

Research Article

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Differences in structure, allergenic protein content and pectate lyase enzyme activity of some Cupressaceae pollen

Bazı Cupressaceae polenlerinde polen yapısı, alerjik protein içeriği ve pektat liyaz enzim aktivitesi bakımından farklılıklar

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Abstract

Objective: Cupressaceae pollen has commonly been reported to be an important aeroallergen and causal factor of spring, autumn and winter pollinosis in many countries. The aim of this study was to compare of the structure and allergenic protein content of *Cupressus arizonica* Greene., *Cupressus sempervirens* L. and *Juniperus oxycedrus* L. pollen in detail and contribute to Cupressaceae pollen allergen diagnosis and therapy studies in Turkey.

Methods: The pollen structure were examined by LM and SEM. Pollen protein content was investigated by Bradford protein assay, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot analysis and two-dimensional polyacrylamide gel electrophoresis (2DE PAGE), respectively. Pectate lyase (PL) enzyme activities

were compared. Immunoblotting was carried out by using extracts of the three taxa pollen collected from Turkey.

Results: All three taxa was found very similar in terms of pollen morphology however, intine thickness was prominently different. *Cupressus arizonica* pollen extracts showed the lowest PL activity. Five sera specific IgE of all allergic subjects showed reaction with only *C. arizonica* pollen extracts.

Conclusions: As a conclusion, the pollen structure, protein function or protein structure and isoforms of allergens could affects allergenic properties of the pollen. This study also may help to improve the Cupressaceae pollen allergen diagnosis and therapy.

Keywords: Allergy; Cupressaceae; Pollen; Isoform; Pectate lyase; Turkey.

Özet

Amaç: Cupressaceae polenlerinin birçok ülkede ilkbahar, sonbahar ve kış polinizasyon dönemleri için önemli aeroalerjen faktör olduğu sıklıkla bildirilmiştir. Bu çalışmanın amacı, *Cupressus arizonica* Greene, *Cupressus sempervirens* L. ve *Juniperus oxycedrus* L. polenlerin yapısını ve alerjenik protein içeriğini karşılaştırmak ve Türkiye’de Cupressaceae polen alerjenlerinin tanısına ve alerji tedavisine katkıda bulunmaktır.

Yöntemler: Polen yapısı LM ve SEM ile incelendi. Polen proteini içeriği, sırasıyla, Bradford protein testi, SDS-PAGE, Western blot analizi ve 2DE PAGE ile araştırıldı. Pektat liyaz enzim aktiviteleri karşılaştırıldı. Immunoblot analizleri çalışılan üç taksona ait polen özütleri kullanılarak gerçekleştirildi.

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Bulgular: Her üç taksonun polen morfolojisi bakımından birbirine çok benzer olduğu ancak intinin kalınlığının taksonlar arasında belirgin olarak farklı olduğu bulundu. *Cupressus arizonica* polen özütlerinde en düşük pektat liyaz aktivitesi görülürken, hastaların beş tanesinde serum spesifik IgE, sadece *C. arizonica* polen ekstraktlarıyla reaksiyon gösterdi.

Sonuç: Polen yapısı, protein fonksiyonu veya protein yapısı ve allerjenlerin izoformları polenlerin allerjenitelemini etkileyebilir. Bu çalışma ayrıca, Cupressaceae polen alerjisi tanısı ve tedavisine katkı sağlayabilir.

Anahtar Kelimeler: Alerji; Cupressaceae; Polen; Isoform; Pektat liyaz; Türkiye.

Introduction

Several cypresses and junipers belonging to the Cupressaceae family represent critical and increasing causes of seasonal allergic diseases in Mediterranean areas, in the southern states of the USA and in Japan. Because of their notable potential to adapt to different edaphic and climatic conditions, cypress trees especially play a prominent role in the design of urban green areas in various regions worldwide. Cupressaceae pollen has commonly been reported to be an important aeroallergen and causal factor of spring, autumn and winter pollinosis in many countries [1–5]. Most of pollen observed in Turkey's atmosphere is formed by Cupressaceae pollen [6–9]. However, there are few studies about the allergenicity of cypresses as their pollen antigens are not commonly used in skin prick tests (SPT) in Allergy Clinics in Turkey [10]. Due to it being commonly in use in Turkey in reforestation in gardens and parks for ornamental purposes and as a wind and noise barrier, and because they produce pollen almost every year, these species are considered allergenic, and it is highly important for diagnosis and treatment of patients to uncover the role of their functional proteins in allergic mechanisms. The pollen morphologies of Cupressaceae are well known and many papers have been devoted to this topic [11–13]. The pollen wall has been seen to consist of a massive intine and a thin exine; the orbicules appear tenuously attached to the surface [14]. *Cupressus sempervirens* L. has a thick intine [13, 15].

Most of the reported investigations on Cupressaceae pollen allergens have been based on the extraction, characterization of water soluble fractions, purification and Western blot analysis. It was presumed that pollen allergens might have expanded via gene duplication since multiple rounds of polyploidy occurred during angiosperm and gymnosperm evolution [16–18]. There is a study indicating that nuclear and cell fusion cause polyploidy in the megagametophyte [19]. This phenomenon brings to mind that

another decisive factor on the allergenicity of allergens could be isoforms. Isoforms are defined as structurally similar proteins that are created as the result of alternative splicing or from similar genes formed from a copied gene and differentiated as the result of evolution. Little changes in the protein structure could result in major changes in the allergenicity as well as enzyme activity. There are a few studies about cypress pollen allergen isoforms using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and immunoblotting. It also has been reported using 2D-PAGE that isoforms of 14 kDa allergen identified in *C. sempervirens* pollen have different allergenicity [20–22].

Pollen extracts from Cupressaceae species have a low protein and high carbohydrate content [23–26]. *Cupressus arizonica* Greene. pollen extracts contain at least six allergenic components with a predominant glycoprotein of 43 kDa (Cup a 1) [23, 27]. There is a high cross reactivity (95% sequence identity) between the major allergens Cup a 1 (43 kDa) obtained from *C. arizonica* and Cup s 1 (43 kDa) obtained from *C. sempervirens* pollen extracts and they have a number of common epitopes [28–30]. In *C. sempervirens* pollen, only two allergens are officially classified in the data bank of the International Union of Immunological Societies (IUIS). Cup s 1 represents the major allergen and is highly cross-reactive with Cup a 1 and Cry j 1, [24] whereas Cup s 3 (34 kDa), which was identified using cDNA cloning and homology sequence analysis, have a low level of protein expression in *C. sempervirens* pollen grains [31]. The pectate lyase (PL) active site of Cup a 1 and Cup s 1 is also found in the major allergen Jun o 1 obtained from *Juniperus oxycedrus* L. PL (EC 4.2.2.2) is an enzyme involved in the maceration and soft rotting of plant tissue. It has been suggested that the expression of PL gene in pollen might relate to a requirement for pectin degradation in the primary cell wall during pollen tube growth [32, 33]. PL action results not only in plant cell wall degradation, but also in the activation of the defense system, most likely through the release of oligogalacturonides from the plant cell wall, which then function as defense elicitors [34–36].

In this study we aimed to compare the pollen structures and allergen content in *C. arizonica*, *C. sempervirens* and *J. oxycedrus* pollen. In the study, the pollen morphological characteristics and PL activity between the three taxa were compared, while the Immunoglobulin E (IgE) reaction profile by immunoblotting due to PL antibody and the 2D-PAGE profile of *Cupressus* and *Juniperus* pollen extracts were examined. We observed different basic spots in *C. arizonica* extracts than the others in 2D-PAGE analysis. However, due to the lack of commercial Cypress extracts in the routine SPT in Turkey we could only perform IgE reactivity using crude pollen extracts. We could not prove if these basic spots are allergenic or not.

Materials and methods

Collection of pollen samples and preparation for analysis

Pollen from some *Cupressus* and *Juniperus* taxa (*C. arizonica*, *C. sempervirens* and *J. oxycedrus*) were directly collected from mature male cones of the trees planted in Ankara from the parks and gardens during the pollen season. The samples dried for 2–3 days after putting them into plastic containers covered with a cover to prevent contamination. The dried samples were sieved through a sieve with 100 µm pore size. The sieved pollen samples stored at –20°C. Purity of pollen samples were checked by a light microscope through 1000 particles prepared by Wodehouse method [36]. The samples with a 98% purity threshold were used for the analysis. After the purity and morphology of the pollen were determined under light microscopy, pollen from the male cones were placed directly on stamps included two-sided adhesive tape to examine in the SEM in detail and then microphotographs were taken. Terminology was adopted from Punt et al. [37, 38].

Protein extraction and quantification

Firstly, the dry *C. arizonica*, *C. sempervirens* and *J. oxycedrus* pollen treated with liquid nitrogen were crushed. Then 0.01 M phosphate buffered saline (PBS) buffer pH 8 was added. Fifty milligram pollen of each taxon 1:10 (w/v) were mixed with PBS buffer. These samples were centrifuged at 14,000 g for 30 min at 4°C and the supernatant was used [20]. The amount of total protein was determined by Bradford method [39].

Analysis of pectate lyase activity

PL activity was assayed spectrophotometrically by measuring the formation of unsaturated products from apple pectin substrate at 232 nm. For three taxa, the standard assay mixture was determined as mentioned by Collmer et al. [40]. Apple pectin was centrifuged at 20,000 g for 30 min. before starting the reaction. The supernatant was added to the reaction mixture and the reaction was carried out at 37°C for 30 min and terminated by the addition of 20 µL of 4 M HCl. The molar extinction coefficient used for the unsaturated product released at A232 nm, was 5200 M⁻¹ cm⁻¹ [40]. One unit of enzyme was defined as the amount of enzyme that forms 1 µmol of 4,5-unsaturated product per minute, under the described assay conditions.

Partial purification of the pectate lyase like protein

The purification was carried out in three stages; Ammonium sulfate precipitation, dialysis and ultrafiltration. Firstly, each pollen extract was saturated by gradual addition of solid ammonium sulfate while stirring at 4°C. After 30 min of equilibration, the precipitated protein was obtained by centrifugation at 12,000×g at 4°C for 12 min. The pellet was resuspended in PBS (pH 8.0). Enzyme activity and protein content were determined after separation of each fraction. Separate trials for each taxon showed that 80% ammonium sulfate precipitation for *C. sempervirens*, 70% ammonium sulfate precipitation for *C. arizonica* and 60% ammonium sulfate precipitation for *J. oxycedrus* was sufficient. After precipitation, dialysis was carried out with special dialysis cassettes to remove salts and other low molecular weight substances. The pellet obtained by ammonium sulfate precipitation was solved in 0.1 M PBS (pH 8.0) and then put on a slide-a-Lyzer G2 20 K MWCO (Thermo Scientific) dialysis cassette dialysed against to 0.1 M PBS (pH 8.0) reaction buffer. This step was repeated for 2 times and the obtained dialysate was concentrated by filtering with Amicon 50 K (Milipore). PL activity of the partial purified proteins according to method mentioned above was also determined. All the reactions were carried out in triplicate and results were reported as mean ± SD.

SDS-PAGE analysis

After extraction for each taxa, the total protein concentrations was measured again with the Bradford method. The crude extract and partially purified extract were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [41] and the gels were stained with Coomassie Brilliant Blue.

Production of polyclonal antibody in rabbits

Two New Zealand rabbits were immunized subcutaneously and intraperitoneally with 1 mg PL enzyme (from *Aspergillus* sp., Megazyme, Lot 111201a) in 2-week intervals. One milliliter of the antigen (PL from *Aspergillus*) diluted with Freund's Complete Adjuvant at a ratio of 1:1 was applied into the rabbits subcutaneously. At 2 week intervals antigens mixed with equal volume of Freund's incomplete adjuvant at the same amount was applied subcutan and intraperitoneal. After the last injection, blood serum samples were collected from the immunized rabbits

after 10 days, antibody levels (serum antibody level from 1/100 dilution ratio to 1/10,000 dilution ratio) in serum were determined by indirect ELISA Method. These serum antibodies have produced in TUBITAK MAM Genetic Engineering and Biotechnology Institute. They are obtained and used in Western blot analysis for PL.

Western blotting analysis

After proteins were loaded to 4–12% ready bis-tris gel (NuPAGE®), in a vertical electrophoresis for each taxa, the gel was run at 120 V for 60 min approximately. The proteins were transferred as dry from the gel to PVDF (polyvinylidene fluoride) membrane (0.2 µm pore size, NuPAGE®) by using iBlot® gel transfer device (Invitrogen), The subsequent phases were carried out using the Western Breeze kit (Invitrogen, Carlsbad, CA, USA). After the blocking, membranes were treated with the PL antibody (TUBITAK MAM, Gebze, Kocaeli, Turkey). The membranes were washed three times and treated with alkaline phosphatase conjugated Anti-Rabbit (secondary antibody solution-WB7105WB). The secondary antibody was removed by washing with concentrated saline solution containing detergent, then it was treated with BCIP/NBT (5-bromo-4-chloro-3'-indolyphosphate/nitro-blue tetrazolium) chromogenic substrate. Chromogenic bands were identified in the Bio-Rad imaging system.

Patients sera

After receiving ethical approval (Ankara University, Faculty of Medicine, Ethical Committee), eight seasonal allergic rhinitis patients (seven female, one male; 22–61 years old) who displayed a positive prick test results for least one Cupressaceae family members and two healthy controls were included into the study. Pollen allergy was established by means of positive SPT and typical case history. Sera were stored at –20°C until use. Demographic data and SPT results for each patients and control group were presented in Table 1.

Immunoblotting for the detection of IgE reactivity

The pollen protein extract analyzed in a 4–14% SDS-PAGE and then transferred onto PVDF membrane. Membranes were blocked with PBS containing 2% milk powder for 1 h at ambient temperature. Membrane was cut into 3 mm

Table 1: Recorded data of each patient for immunoblotting.

Patient no.	Gender	Age	Skin prick test	
			<i>Betula</i>	Cupressaceae
1	M	27	11×7	3×3
2	F	25	4×3	6×5
3	F	39	11×11	5×5
4	F	22	3×2	5×5
5	F	61	–	4×4
6	F	28	5×3	4×4
7	F (Control)	26	–	–
8	M (Control)	30	–	–

wide strips that individually incubated with 1/30 diluted patient sera in tris buffered saline (TBS) + 0.1% skim milk powder (overnight). After the incubation, the membrane was washed and then incubated for 1 h with 1:1000 dilution of Mouse anti-human IgE conjugated with alkaline phosphatase (Sigma, A3076) in TBS. The membrane was washed again and treated by the BCIP-NBT. Substrate kit (Bio-Rad).

2D-PAGE analysis

Before isoelectric focusing, all three extract were precipitated by 25% trichloroacetic acid (TCA) (v/v) and washed by ice-cold acetone. Precipitated samples were resuspended by rehydration buffer (includes 8 M urea, 2% CHAPS, 50 mM DTT, 0.2% ampholyte, 0.001% Bromophenol Blue). Separation of the proteins were performed in three steps using Protean i12 IEF Cell (Bio-Rad Laboratories, Hercules, CA, USA) by using PG strips (7 cm, pH 3–10). After the first separation, of the proteins at the second dimension was carried out with 14% SDS-PAGE using Mini-Protean Tetra Mini Gel (Bio-Rad Laboratories, Hercules, CA, USA), at 150 V constant voltage for 2 h. Then the gels were stained with Coomassie Brilliant Blue.

Results

Pollen structure

The main palynological structure of Turkish Cupressaceae taxa are summarized in Table 2 and they are shown in Figure 1. The pollen grains of the *Cupressus* taxa are apolar and spheroidal with polar and equatorial axes of 25.18–32.5 µm. The pollen of *J. oxycedrus* is spheroidal

Table 2: Pollen measurements of the species.

Taxa	Diameter (µm)			Intine thickness (µm) (outer-middle-inner layers)			Intine/diameter (%)
	Min	Max	Mean	Min	Max	Mean	Mean
<i>C. arizonica</i>	26.37	30.62	29.25	0.25–3.06–0.56	0.37–5.62–1.25	0.31–4.48–0.86	38.9
<i>C. sempervirens</i>	25.18	32.5	28.69	0.35–2.5–0.54	0.41–4.68–0.93	0.38–3.37–0.75	31.4
<i>J. oxycedrus</i>	23.62	31.31	28.43	0.16–1.68–0.18	0.20–2.59–0.54	0.18–2.16–0.36	19.0

Taxa	Protoplasma diameter (µm)	Ornamentation	Orbicular	
			Diameter	Number (in 5 µm ²)
<i>C. arizonica</i>	16	Rugulate-granulate	0.5	52
<i>C. sempervirens</i>	18.3	Rugulate-granulate	0.53	43
<i>J. oxycedrus</i>	18.81	Rugulate-granulate	0.49	57

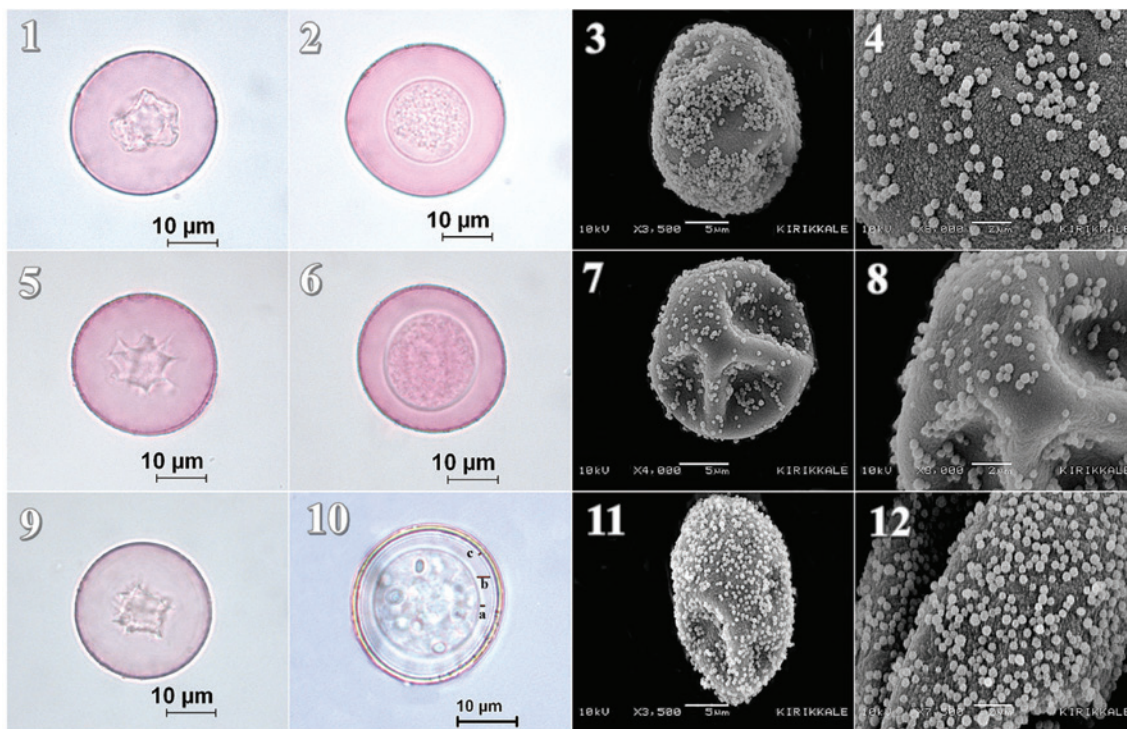


Figure 1: LM and SEM microphotographs of studied taxa. 1–2.

LM microphotographs of *C. arizonica*, 3–4. SEM microphotographs of *C. arizonica*, 5–6. LM microphotographs of *C. sempervirens*, 7–8. SEM microphotographs of *C. sempervirens*. 9–10. LM microphotographs of *J. oxycedrus* (a: inner intine; b: middle intine; c: outer intine layers), 11–12. SEM microphotographs of *J. oxycedrus*.

with polar and equatorial axes of 23.62–31.31 µm. *Cupressus arizonica* showed the highest values. In general, the pollen grains of *Cupressus sempervirens* and *J. oxycedrus* have similar values. The pollen grains are inaperturate. They have a distinctive pseudopore and a 10 × 15 µm size. The exine is a weakened 0.75 µm thick. Intine is 3.70 µm thick on average. The thickest intine layers were observed in *C. arizonica* pollen grains. *Juniperus oxycedrus* have a low intine thickness. The percentages of intine thickness

in relation to pollen grains diameter were calculated. The intine covered a greater percentage of the pollen grains diameter with a mean of 30%, ranging from a minimum of 19% in *J. oxycedrus* to a maximum of 38.9% in *C. arizonica*. The exine is rugulate-granulate with microechinate orbicules. Orbicular size varies between 0.30 and 0.60 µm in *Cupressus* and 0.40–0.59 µm in *J. oxycedrus*. The number of orbicules in 5 µm² are 33–100 in *Cupressus*, 39–87 in *J. oxycedrus*.

Detection of pectate lyase activities in pollen extracts

Bradford protein assay revealed that the highest total amount of protein was found in *J. oxycedrus* (342 µg/mL) pollen. The next highest was detected in pollen of *C. sempervirens* (225 µg/mL) and the lowest amount was found in *C. arizonica* (223 µg/mL). Two different substrate concentrations (0.30 and 0.50% apple pectin) were tested for PL activity in the pollen extracts at 37°C. All three pollen taxa showed PL activity. The highest PL activity was detected in the presence of 0.30% apple pectin in *C. sempervirens* and in the presence of 0.50% substrate in *J. oxycedrus*. Increasing the concentration of the substrate showed a positive effect and the enzyme activity also increased in

C. arizonica and *J. oxycedrus* pollen. However, it caused a decrease of almost half in *C. sempervirens* pollen (Figure 2, Table 3).

While the highest specific enzyme activity in the presence of 0.30% substrate was still in *C. sempervirens* pollen, in the presence of 0.50% substrate it was found in *C. arizonica*. Although the highest enzyme activity in the presence of 0.50% was detected in *J. oxycedrus*, since it had the highest total protein concentration, specific activity was found to be lower in *J. oxycedrus* pollen than in *C. arizonica* pollen. The PL enzyme activity values of *J. oxycedrus* and *C. arizonica* in both the substrate concentrations were found to be very similar. As a result, the concentration of substrate saturation varies according to the taxa (Figure 2).

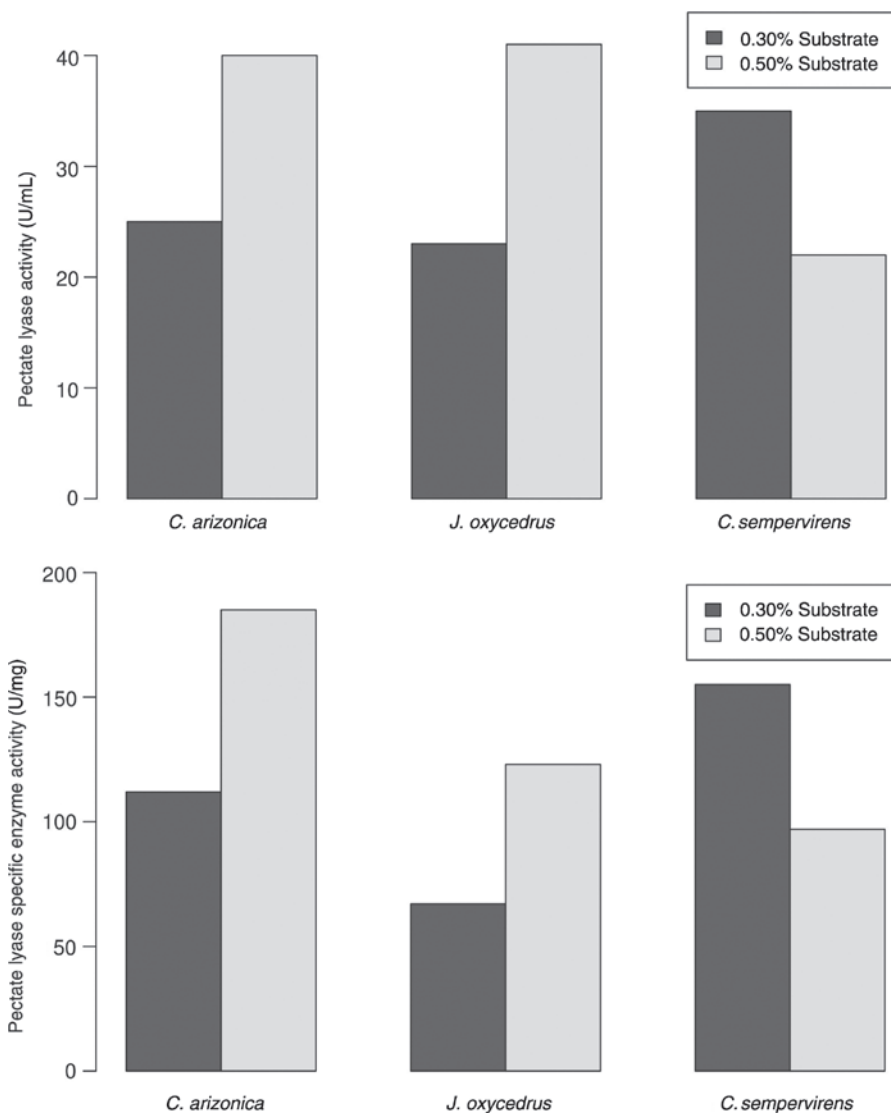


Figure 2: Values of enzyme activity in two different substrate concentration.

Table 3: Partial purification results of pectate lyase (PL) from the studied taxa.

Taxa	Total protein (mg/mL)	Enzyme activity (U/mL)	Specific enzyme activity (U/mg)	Purification fold	Yield (%)
Before partial purification					
<i>Cupressus arizonica</i>	0.223 ± 0.0	25 ± 0.2	112 ± 0.7	1	100
<i>Juniperus oxycedrus</i>	0.342 ± 0.0	23 ± 0.1	67 ± 0.3	1	100
<i>Cupressus sempervirens</i>	0.225 ± 0.0	35 ± 0.3	155 ± 0.9	1	100
After partial purification					
<i>Cupressus arizonica</i>	0.182 ± 0.0	6 ± 0.1	33 ± 0.2	0.29	24
<i>Juniperus oxycedrus</i>	0.064 ± 0.0	12 ± 0.2	187 ± 0.4	2.79	52
<i>Cupressus sempervirens</i>	0.17 ± 0.0	16 ± 0.2	94 ± 0.2	0.6	46

After partial purification, the total amounts of proteins and enzyme activities were remeasured in the presence of 0.30% substrate. The highest total amount of protein was observed in *C. arizonica*. The highest enzyme activity was detected in *C. sempervirens* pollen. Despite the reduction in the amount of total protein after partial purification, the enzyme activity of *J. oxycedrus* pollen was measured as 12 ± 0.2 U/mg. Accordingly, the specific activity (187 ± 0.4 U/mg) was considerably higher than the value of other taxa (Table 3). Purification yield of PL from *C. arizonica* crude extract was 0.29-fold, which is lower than that of *C. sempervirens* (0.6-fold). The partial purification of PL by 2.79-fold yielded an activity of 187 ± 0.4 U/mg from *J. oxycedrus* pollen extracts (Table 3).

Comparison of the crude extracts protein profile for *C. arizonica* in Figure 3A and gel image in Figure 3B. showed that Cup a 1 protein (~43 kDa) was precipitated by 70% ammonium sulfate. For *J. oxycedrus* in Figure 3C,

it was clearly seen that proteins with a higher molecular weight than 70 kDa and proteins with a lower molecular weight than 43 kDa (Jun o 1) were precipitated by 60% ammonium sulfate. For *C. sempervirens* pollen, three protein bands were observed in Figure 3D. Approximately 86 kDa, 70 kDa and 43 kDa (Cup s 1) protein bands were precipitated by 80% ammonium sulfate.

Pollen proteins' profiles of *Cupressus arizonica*, *Cupressus sempervirens* and *Juniperus oxycedrus*

As a result of the extraction performed with buffer PBS (pH=8) seven distinct protein bands including 106 kDa, 95.3 kDa, 86.7 kDa, 80.8 kDa, 63.8 kDa, 42.4 kDa and 37 kDa, were observed in *C. arizonica* pollen. Nine protein bands including 106 kDa, 95.3 kDa, 86.7 kDa, 80.8 kDa,

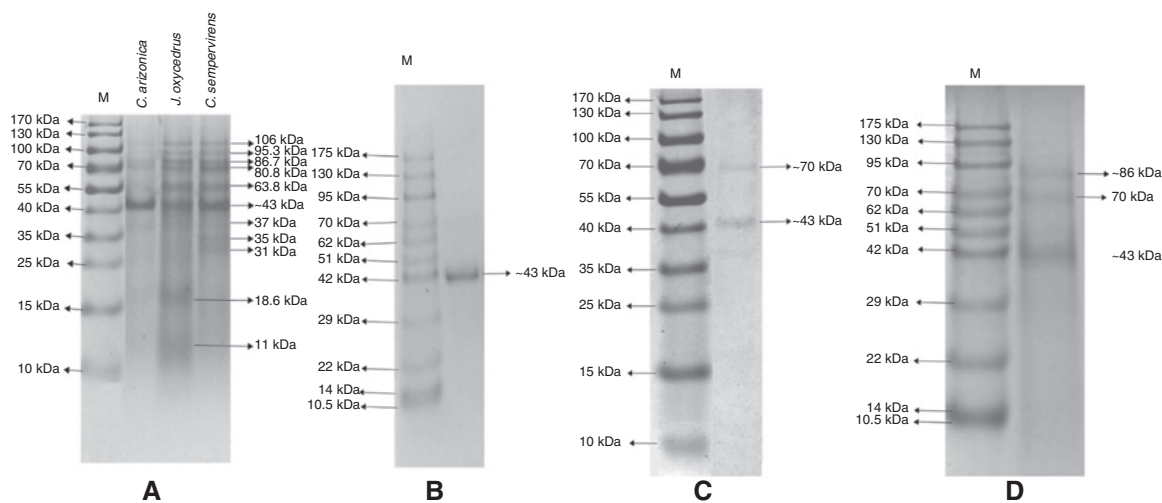


Figure 3: SDS PAGE analysis before and after partial purification. (A) Crude extracts protein profiles for studied taxa. (B) *Cupressus arizonica* pollen extract + 70% ammonium sulfate precipitation + dialysis + ultrafiltration. (C) *Juniperus oxycedrus* pollen extract 60% ammonium sulfate precipitation + dialysis + ultrafiltration. (D) *Cupressus sempervirens* pollen extract 80% ammonium sulfate precipitation + dialysis + ultrafiltration.

63.8 kDa, 42.4 kDa, 37 kDa, 18.6 kDa and 11 kDa were observed in *J. oxycedrus* pollen. In *C. sempervirens* pollen, too, nine protein bands – 106 kDa, 95.3 kDa, 86.7 kDa, 80.8 kDa, 63.8 kDa, 42.4 kDa, 37 kDa, 35 kDa and 31 kDa – were observed. The molecular weight of protein bands was measured by a program called “Image Lab Software” (Biorad).

Western blotting

The dilution ratio was determined as 1/400 to achieve the required primary antibody levels. Western blot was carried out with page ruler 4–12% bis-tris gel protein marker and the proteins were transferred to a PVDF membrane. The gel image is given in Figure 4A. Western blot analysis revealed the PL-like proteins. The approximately 43 kDa molecular weight protein band seemed to be quite intense in all three taxa. Two protein bands (approximately 37 kDa and 35 kDa) appeared in two distinct bands in *C. sempervirens* pollen as shown in Figure 4A. Also, two bands greater than 43 kDa were clearly observed in the Western blot from partially purified pollen extracts of *J. oxycedrus*. However, only one protein band (43 kDa) was observed in the blot from *C. arizonica* pollen proteins (Figure 4A).

Immunoblotting for the detection of IgE reactivity

PVDF membranes of three taxa were incubated with individual sera. IgE reactivity was observed only in the *C. arizonica* blot. Figure 4B demonstrates that among eight tested patients, 5 (63%) showed a positive IgE response to the 43 kDa allergen. IgE of all allergic patients (100%) also showed a weak IgE-binding to the band at approximately 14 kDa protein.

2D-PAGE analysis

After separation of pollen protein extract of *C. arizonica*, *C. sempervirens* and *J. oxycedrus* in 2D electrophoresis, more than one distinct protein spot was identified in a wide range of molecular masses and isoelectric points (Figure 5). In *C. arizonica* gel, although the spots were not clear, a pattern with three different spots were observed with molecular masses ranging from 37 to 50 kDa (Figure 5A). Several basic spots were detected in *C. sempervirens* gel, with a wide range of pH between 4 and 8 and molecular mass between 25 and 50 kDa (Figure 5B). Lastly, in the *J. oxycedrus* gel, several spots were again observed but they were distributed between approximately pI 4–5

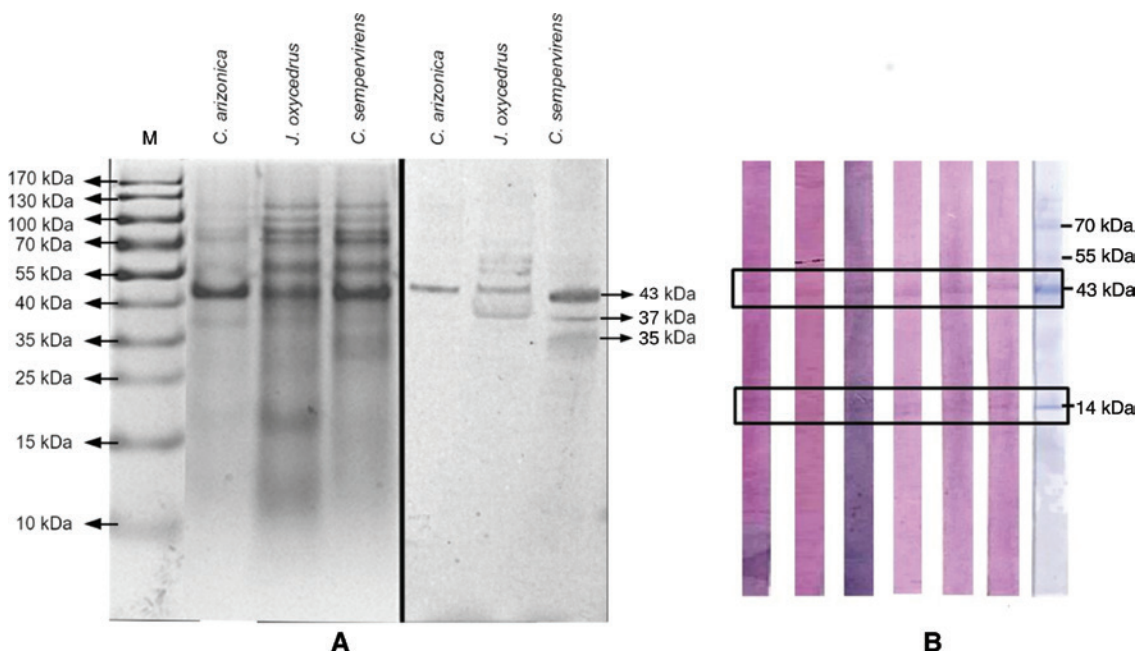


Figure 4: Gel image and IgE reactivity.

(A) Comparison of SDS PAGE analysis of the crude extracts and Western blot analysis of the three type pollen extracts with using pectate lyase specific antibody. (B) IgE reactivity to *C. arizonica* pollen extracts. The eight sera were provided by the same center and included seven females and one male with a mean age of 35.8 (range 22–61), (see Table 1).

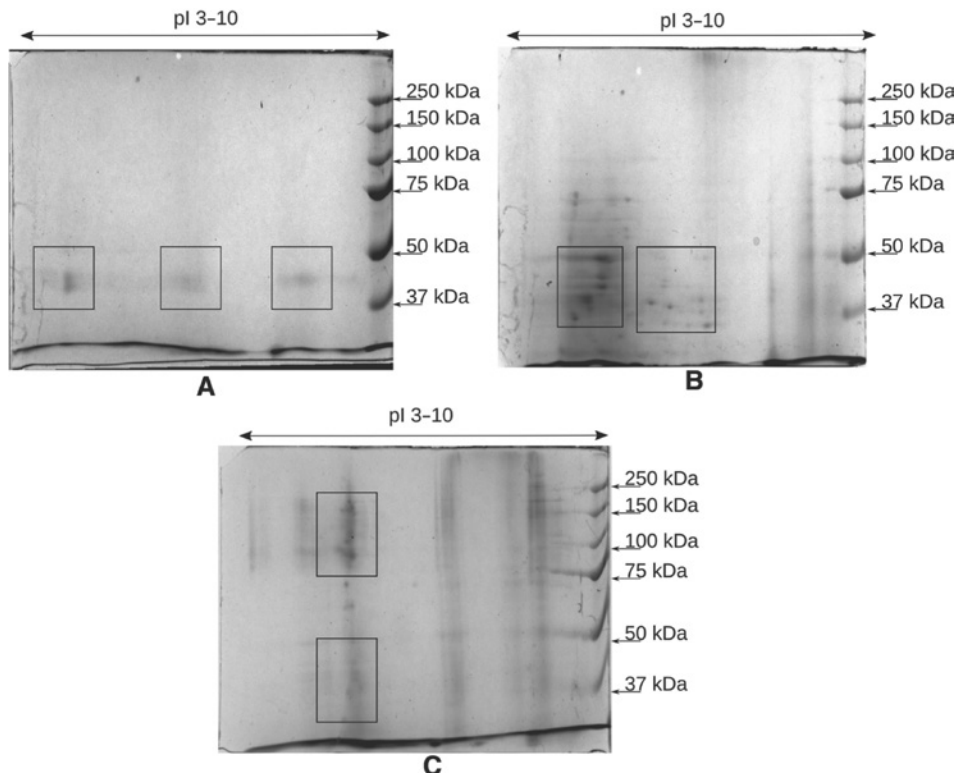


Figure 5: After separation of pollen protein extract of *C. arizonica*, *C. sempervirens* and *J. oxycedrus* in 2D electrophoresis, more than one distinct protein spot was identified in a wide range of molecular masses and isoelectric points.

2D PAGE profile for *C. arizonica* (A), *C. sempervirens* (B) and *J. oxycedrus* (C) pollen extracts (M: marker).

and the 25–250 kDa range (Figure 5C). Three protein spots exhibited similar molecular mass (approximately 43 kDa) and different pI (4–4.5, 6.5 and 9, respectively) in *C. arizonica*. Figure 5C shows that two distinct areas might be related to the 43 kDa allergen protein as pI 5 and pI 6.5 in *C. sempervirens*. However in Figure 5C, it is seen that there were a few obscure spots (pI 5.5) which could be related to the 43 kDa allergen protein.

Discussion

All three taxa were closely similar in terms of pollen morphology. Kurmann and Zawada [14] stated that pollen grains are monoporate, subspheroidal, that ornamentation is microverrucate in all species in Cupressaceae family, as well as that there are a lot of orbicules (gemma) in pollen surface and that it is difficult to distinguish the orbicule using LM. In a study about exine degradation in *Cupressus* and *Juniperus*, it was reported that the small pores did not have the classic aperture and the pores of this type have been named “pseudopores” by many researchers [42]. Also, by using transmission microscopy, the pollen wall of Cupressaceae was seen to consist of

a massive intine and a thin exine formed by a layered, electron-dense endexine and a thinner ectexine made of orbicules which appear sparsely attached to the surface [14]. In the current study, we examined three taxa under light microscopy and scanning electron microscopy and it was observed that the apertures were pseudopores. Pollen grains were isopolar and spheroidal. Ornamentation was rugulate-granulate. Three layers of intine were observed in the pollen grain wall of *Cupressus* species: a very thin outer layer, a very thick homogeneous middle layer and a thin inner layer. The thickest outer layer and the highest enzyme activity was observed in *C. sempervirens* pollen. It has been demonstrated that pectin, and the particularly the outer layer, play an important role in hydration and early pollen germination both in gymnosperms and angiosperms [15, 36]. In this case, we could say that there is a relationship between PL activity and the outer layer thickness, and if the outer layer of pollen is thicker, the enzyme activity could be higher than the other. No considerable differences were found in *C. arizonica* and *C. sempervirens* pollen grain diameters measured in our study (25–32 μm) when compared with the grain dimensions reported by Danti et al. [43] (24–33.2 μm) and Caiola et al. [13] (23.1–26.4 μm). The most obvious difference between the taxa

was that there were more orbicules in $5 \mu\text{m}^2$ in *J. oxycedrus* pollen grains than in the others.

The most important feature of Cupressaceae pollen is that it contains a low amount of protein and a higher amount of carbohydrate. This makes it difficult to achieve high levels of protein extraction from pollen. In our study, we determined that the most efficient extraction method was crushing pollen in liquid nitrogen and treating with PBS (pH 8.0) buffer. Different extraction experiments were carried out and the total amount of protein varied in the pollen of *C. arizonica*, with 170–223 mg/mL, in *C. sempervirens* pollen, with 180–225 mg/mL, while in *J. oxycedrus* pollen it was 270–342 mg/mL.

PL enzyme in pollen grains is involved in the development and extension of the pollen tube [33, 44] and most pollen proteins in Cupressaceae are known to be in the PL protein family [24, 45, 46]. Arilla et al. [24], defined the PL activity as 1.200 U/mg obtained from completely purified Cup a 1 by chromatographic methods. The same researchers also found the PL activity to be 725 U/mg obtained from completely purified Cup s 1 [25]. In our study, the results from the partial purification showed that the highest PL activity among the taxa was found in *J. oxycedrus*. In contrast, the lowest PL activity was determined in *C. arizonica* pollen. Also, it was found relatively lower (33 ± 0.2 U/mg) than the chromatographic purification values reported by Arilla et al. [24]. We agreed that these three taxa were very similar based on the pollen morphology but we could distinguish them due to their PL activities.

According to SDS-PAGE analysis, 7, 9 and 9 protein bands were detected in *C. arizonica*, *J. oxycedrus* and *C. sempervirens* pollen, respectively. We found the major allergen protein band to be defined as 43 kDa in all pollen types, and measured its enzymatic activities. *Cupressus sempervirens* and *J. oxycedrus* pollen extracts have similar PL activity. The major allergen protein band was seen in high density in *C. arizonica* pollen extracts; however, it was found to have lower PL enzyme activity. A protein band of approximately 35 kDa was detected in the pollen of *C. sempervirens*, which could be Cup s 3 allergen protein [47]. We also performed the Western blot with 1/400 diluted PL antibody to detect the protein bands showing PL activity and observed a reaction with only 43 kDa in common with all taxa. In *C. sempervirens* and *J. oxycedrus*, protein bands of approximately 37 kDa and 35 kDa were also detected. We could not observe any protein band about 14 kDa in the Western blot. Although this protein was observed in *C. arizonica* extracts that was treated with patient sera which is sensitive to Cupressaceae, this protein did not have PL activity in Western blot analysis. These results are compatible with

the study by Shahali et al. [20]. Likewise, the protein of about 18 kDa obtained from *J. oxycedrus* pollen in SDS-PAGE, could not be visualized on the Western blot. This protein has no PL activity and should be calmodulin like the protein Jun o 2 (17 kDa) [48].

In our immunoblot assays three taxa were treated with the patient sera but we observed reaction only in *C. arizonica* extracts. We detected two patterns of sensitization in patients that can be clearly distinguished: while the first one represented IgE reactivity to 43 kDa, the second one was liable to a weak reactivity of 14 kDa. The immunoblot assays in the literature have demonstrated that pollen extracts of *J. oxycedrus*, *J. ashei*, *C. arizonica*, *C. sempervirens*, *Parietaria judaica*, *Olea europaea* and *Lolium perenne* showed a high cross-reactivity [3, 30, 49, 50].

According to our results, *C. sempervirens* showed a wider diversity of protein bands whereas *C. arizonica* showed a higher content of the major 43 kDa allergen. Cup a 1 is a quite polymorphic molecule with multiple protein spots identified in 2-dimensional electrophoresis, and pI ranging from 6.5 to 10. The close series of these spots presumably correspond to various isoforms of the same protein in *C. arizonica* pollen extract.

In this study we found that *C. arizonica* pollen extracts show the lowest PL activity. To reveal the cause of this result, 2D-PAGE was applied to all three extracts for checking the isoform proteins which did not show PL enzyme activity. The 2D-PAGE analysis confirmed that there are three different isoelectronic spots (pI 4–9) with 43 kDa in *C. arizonica* pollen extracts. We were not able to observe any basic (alkaline) spot with 43 kDa in *C. sempervirens* and *J. oxycedrus* pollen extracts. In addition, it was reported that numerous proteins of 94 and 75 kDa (neutral spots, pI 3.5–8.5) were shown to be IgE reactive and to exhibit numerous isoforms, as revealed in 2-DE immunoblotting in *C. sempervirens* pollen [51]. According to immunoblotting results we detected positive IgE reactivity only in *C. arizonica* pollen extracts. This could be as a result of the basic isoforms of Cup a 1 (43 kDa) isoforms. This heterogeneity has been previously reported for Bet v 1 isoforms as well [52, 53]. In summary, we evaluated three Cupressaceae pollen allergen and their potential allergen isoform proteins due to their pollen morphological features, PL enzyme activities, Western blotting results and 2D-PAGE analysis. The limited side of this study was carrying out the immunoblotting analysis with a few patients. For further studies, developing of a suitable purification, and more efficient immunoblotting (since the antigen is not present in the standard SPT panel) with 2D-PAGE analysis with a proteomic approach are currently planned to demonstrate the characteristics of isoforms in Cypress pollen allergens

clearly. The data presented here may help to improve the Cupressaceae pollen allergen diagnosis and therapy.

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