO) wall bound fractions. Activity of the first fraction was measured using guaiacol as an electron donor and of the two later which are supposed to be more related to the lignification of the cells, was measured using syringaldazine as an electron donor. Samples (1 g fresh weight) were homogenized in 50 m*M* Tris-maleate buffer (pH = 6.0) and centrifuged at 1000 × *g* for 10 min at 2 °C. The supernatant was re-centrifuged at 18,000 × *g* for 20 min at 2 °C. This second supernatant was used to assay soluble PO. Pellets of the first and the second centrifugations were pooled, incubated with 0.2 *M* $CaCl_2$ for 2 h at room temperature, and then centrifuged at 18,000 × *g* for 20 min at 2 °C. The supernatant was used to measure the activity of IPO. The pellet was used directly for assay of CPO. Activity of SPO fraction was assayed in 100 m*M* Na-phosphate buffer (pH = 6.1) containing 28 m*M* guaiacol and 5 m*M* H_2O_2. The increase in the absorbance was recorded at 470 nm. For IPO and CPO assay, the final reaction mixture (3 ml) contained 41.6 nm syringaldazine, 40 m*M* Tris-maleate buffer (pH = 6.0) and 16 m*M* H_2O_2. Activity of IPO was expressed as the increase in absorbance at 530 nm per min per mg protein and activity of CPO was expressed as the increase in absorbance at 530 nm against cell wall dry weight (MORITA et al. 2006).

Protein content

The amount of total protein in the supernatant was assayed using the Bradford method (BRADFORD 1976) with bovine serum albumin (BSA) as a standard.

Lignin content

Cell wall preparations were obtained by homogenization of frozen sample (with liquid N_2) in 50 m*M* Na-phosphate buffer (pH = 6.8) with mortar followed by sequential washing of the pellet with EtOH, $CHCl_3$–MeOH (2 : 1 v/v) and acetone, and then drying in air (GHANATI et al. 2005). Lignin

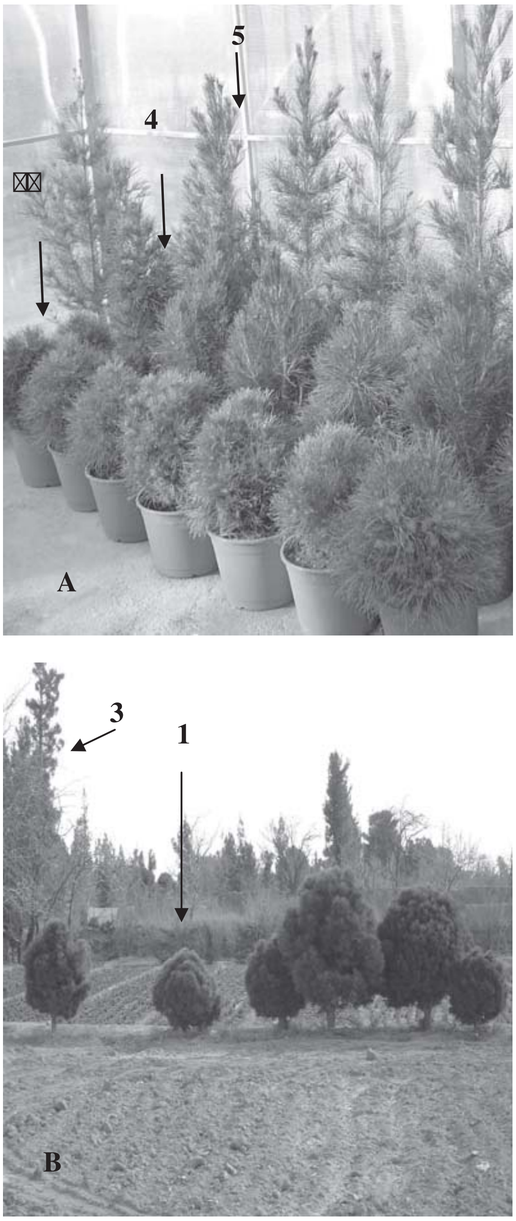


Fig. 1
Apparent shapes of wild type (3) and its species nova; conical type (2) and ball shaped type (1)

A — three years old samples; B — 12–20 years old ball shaped and wild type in the nature

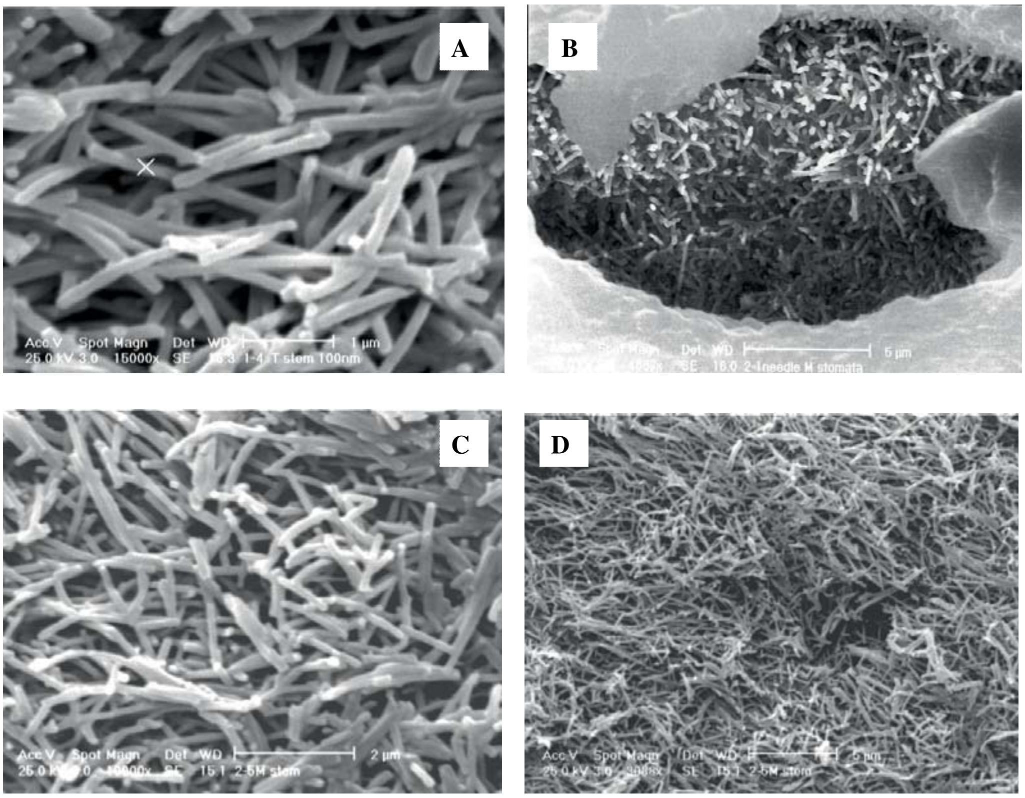


Fig. 2
Scanning electron micrographs of wax on stem of wild type and its two species nova
A, B — tubular crystalloids on stem and exterior epistomatal chambers of wild type. Similar shape and pattern of stem wax crystalloids were observed in conical type (C) and ball shaped type (D) as well

A × 15,000; B × 4683

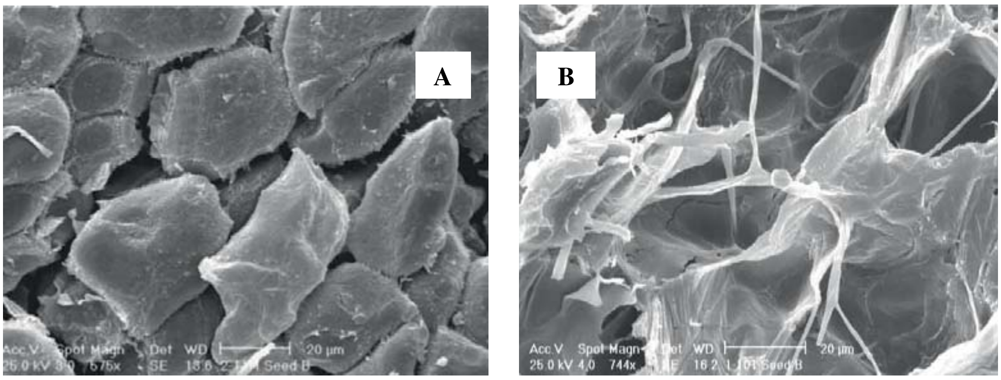


Fig. 3
Scanning electron micrographs of crust like wax on the surface of seeds of wild type and conical type (A) and membranous platelets crystalloid on the seed surface of ball shaped type (B)

A, B × 10,000

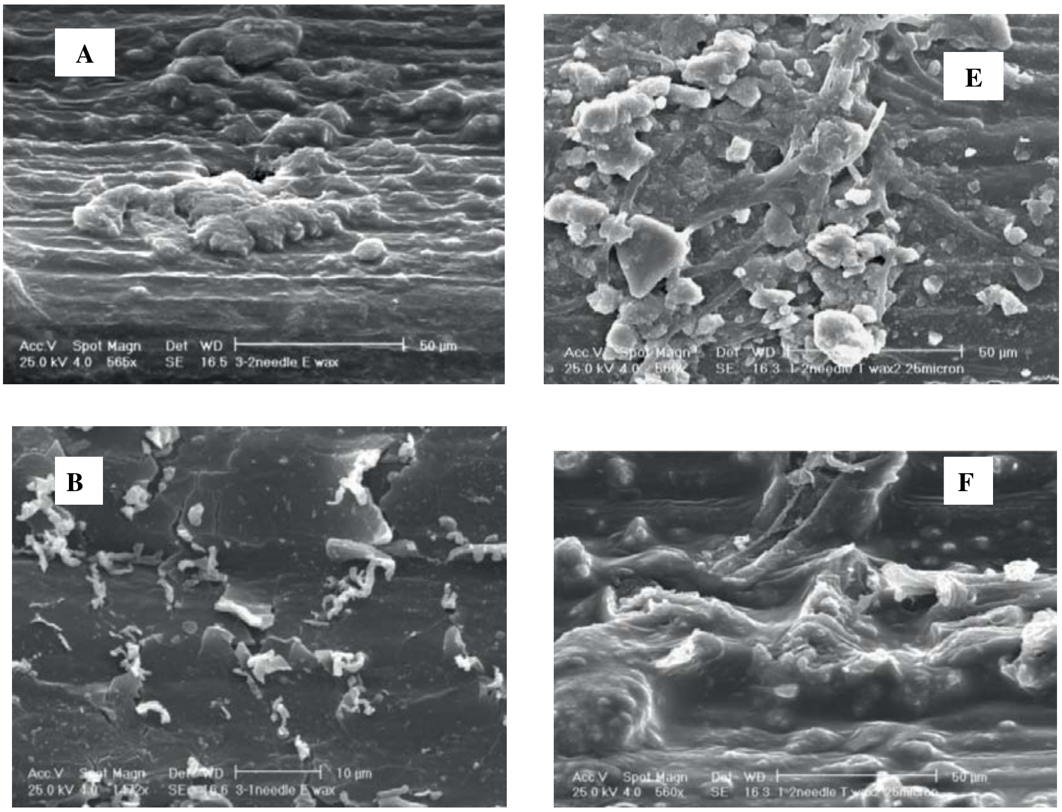
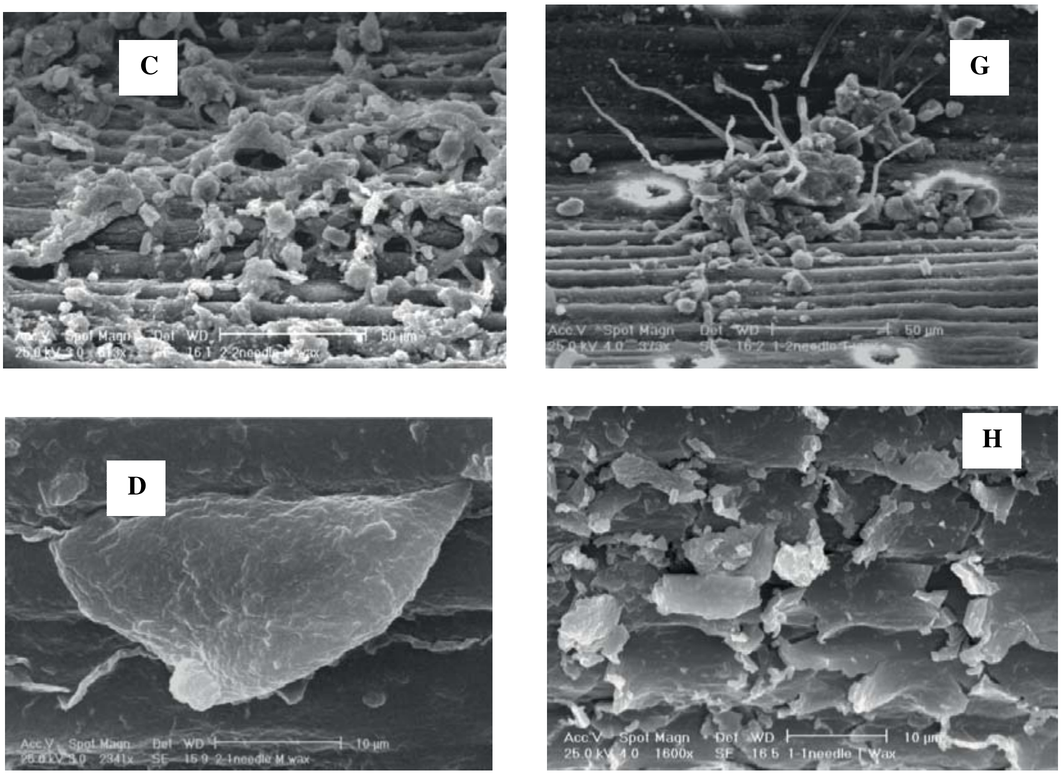


Fig. 4
Characteristics of wax on the needles of wild type and its two species nova observed by Scanning electron A, B — amorphous wax and fissured layer of wax on adaxial and abaxial surfaces (respectively, in tandem) scattered amorphous wax on adaxial and abaxial surfaces of conical type needles (respectively, in tandem); crystalloids (E) soft, honey like wax (F) mass amorphous outward which tubular crystalloids are emerged
A, C, E, F × 600; B × 500; D, G × 400; H × 800

content was determined using a modified acetyl bromide procedure (IIYAMA & WALLIS 1990). In brief, 6 mg of a finally powdered wall preparation was treated with a mixture (total of 2.5 ml) of 25% (w/w) AcBr in HOAc and 0.1 ml of 70% $HCLO_4$ at 70 °C for 30 min which shacking at 10 min intervals. After cooling with ice, the digestion mixture was transferred to a 25 ml volumetric flask containing 5 ml of 2 *M* NaOH and 6 ml HOAc and made up to 25 ml. Lignin content was determined by measuring the absorbance at 280 nm using a specific absorption coefficient of 20 g^{-1} L cm^{-1} (IIYAMA & WALLIS 1990).

Results

Observation of epicuticular wax layer of stem by SEM showed that stem of wild type was covered by a continuous and dense layer of uniform but randomly-oriented, tubular crystalloids with 200 nm in diameter and 1–2 µm in length (Fig. 2A). Wax on the exterior surfaces of epistomatal chambers of wild type also was very similar to that of the stem of it (Fig. 1B). Shape of wax crystals and pattern of their distribution on the stem of conical and ball shaped



micrographs
of needles of wild type; C, D — interconnected granules of wax with different dimensions of 2–20 µm, and E–G — different shapes of wax on abaxial surface wax of ball shaped type: the aggregated amorphous (G); H — crust like components of wax on abaxial surface of ball shaped type

types were identical to that of the wild type (Fig. 2C, D).

Shape and pattern of distribution of wax on seeds and needles of studied types however, showed distinct differences. Wax on the surface of seeds of wild type and conical type were composed of crust like structures (with more than 10 µm in diameter) (Fig. 3A), but wax on the seeds of ball shaped type was composed of membranous platelet crystalloids with some connections (Fig. 3B).

On the adaxial surfaces of needle of wild type, wax was amorphous with diverse size from very small pieces (aggregated), to scattered masses of ca. 20 µm (Fig. 4A). Abaxial surface wax however, bore cracking and separating in some places forming fissured layer with a thickness of 1–1.2 µm (Fig. 4B).

Adaxial surface of needle of conical type, was covered with interconnected granules of wax. The approximate dimensions of these granules were 2–20 µm (Fig. 4C). Abaxial surface however, was covered with scattered amorphous wax with dimension of 10–35 µm (Fig. 4D).

On adaxial surface of needle of ball shaped type, epicuticular waxes often were seen in three shapes: rock-shaped (or amorphous) with

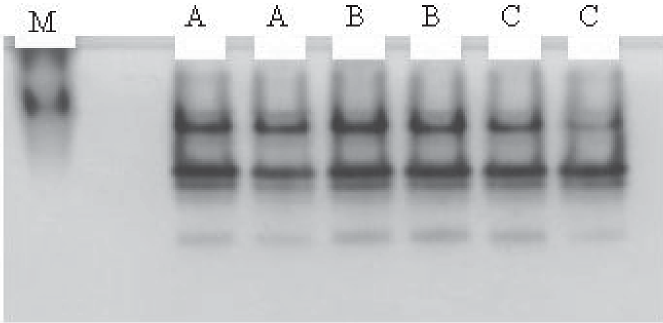


Fig. 5
PAGE of peroxidase isozymes of wild type and its two species nova on native gel (12.5%)
A — wild type; B — conical type; C — ball shaped type
M — peroxidase marker of horseradish

maximum dimension of 30 µm which were aggregated in some places (Fig. 4E), soft, honey like waxes (Fig. 4F), and the masses of amorphous wax (ca. 75 µm in diameter) from which vigorous tubular crystalloids were emerged. The tubules were 40–70 µm in length and 2–6 µm in diameter at the base (Fig. 4G).

The wax of the abaxial surface of needle of ball shaped type was fissured and composed of crust like fragments with 10–30 µm in diameter (Fig. 4H).

Electrophoretic analysis of peroxidase isozymes by one-dimensional protein patterns showed no differences between wild type and two species nova (Fig. 5).

There was no significant differences between the activity of soluble peroxidase (SPO) of wild type and conical type, however, statistically significant differences were observed between SPO of these pines with that of ball shaped type (Fig. 6A). The activity of covalently wall-bound peroxidase (CPO) was identical in all three studied types, however, the activity of ionically wall-bound fractions (IPO) showed again significant differences between all three types (Fig. 6B, C). There was no differences between protein contents of needles of studied types (Fig. 6D).

A significant increasing order was observed in lignin content of stems of wild type, conical and ball shaped type, respectively (Fig. 7). However, lignin content of needles of wild type was higher than those of two species nova (Fig. 7).

Discussion

The investigations confirmed the existence of substantial differences between the analyzed species in the aspect of structure and either amount of the wax layer. The most distinct differences were related to adaxial and abaxial surfaces of needles of ball shaped type which were covered with dense crystalloids of wax in comparison with that of the two other types. Ultrastructure of wax in this species nova was also more diverse than the two other types, due to the presence of tubules as the prevalent structures on adaxial and fissured layers on the abaxial surfaces.

Some of the observed structures of these types were not exactly assigned to any of the types classified by BARTHLOTT et al. (1998). Interconnected granules on the adaxial surface of conical pine needles, fractured and curved radial tubules which came out from masses of amorphous wax, membranous platelets on the seed surface of ball shaped type as well as crust layer on the seed surface of wild type and conical type are certain examples of the features which are novel and can not be included in BARTHLOTT et al. (1998) classifications.

The stem surfaces were covered by tubular crystals similar to Nonacosanol-tubules, had been the characteristic tubule shape described previously for gymnosperm surfaces (BARTHLOTT et al. 1998; WEN et al. 2006). These tubules had a constant diameter, length and no

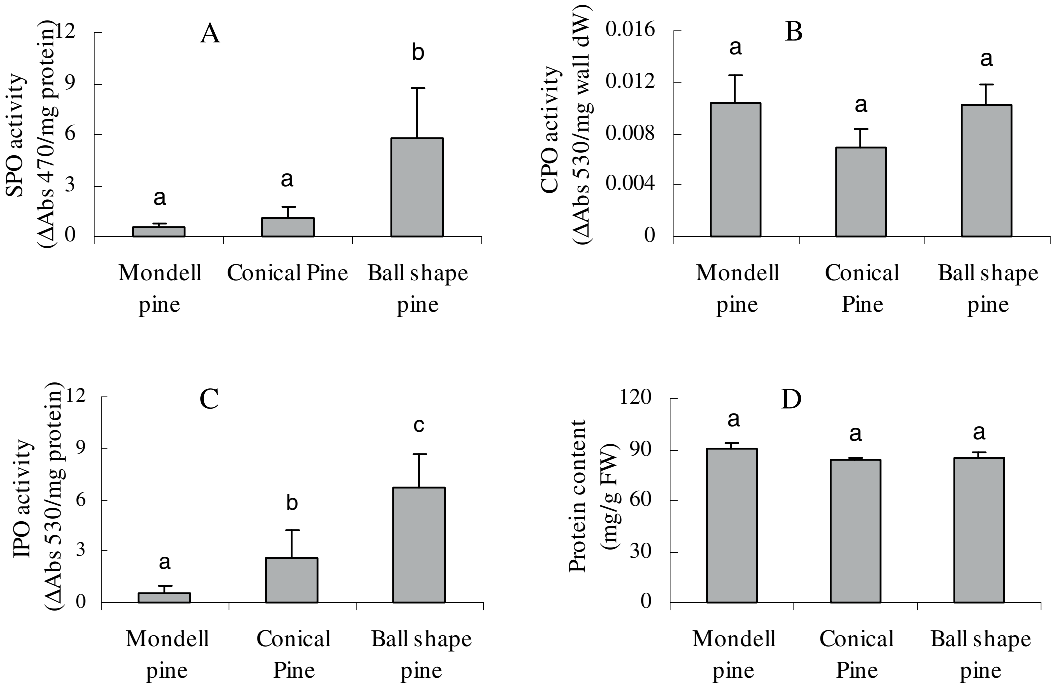


Fig. 6
Activity of different fractions of peroxidase in needles of wild type and its two species nova
A — soluble peroxidase (SPO); B — covalently wall-bound peroxidase (CPO); C — ionically wall-bound peroxidase (IPO); D — total protein

Data show means of three different experiments in triplicate ± SE. Signs with different letters in each group indicate significant differences at $P < 0.05$ according to Duncan test

orientation pattern and were so dense that the smooth and continuous surfaces of cuticle were seed surface of ball shaped type had a different crystalloid with the two others. It had a structure similar to membranous platelets, but the others had a thick crust layer.

Morphology, chemical composition, the amount and distribution of wax layer not only on is related to plant taxon, but also to geographical location, environmental conditions, and stage of development (KINNUNEN 1999; CAMERON et al. 2002; TOMASZEWSKI 2004; BRINGE et al. 2005; MEDINA et al. 2006). The influence of abiotic environmental factors on the morphology of the wax has been already mentioned by many studies (SCHREIBER & RIEDERER 1996; RIEDERER & SCHREIBER 2001; KOCH et al. 2006). Accumulation of higher amounts of epicuticular waxes on leaf surfaces could be interpreted as micromorphological adaptation to environmental stress (BRINGE et al. 2005; KOCH et al. 2006). Development of waxes is determined genetically and modified by many environmental factors, such as light quantity and quality, day length, temperature and relative humidity (KINNUNEN 1999).

Regard to the fact that sample pines studied in the present work were collected from only one geographical location and environmental conditions in terms of soil type, precipitation, temperature, light, humidity and so on were identical for all, it can be suggested that observed differences in their morphology and wax micromorphology are of relevance for differences in their genetic profile. The later may reflect in turn to the adaptation of plants to different conditions that forced them during long periods of accommodations.

The isozyme technique in many studies has proved to be useful in supporting the taxonomical species identification and genetic in-

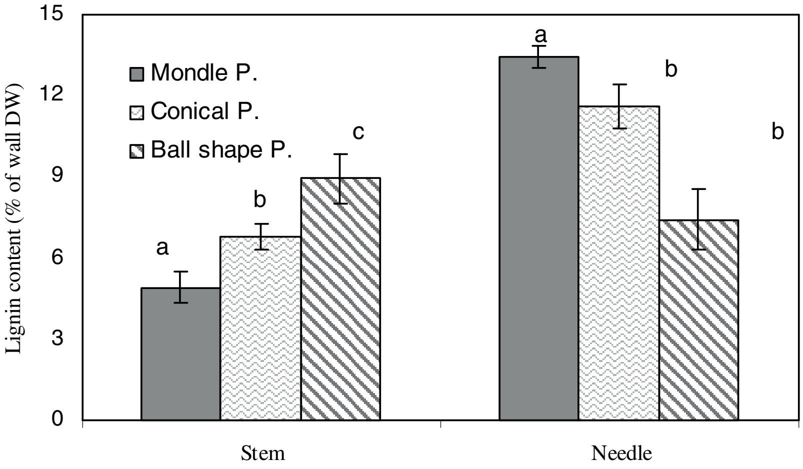


Fig. 7
Lignin content of stems and needles of wild type and its two species nova. Data show means of three different experiments in triplicate ± SE. Signs with different letters in each group indicate significant differences at $P < 0.05$ according to Duncan test

vestigation in plant population (PASHKOULOV et al. 1995; APAVATJRUT et al. 1999). Using only peroxidase system, some tree species (such as *Ulmus* and *Boesenbergia* genera etc.) were separated successfully by the polymorphic isozyme loci (PUCCINELLI et al. 1998; GIJZEN et al. 1999; AGUSTÍN et al. 2000; SYROS et al. 2002; VANIJAJIVA et al. 2003; HATZILAZAROU et al. 2005). Most higher plants possess a large number of peroxidase isoenzymes, which are encoded by multigene families. Several physiological functions for peroxidases in plants have been reported, i.e., removal of H_2O_2, oxidation of toxic reductants, biosynthesis and degradation of lignin in cell walls, auxin catabolism, defensive responses to wounding and defense against pathogen or insect attack as well as physiological changes caused in plants by high temperature stress (SHIVAKUMAR et al. 2003; YOSHIDA et al. 2003; GULEN & ERIS 2004). Electrophoresis pattern of peroxidase isozymes of three studied pines were identical therefore, it can not be used for characterization and distinguishing Mondell pine and its two species nova from each other, but the differences in the activity of SPO and IPO, in particular between wild type and ball shaped type, can reasonably explain their difference in apparent shapes. Increase of wall-bound peroxidases have been proposed to be of relevance for higher amounts of lignification of cell walls (GHANATI et al. 2005). Dwarfism and special shape of ball shaped type may be resulted from higher amounts of lignin which was more pronounced in cell walls of stem of this pine, compared to those of conical type and wild type. Regard to the role of some peroxidase isozymes in auxin catabolism focusing on shoot apex of studied types and even electrophoresis of peroxidase isozymes of this part of plants may be more helpful providing us more reliable discrimination. Using DNA markers, cytogenetic studies (for probability of polyploidy incidence) as well as studying the enzymes which contribute to the synthesis and polymerization of fatty acids for wax (e.g., esterase) is also suggested.

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Manuscript received: December 3rd, 2007/revised version: May 5th, 2008.