S. Barberini[1], G. Della Rocca[1], R. Danti[1], D. Zanoni[2], B. Mori[1], R. Ariano[3], G. Mistrello[2]

Different allergenicity of pollen extracts of three Mediterranean cypress species accounted for cytological observations

[1]Istituto per la Protezione Sostenibile delle Piante. IPSP-CNR, Sesto Fiorentino, Italy
[2]Lofarma S.p.A., R & D, Milano, Italy
[3]Azienda Sanitaria Imperiese (ASL 1 - Liguria) Bordighera, Italy.

Key words

Cupressaceae; Cupressus macrocarpa; Skin Prick Test; Pollen Cytology; pollinosis

Corresponding author
Gianni Mistrello
Research Department
Lofarma S.p.A. Italy
Viale Cassala, 40
20143 Milano, Italy
Phone: +39 02 581 98 225
Fax: +39 02 581 98 302
E-mail: misgia@lofarma.it

Summary

Background*. Cypresses play an important role in the urban landscape of the Mediterranean region, releasing a huge amount of allergenic airborne pollen which causes a specific pollinosis in exposed people.* ***Objective.*** *The aim of this work is to evaluate,* in vivo *and* in vitro, *the potential allergenicity of* Cupressus macrocarpa *pollen, and to compare it with the allergenicity observed for* C. arizonica *and* C. sempervirens. ***Methods****. Pollen extracts of the three species were prepared, to determine their protein profile through SDS PAGE analysis and to evaluate their allergenic potential through EAST inhibition assays and SPT. Pollen grain composition was evaluated using a cytochemical approach with optical microscopy.* ***Results****. SDS PAGE, EAST inhibition and SPT indicated the higher allergenic potential of* C. arizonica *compared to* C. sempervirens *and* C. macrocarpa. *No significant differences in allergenic potential were found between the latter two species. Cytochemical observations reveal higher* β*-glucans and protein content in the intine of* C. arizonica *during hydration.* ***Conclusion****. The higher protein content found in* C. arizonica *pollen grains extract may be due to higher enzyme activity leading to the movement of* β*-glucans and pectins from the intine to the partially developed pollen cell wall during hydration. This could explain the higher potential allergenicity of* C.arizonica *in respect to* C. macrocarpa *and* C. sempervirens.

Introduction

The genus *Cupressus* is one of the largest genera in the Cupressaceae family and includes species that are mainly spread throughout the temperate regions of the Northern hemisphere. In the Mediterranean area, Italian cypress (*Cupressus sempervirens*) is the most common species and has been traditionally cultivated as an ornamental tree, becoming a typical feature of both urban and rural landscapes. Besides Italian cypress, *C. macrocarpa* and *C. arizonica* are two exotic species (native of Northern and Central Americas) which have been widely planted in Europe for decades due to their aesthetic relevance and marked adaptability to different climatic and soil conditions.

These three cypress species influence the pollen map of many cities (1). Cypresses generally release a large amount of anemophilous pollen, and have been recognized to be responsible for a significant component of total annual airborne pollen of Mediterranean areas (2,3). According to recent surveys, sensitization to cypress pollen has increased in central Italy (4), in southern France where it reached more than 20% (5) and in Turkey (6).

The early pollination period (January to March) of Cypress trees makes precise diagnoses difficult because allergic symptoms can be confused with seasonal illness such as flu or common colds. The real incidence of this allergy may therefore be greatly un-

derestimated, despite the availability of more powerful (effective) Cypress pollen extracts which facilitate diagnosis (7,8). Reported divergence in allergenic responses to pollen extracts of different cypress species may be related to the amount of allergens present in the interior wall (intine) of pollen grains, as the pollen exterior wall (exine) is discarded during aqueous extract preparation. It has been demonstrated that the intine contains pectic substances with protein inclusions and a dominant cellulosic component (polymeric carbohydrates) (9); both proteins and carbohydrates could be involved in the reaction with IgE (Immunoglobulin type E) (10).

The main protein allergens of *C. arizonica* and *C. sempervirens* pollen grains have been characterized, showing four main groups of molecular families with diverse biological functions: pectate lyase, polygalacturonase, thaumatin-like proteins and calcium-binding proteins (7,11,12,13,14). Among them, Cup a1 and Cup s1, a 43-45 kDa proteins belonging to pectate-lyase family, are currently recognized as the major cypress pollen allergens (15). The cross-reactivity within cupressaceae family has been demonstrated, in particular between *C. arizonica* and *C. sempervirens* (16); however, no information is available in the literature regarding the potential allergenicity of *C. macrocarpa* pollen and its role in inducing allergies.

The aim of this work is to evaluate, *in vivo* and *in vitro*, the potential allergenicity of *C. macrocarpa* pollen, and to compare it with the allergenicity observed for the more studied *C. sempervirens* and *C. arizonica*. The pollen of the three *Cupressus* species was also observed using transmitted-light and fluorescence microscopy to investigate potential cytological aspects related to allergenicity. Information derived from pollen cytology could provide a new key of interpretation of their potential allergenic effects.

Materials and Methods

Pollen collecting and storage

Cupressus arizonica, *C. sempervirens* and *C. macrocarpa* (Ca, Cs and Cm) pollen was kindly supplied by the Institute for Sustainable Plant Protection (IPSP) of CNR, Florence, Italy. Pollen was obtained from several adult trees belonging to the IPSP-CNR cypress germplasm collection. Five twigs per tree bearing mature microsporophylls (17) were randomly cut, placed in plastic bags and processed in the laboratory within 24 hours. They were arranged in vases placed on a sheet of wrapping paper and maintained at 25°C to collect the shed pollen. The collected pollen was cleaned with a 300 µm sieve and then dehydrated at room temperature under a vacuum using silica gel, until it reached a relative humidity of 30-35%. The pollen was then stored at -20°C in hermetic plastic tubes until analysed.

Preparation of Cypress extracts

Potential cross species contamination of the collected pollen was assessed using light microscopy, with a purity of > 99% being observed in all of the samples. Pollen samples of the three cypress species were defatted separately, before 5% (w/v) aqueous extraction in 0.125 M NH_4HCO_3 for 4h under stirring at 4°C. Suspensions were centrifuged at 20,000 *g* for 1 hour at 20°C and supernatants were dialyzed against distilled water. Pollen extracts were separately then treated with ammonium sulphate in order to obtain precipitated (PPT). To achieve protein precipitation, ammonium sulfate was slowly added to obtain an 80% saturated solution. After 4 h of stirring at 4°C, precipitated proteins from each extract were recovered by centrifugation at 20,000 *g* for 1 hour at 4°C, re-dissolved in 1/10 of the initial volume with water, then dialyzed extensively against water to eliminate the residual salt and finally against 0.05 M NH_4HCO_3. Protein content of both extracts was determined according to a procedure previously described (18) using the commercial Bio-Rad Protein Assay Dye Reagent (Bio-Rad, Milan, Italy) and BSA reference standard. All extracts were then lyophilized and stored at 4°C until use.

SDS-PAGE analysis

Electrophoresis of Ca, Cs and Cm extracts was carried out in a 10% polyacrylamide precast Nupage Bis-Tris gel according to the manufacturer's instructions (Novex, Prodotti Gianni, Milan, Italy) at 180 mA for 1 h. Thirty µg of each extract was loaded per 1 cm^2 of gel. The resolved proteins were stained with Coomassie Brillant Blue.

EAST-inhibition

Extracts of Ca, Cs or Cm (initial concentration of 0.05 mg/l) were diluted two-fold in PBS-1% BSA and used for the EAST analysis. Fifty µl of each extract dilution were added in tubes to 50 µl of a pool of sera from cypress allergic subjects, and incubated 2 hours at room temperature. The sera pool of non allergic individuals was also incubated and used as a negative control. Subsequently, beads coated with Ca extract prepared following a method previously described (7), were added to each tube and incubated overnight. After several washings, specific IgE bounded to the bead was detected by 30 min of incubation with goat peroxidase anti-human IgE. A colorimetric reaction was developed by adding TMB-H_2O_2, then stopped after 20 min with HCl. Absorbance was measured at 450 nm (PowerWave microplate reader, Bio-Tek Instruments Inc., Winooski, USA).

Data were expressed as percentage of inhibition in respect to maximum binding obtained without the inhibitor. To compare the relative allergenic potency, the C_{50} (defined as the volume of extract able to inhibit IgE binding by 50% of Ca, Cs or Cm extracts) was calculated.

Human sera and skin prick test

Cypress pollen allergic human sera were provided by Dr. Ariano (Modulo di Allergologia, Bordighera Hospital, Imperia, Italy). Patients referring clinical allergic symptoms were previously subjected to SPT with commercial products (Lofarma S.p.A., Milan, Italy), consisting of a mixture of Ca and Cs pollen extracts. A total of 38 patients (male 20, average age 33.5; female 18, average age 30.3) were included in the study. All these patients were negative for Birch and Grass. Some of them were also positive to mites or Parietaria. Their sera were harvested and used as pool for EAST inhibition experiments. Pool of sera from non allergic patients was used as a negative control. Positive patients were enrolled after having given their informed consent and SPT were performed once more, separately, with Ca Cs and Cm extracts. All the SPT preparations were diluted in saline solution, with 50% glycerol histamine solution (0.1%) used as a positive control.
All the patients subjected to SPT are considered habitually and equally exposed to pollen of the three cypress species because of their widespread distribution in Liguria, the region where the sampled human sera come from.

Cytochemistry

Stored pollen was first kept at room temperature for 10 min and hydrated in 2% sucrose water solution (25,000 grains/µl) before analysis. Pollen composition was determined after addition of different dyes to the hydration solution: calcofluor to highlight β-glucans (19), and aniline blue black and a combination of aniline blue black with neutral red for protein staining (20). Observations were carried-out 1-2 min after exine breakage due to hydration and swelling of the intine layer, and repeated at 10 min intervals for 1 hour.
All observations were carried out using transmitted-light and fluorescent microscopy utilizing a Laborlux S (Leitz) combined with a digital camera and dedicated software (Digital Sight DA-5M, Nikon). The excitation wavelength for calcofluor was 405 nm. For all species, measurements were performed on seven replicates of 50 grains each.

Statistical analyses

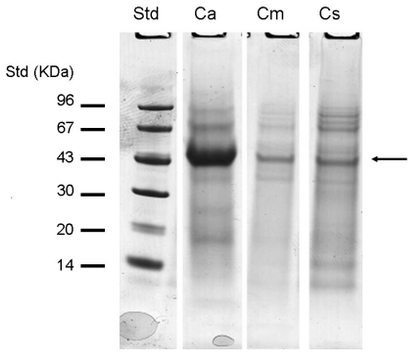
In SPT, comparison of wheal diameter from patients treated with the three different extracts was performed using the Mann-Whitney test. Statistical significance was assumed at $p = 0.01$, and data are expressed as mean ± SD.

Results

SDS-PAGE analysis

The SDS profile of the three *Cupressus* species extracts is shown in **figure 1**. A similar profile appears between Cs and Cm extracts in contrast to the Ca extract; in particular as concerning the concentration of Cup a 1-like (protein band of 43 kDa) that is considered to be the major allergen. Other components seem to be present but it is difficult to identify them (for example polcalcin, thaumatin-like) because there is not yet a general consensus on their molecular dimension in different Cupressus species. Perhaps the component at 37 kDa could be the polygalaturonase.

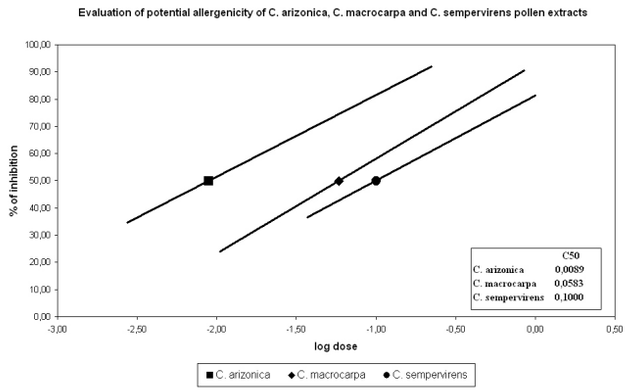
Figure 1 *- SDS-PAGE profile of Cs, Cm and Cs pollen extracts. Lanes: Std., molecular weights standard; Ca, Cupressus arizonica; Cm, Cupressus macrocarpa; Cs, Cupressus sempervirens. Arrow indicates the major allergene Cup a 1 (mw ~43 KDa).*



EAST-inhibition

In EAST-inhibition experiments (**figure 2**) the C_{50} value is calculated for different extracts; the lower the C_{50} value the higher the allergenic potential of the particular extract. The C_{50} values of the Cypress extracts used in this study are 0.058 for *C. macrocarpa* and 0.100 for *C. sempervirens*, both values being an order of magnitude greater than *C. arizonica* where $C_{50} = 0.0089$, confirming that the allergenic potency expressed by Ca is significantly greater than those of Cs and Cm. No significant difference was observed between the allergenic potential of Cs and Cm. Results (not shown) were very similar using Cm coated-beads, confirming the allergenic relevance of Cup a1 or Cup a1-like allergens.

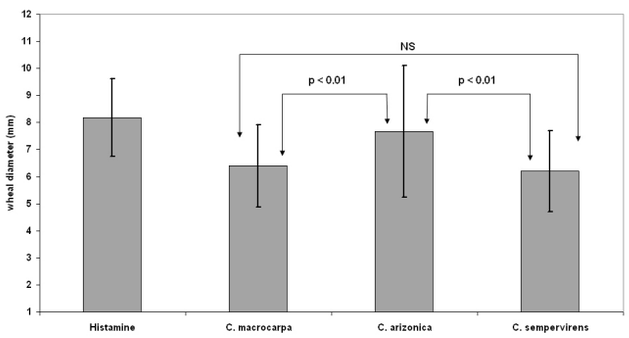
Figure 2 - *Comparison of allergenic potency among the three Cupressus extracts (Ca, Cupressus arizonica; Cm, Cupressus macrocarpa; Cs, Cupressus sempervirens). The corresponding C_{50} value are showed for each species.*



Skin prick test

As shown in **figure 3**, the cutaneous reaction induced by SPT of Ca (7.8 ± 1.9 mm) extract is significantly higher ($p < 0.01$) than those observed using both Cs and Cm extracts (6.1 ± 1.3 mm and 6.3 ± 1.5 mm, respectively). According to the Mann-Whitney rank sum test, no significant difference resulted between Cs and Cm extracts.

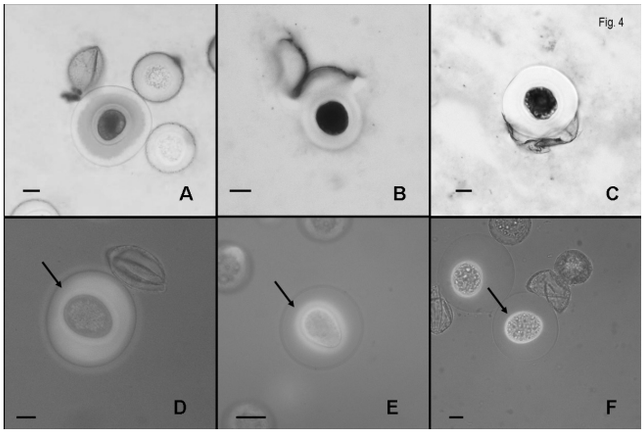
Figure 3 - *Cutaneous reactivity (SPT) of Cm, Ca and Cs extracts. Histamine solution (0.1%) was used as positive control. P values indicates significant differences among groups; NS, not significant.*



Cytochemistry

Differences in the dye staining pattern of pollen grains were observed among the three cypress species. Immediately after hydration, Ca pollen exhibited a deep stain with both calcofluor and aniline blue black in the middle intine, indicating the presence of β-glucans and proteins in this layer, but not in the inner intine which was not stained (**figure 4A** and **4D**).
In contrast, the inner intine layer of both Cm and Cs pollen grains were deeply stained with calcofluor (**figure 4E** and **4F**), revealing the presence of β-glucans in the layer but not in the middle intine, while aniline blue black stained only the cytoplasm of the pollen nucleus (**figure 4B** and **4C**).

Figure 4 - *Pollen grains of C. arizonica (A, D), C. macrocarpa (B, E) and C. sempervirens (C,F) stained with aniline blue black (A, B, C) or calcofluor (D, E, F). Visible light or fluorescence light plus visible light were used to observe the intine staining with aniline blue black or calcofluor, respectively.*



However, 60 minutes after hydration and calcofluor staining, the composition of middle and inner intine of Ca pollen grains changed, showing a fluorescence signal similar to that observed in the pollen grains of Cm and Ca which did not change their staining pattern over time (**figure 5**). To more effectively highlight the eventual change in pollen grain protein content of the three cypress species, aniline blue black and neutral red were combined to monitor the dynamic phase occurring after pollen grain swelling. As illustrated in **figure 6**, only *C. arizonica* proteins were deeply stained in the middle intine layer after 60 minutes of swelling before intine dissolution.

Figure 5 *- Pollen grains of C. arizonica after 60 min of hydration and treatment with calcofluor, showing an even fluorescence in the middle and inner intine layers (bar 10 µm).*

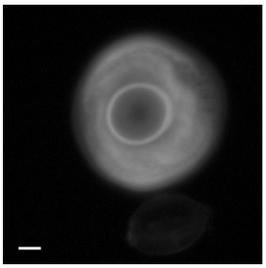
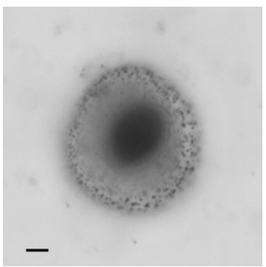


Figure 6 *- Pollen grains of C. arizonica after 60 min of treatment with neutral red plus aniline blue black (bar 10 µm).*



Discussion

This is the first study reporting the allergenic profile of *C. macrocarpa*, a species imported from California and widely planted in parks and gardens in urban and periurban areas of southern Europe for its ornamental value.

In Liguria region (were the SPT / in vivo tests were performed) the three species of cypress are evenly spread (official data on their distribution does not exist because they are not included in forest inventory) and can be observed as single trees or groups in parks and gardens due to their peculiar ornamental value. *C. macrocarpa* is frequently present on the seashore / seafront for its marked tolerance to salty aerosol; *C. sempervirens* has been more commonly planted in the inland countryside, also to form little groves, and *C. arizonica* has met particular interest for the colour of its grey-green foliage, especially to make hedges.

Cupressaceae pollen has been identified as a source of increasing pollinosis in Mediterranean countries, and it is responsible for winter allergy during a period of the year when no other allergenic plants are flowering (1). Since pollen allergy has a pronounced clinical impact and respiratory allergic reactions induced by pollens are increasing (2), the study of the most abundant cypress species is of great significance. This study takes into consideration the three most widespread cypress species in the Mediterranean basin that are responsible for high airborne pollen dispersion.

Our results demonstrate the great allergenic activity possessed by Ca pollen grain extract compared to extracts of Cs and Cm, illustrated by the very low C_{50} value in the EAST inhibition test. The C_{50} value of the Cm extract was six-fold lower than the allergenic potential of Ca, and twofold higher than Cs (but no significant differences were observed between Cm and Cs). These results are consistent with those previously known (7) regarding the allergenic potential of *C. arizonica* pollen. Confirmation of these findings was obtained through *in vivo* evaluation of cutaneous reactivity on volunteers by specific SPT. This revealed that the diameter of wheals induced by Cm and Cs extracts were similar and significantly smaller than those provoked by Ca extract ($p < 0.01$). Hence the allergenicity of *C. macrocarpa* and *C. sempervirens* extracts are similar to one another, but significantly lower than that of *C. arizonica*: one possible explanation for this difference may be due to the different amount of the allergen Cup a 1 contained in the pollen extracts of the three cypress species, as revealed by the SDS-PAGE profile. Pollen of Ca exhibited greater concentrations of Cup a 1, whereas in Cm and Cs the Cup a 1-like component band appeared to be less intense. Therefore, the higher content of Cup a 1 seems to form the basis of the higher allergenic potential showed by Ca relative to Cs and Cm. On the other hand, Immunoblotting experiment (data not shown) using the pool sera of patients evidenced the presence of band at about 43 kDa, more intense in case of Ca in respect to Cm and Cs extracts, and another, more slightly, at about 37 kDa, much probably polygalacturonase, suggesting that other eventual components (polcalcin or thaumatin-like) are not of relevance at least on the basis of current scientific knowledge.

Allergic symptoms (asthma and rhinitis in particular) start after pollen inhalation, when grains come in contact with the nasal mucosa. The humid conditions of the respiratory tract lead to pollen hydration and exine breakage due to the swelling of the intine, similarly to what observed in pollen grains when hydrated in vitro. It was found that during pollen hydration, the protein Cry j 1, the major allergen of *Cryptomeria japonica* (a species belonging to *Cupressaceae*), quickly migrated to the vac-

uole through the intine (21). Furthermore, TEM observations of *C. arizonica* pollen revealed that specific epitopes (glucidic or proteic) were localized in the pollen intine of fixed samples, but not in the hydrated samples (10).

Our observations during the whole hydrating process indicated a different dynamic change in cell wall composition depending on the species. As evidenced by the aniline blue black staining, the protein content, including eventual allergens, was more abundant at the beginning of the pollen grain hydration in the middle intine of *C. arizonica* pollen grains compared to those of *C. macrocarpa* and *C. sempervirens*. The β-glucans content in the middle intine at the beginning of the hydration process was also clearly evident in *C. arizonica* pollen grains stained with calcolfuor. During the first 20 minutes of pollen hydration, the observed dynamic 'dissolution' of β-glucans in the middle intine and their concentration in the inner intine imply enzymatic activity in *C. arizonica* pollen that eventually involves allergenic proteins such as Cup a 1. In contrast, β-glucans in Cs and Cm are clearly already present in the inner intine by deep staining with Calcofluor at the beginning of hydration.

It is significant that Cup a 1 belongs to the pectate lyase family and that these proteins are involved in cell wall degradation (22). As pectins were present in all the intine layers (ruthenium red staining (9)), our hypothesis is that proteins in the middle intine of *C. arizonica* (indicated by aniline blue black staining) could be related to intensive enzyme activity, which leads to the complete construction of the cell wall (inner layer) that is only partially formed at the beginning of hydration. This has not been observed in the pollen of *C. macrocarpa* and *C. sempervirens* that have a cell wall that is already fully structured before pollen grain hydration. The pollen extracts used in this study were based on the preparation of a water suspension in which pollen is kept for 4h, thus permitting pollen hydration and swelling accompanied by all the enzymatic processes that lead to the movement of β-glucans from the middle to the inner intine, as observed during hydration. Therefore, the higher amount of proteins contained in the pollen of *C. arizonica* during hydration could be also found in the pollen extract.

Cytological similarities between *C. sempervirens* and *C. macrocarpa* pollen grains were previously evidenced, although *C. macrocarpa* is considered phylogenetically closer to *C. arizonica* (9,23). These cytological observations evidenced some differences among cypress species in the protein content of pollen grains during hydration that may account for the increased potential allergenic activity observed in *C. arizonica* pollen extract, in respect to that of *C. sempervirens* and *C. macrocarpa*.

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